RESEARCH ARTICLE SUMMARY

PLANT SCIENCE

A specialized metabolic network selectively modulates *Arabidopsis* root microbiota

Ancheng C. Huang*, Ting Jiang*, Yong-Xin Liu, Yue-Chen Bai, James Reed, Baoyuan Qu, Alain Goossens, Hans-Wilhelm Nützmann, Yang Bai†, Anne Osbourn†

INTRODUCTION: Specialized metabolism is a feature of plant evolution and adaption. Plant-specialized metabolites have ecological functions, mediating interactions between plants and their environments. Although microbes can have diverse effects on plant growth and fitness, how plants assemble and modulate their microbiota remains unclear. Understanding the factors and mechanisms underlying this process will open up avenues for engineering plant microbiota for sustainable agriculture. Plants are estimated to use ~20% of their photosynthesized carbon to make root-derived organic molecules. However, whether (and if so, which) specialized metabolites can direct the assembly of specific root microbiota is not known.
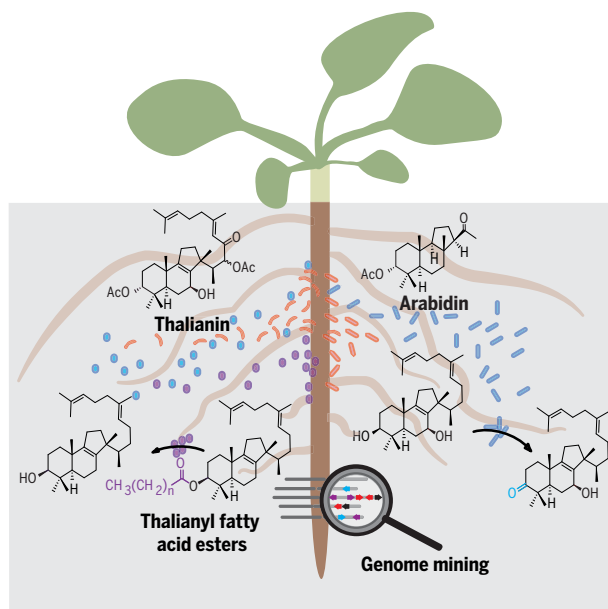
RATIONALE: Triterpenes are plant-specialized metabolites that have functions in plant defense and signaling and also have antimicrobial activities. They are one of the largest and most structurally diverse families of plant natural products. The genome of the small mustard plant *Arabidopsis thaliana* harbors four root-expressed triterpene biosynthetic gene clusters that encode unknown triterpene biosynthetic pathways. Plant biosynthetic gene clustering is likely to be a result of strong selection pressure during evolution with associated production of small molecules of biological and ecological importance. Several of these clustered *Arabidopsis* genes have been implicated in defense against root pathogens, further suggesting that metabolites derived from these triterpene biosynthetic gene clusters may modulate the *Arabidopsis* root microbiota.

RESULTS: We have elucidated a specialized metabolic network expressed in the roots of *A. thaliana* that consists of functionally divergent triterpene biosynthetic gene clusters connected by scattered genes outside the clusters that encode promiscuous acyltransferases and alcohol dehydrogenases. This metabolic network has a latent capacity for synthesizing more than 50 previously unknown root metabolites. This is a relatively large number considering the total number of nonvolatile root metabolites that we detected (approximately 300). We characterized three divergent pathways for the biosynthesis of root triterpene metabolites: thalianin, thalianyl fatty acid esters, and arabidin. Analysis of the root microbiota of *A. thaliana* mutants disrupted in the biosynthesis of these compounds revealed shifts in the composition and diversity of their root microbiota compared with those of the wild type. Comparison with the root bacterial profiles of the taxonomically remote species rice and wheat supports a role for this specialized triterpene biosynthetic network in mediating the establishment of an *Arabidopsis*-specific microbiota. We next tested the activity of purified or synthesized *Arabidopsis* root triterpenes and representative triterpene cocktails in vitro toward 19 taxonomically diverse bacterial strains isolated from the *A. thaliana* root microbiota. We found that these compounds could indeed selectively modulate the growth of these bacteria, examples of both positive and negative modulation being evident. The modulation effects of the various triterpenes on the growth of different bacterial strains correlated with the relative differential abundance of the differential bacterial genera in the roots of *A. thaliana* Col-0 and triterpene mutant lines. Moreover, some root bacteria were found to be able to selectively metabolize certain triterpenes (such as thalianyl fatty acid esters) and use the breakdown products such as palmitic acid as carbon sources for proliferation.

CONCLUSION: We demonstrate that *A. thaliana* produces a range of specialized triterpenes that direct the assembly and maintenance of an *A. thaliana*–specific microbiota, enabling it to shape and tailor the microbial community within and around its roots to its own purposes. We speculate that metabolic diversification within the plant kingdom may provide a basis for communication and recognition that enables the sculpting of microbiota tailored to the needs of the host and that this may in part explain the existence of plant-specialized metabolism. Our study opens up opportunities for engineering root microbiota and further paves the way for investigating the functions of root microbiota in plant growth and health. ∎

Dynamic modulation of the *Arabidopsis* root microbiota by specialized triterpene metabolites derived from biosynthetic gene clusters. The specialized triterpenes thalianin, thalianyl fatty acid esters, and arabidin selectively modulate *A. thaliana* root microbiota members by promoting (indicated with the orange and purple bacteria) or inhibiting (indicated with the blue bacteria) the growth of different bacterial taxa and, in some cases, by serving as carbon sources (purple bacteria). These triterpenes are products of pathways encoded by biosynthetic gene clusters and nonclustered genes. Colored arrows indicate genes encoding different types of enzymes: black, triterpene synthase; red, cytochrome P450s; purple, acyltransferases; and blue, alcohol dehydrogenases. The dynamic modulation of root bacteria mediated by these specialized triterpenes contributes to the assembly of an *A. thaliana*–specific root microbiota.

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A specialized metabolic network selectively modulates *Arabidopsis* root microbiota

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Plant specialized metabolites have ecological functions, yet the presence of numerous uncharacterized biosynthetic genes in plant genomes suggests that many molecules remain unknown. We discovered a triterpene biosynthetic network in the roots of the small mustard plant *Arabidopsis thaliana*. Collectively, we have elucidated and reconstituted three divergent pathways for the biosynthesis of root triterpenes, namely thalianin (seven steps), thalianyl medium-chain fatty acid esters (three steps), and arabidin (five steps). *A. thaliana* mutants disrupted in the biosynthesis of these compounds have altered root microbiota. In vitro bioassays with purified compounds reveal selective growth modulation activities of pathway metabolites toward root microbiota members and their biochemical transformation and utilization by bacteria, supporting a role for this biosynthetic network in shaping an *Arabidopsis*-specific root microbial community.

Plants have evolved the ability to produce a vast array of specialized metabolites. This metabolic diversification is likely to have been driven by the need to adapt to different environmental niches (*1*). One of the key environmental factors that influences plant health and fitness is the root microbiota (*2–4*), yet mechanisms underpinning the establishment of specific root microbiota and how plants direct the microbial communities around their roots remain elusive. Plants are estimated to use ~20% of their photosynthesized carbon to make root-derived organic molecules, stimulating the formation of distinctive root microbiota from surrounding soil (*5–8*). However, whether and which specialized metabolites produced by plants can modulate root microbiota is not known. The presence of a substantial number of uncharacterized root-expressed biosynthetic genes in plant genomes also reveals our incomplete understanding of these communication processes.

Triterpenes are specialized plant metabolites that have important functions in plant defense and signaling. They are one of the largest and most structurally diverse families of plant natural products and many have been shown to possess antifungal and antibacterial activities, suggesting potential roles in mediating interactions between the producing plants and microbes (*9–11*). Triterpenes are synthesized by means of the mevalonate pathway, the first committed step being carried out by enzymes known as triterpene synthases (TTSs), which collectively are able to make a diverse array of different triterpene scaffolds (*12*). There are 13 predicted TTS genes in the genome of *A. thaliana* accession Col-0, five of which (*MRN*, *THAS*, *ABDS*, *PEN3*, and *BARS*) are located in a total of four plant biosynthetic gene clusters on chromosomes 4 and 5 (Fig. 1A).

Plant biosynthetic gene clusters represent evolutionary genomic hot spots under strong selection pressure (*13*), with the potential to encode metabolites of ecological importance (*14*). Moreover, most genes within these four *Arabidopsis* biosynthetic gene clusters are root-expressed (Fig. 1B), and the arabidiol gene cluster has been implicated in defense against the root-rot pathogen *Pythium irregulare*, suggesting that the derived metabolites might play a role in root-microbe interactions (*15–18*). The TTSs of the *A. thaliana* biosynthetic gene clusters appear to share a common ancestor but have functionally diverged and form a monophyletic clade that is distinct from other *A. thaliana* TTSs (fig. S1) (*15*). These clustered TTSs channel primary metabolism into specialized metabolism by converting the ubiquitous precursor 2,3-oxidosqualene into differ-

ent triterpene scaffolds, specifically marneral/marnerol **M1/M2** (*19*), thalianol **T1** (*20*), arabidiol **A1** (*21*), tirucalladienol **Tr1** (*22*), and baruol **B1** (*23*) through the corresponding bi/tri/tetra-cyclic carbocations, respectively (fig. S1). Four of the 13 cytochrome P450 (CYP) genes in these cluster regions [*At5g48000* (*THAH*), *At4g15330* (*CYP705A1*), *At5g62590* (*MRO*), and *At5g36110* (*CYP716A1*) belonging to the CYP708, CYP705, CYP71, and CYP716 families, respectively] have previously been shown to convert **T1**, **A1**, **M1/M2**, and **Tr1** primarily to 3β,7β-thalianiol **T2**, 14-apo-arabidiol **A2**, 23-hydroxy-marner-al/ol **M3/M4**, and unknown products, respectively (Fig. 2) (*18*, *24*). However, numerous other uncharacterized genes with predicted functions in specialized metabolism are present in these biosynthetic gene clusters and are coexpressed with the characterized TTS and CYP genes in *A. thaliana* roots (Fig. 1B and fig. S2) (*15*, *18*). Here, we elucidate three biosynthetic pathways derived from the thalianol and arabidiol gene clusters and show that these major root-specialized metabolites play important roles in selectively modulating *Arabidopsis* root bacteria both in vivo and in vitro and contribute substantially to the establishment of an *Arabidopsis*-specific root microbial community.

Elucidation of the thalianol gene cluster–derived pathways

The thalianol biosynthetic gene cluster in *A. thaliana* contains four coexpressed genes, of which the functions of two (the TTS *THAS* and the CYP *THAH*) have been characterized (*15*, *24*). A second CYP (*THAO*, *At5g47990*) that belongs to the CYP705 family mediates a third step in the pathway, but the precise structure of the resulting product has not been determined (*15*). A fourth gene (*THAA1*, *At5g47980*) that is coexpressed with the other pathway genes is predicted to encode an acyltransferase belonging to the BAHD family, but its function is unknown (*15*, *25*). We used *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana* leaves to investigate the functions of the cluster genes *THAO* and *THAA1* (*26–28*). We first coexpressed *THAS* with the three other cluster genes encoding the hydroxylase (*THAH*), CYP705A5 (*THAO*), and acyltransferase (*THAA1*) in the leaves of *N. benthamiana* in a combinatorial fashion (table S1). We found that THAO alone could modify thalianol to give two new products—3β,16-thaliandiol **T3** and 16-keto-thalianol **T4**, respectively—when coexpressed with *THAS* (Fig. 2 and fig. S3). Coexpression of *THAO* and *THAH* with *THAS* gave 16-keto-3β,7β-thaliandiol **T5** and 16-keto-3β,7β,15-thaliantriol **T6** as the main products, instead of the desaturated thaliandiol proposed previously (Fig. 2 and fig. S3) (*15*). Furthermore, we found that *THAA1* encodes an acyltransferase that was functional only when coexpressed with all three other cluster genes—*THAS*, *THAH*, and *THAO*—yielding a new product identified as 3β,7β-dihydroxy-16-keto-thalian-15-yl acetate **T7**, along with its $C_{17}=C_{18}$ cis isomer ***cis*-T7** as the minor product (Fig. 2 and fig. S3).
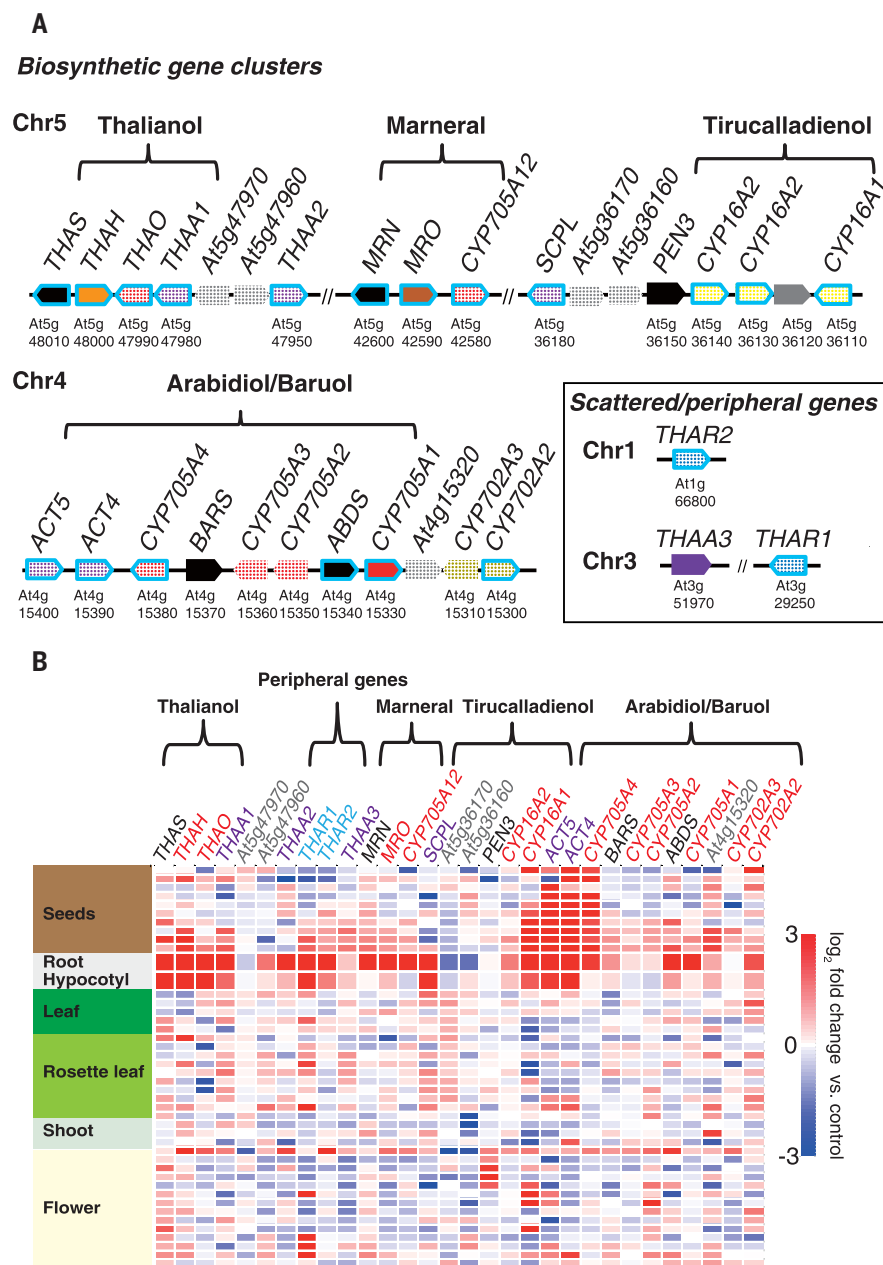
A

Biosynthetic gene clusters

Fig. 1. Identification of a root-expressed triterpene biosynthetic network in *A. thaliana*. (**A**) The four *A. thaliana* triterpene biosynthetic gene clusters and peripheral genes. Genes encoding previously characterized enzymes are shown in solid color, and uncharacterized ones are dotted. Black, TTSs; red, CYP705 family; orange, CYP708 family; brown, CYP71 family; yellow, CYP716 family; dark yellow, CYP702 family; purple, acyltransferases (ACTs); blue, alcohol dehydrogenase (ALDHs); gray, nonbiosynthetic genes. The scattered/peripheral genes identified as part of this network are also shown. Coexpressed genes have light blue borders. PEN3 was previously shown to be coexpressed with other cluster genes in roots (*18*). (**B**) Expression profiles of the triterpene cluster genes and additional scattered (peripheral) biosynthetic genes identified as part of this study in different *A. thaliana* tissues. The relative expression indicates the log2 ratio of a tissue's expression level to control signal—typically, the median of different tissues (*45*). The heatmap was generated by using microarray expression data from the eFP browser (*45*). For clarity, expression profiles for the same tissue across different developmental stages are labeled with the tissue names only. A more detailed heatmap can be found in fig. S2.

This suggests that THAA1 functions after THAS, THAH, and THAO to add an acetate group on the C15 position of the thalianol scaffold after a hydroxy moiety is installed at this position. We also identified another BAHD acyltransferase gene (*THAA2, At5g47950*) in close proximity to *THAA1* on chromosome five that was coexpressed with the thalianol cluster genes (Fig. 1). In contrast to THAA1, THAA2 is promiscuous and can act on the C3 hydroxy moiety

of different thalianol-derived compounds (**T1** to **T7**) to introduce an acetyl group (**T11** to **T17**) when coexpressed with subsets of the thalianol cluster genes (Fig. 2, fig. S4, and table S1). Our results indicate that the four thalianol cluster genes and the nearby coexpressed gene *THAA2* are functional and yield 7β-hydroxy-16-keto-thalian-3β-15-yl diacetate **T17** when coexpressed in *N. benthamiana*.

Identification of thalianol-derived root metabolites in *Arabidopsis*

We next performed targeted metabolomic analysis to identify thalianol-derived metabolites in *A. thaliana*. Metabolic profiling identified seven major thalianol-derived products (**T1**, **T2**, **T9**, **T10**, and **T18a** to **T18c**) in root extracts from the wild-type Col-0 accession and the *THAS* over-expression line (*thas-oe*) that are absent in those from the *THAS* mutants (*thas-ko1* and *thas-ko2*) (Fig. 3, A to C, and table S2). Mutation of *THAH* (*thah-ko*) led to accumulation of 3β,15-thaliandiol **T3** and 16-keto-thalianol **T4**, whereas mutation of *THAO* (*thao-ko*) resulted in elevated levels of 3β,7β-thaliandiol **T2** and absence of **T3** and **T4** (Fig. 3, A and B, and fig. S5), indicating that THAO functions as a C16 oxidase of thalianol in *A. thaliana*. We were unable to detect **T5** to **T7** or **T17** in either Col-0 wild-type or *thas-oe* root extracts, but we did detect two compounds **T9** and **T10**, which are potential isomers of **T7** and **T17** (fig. S5). Compound **T10** was absent in root extracts from mutants of all thalianol cluster genes and *THAA2* (*thaa2-ko* and *thaa2-crispr*), whereas **T9** was still detected in those of *thaa2-ko* and *thaa2-crispr*, suggesting that **T9** and **T10** are downstream products of the thalianol gene cluster and that THAA2 may act after **T9** to form **T10** (Fig. 3, A to C, and fig. S5). Targeted metabolomics analysis also revealed the presence of thalianol-derived medium-chain saturated triterpene fatty acid esters (TFAEs) in the root extracts of Col-0 and *thas-oe*, including thalianyl palmitate (16:0, **T18a**), myristate (14:0, **T18b**), and laurate (12:0, **T18c**) (Fig. 3, A and B). Compounds **T18a** and **T18b** were also detected in the roots of other mutant lines *thah-ko*, *thao-ko*, *thaa1-crispr*, *thaa2-ko*, and *thaa2-crispr* except *thas-ko1* and *thas-ko2*, suggesting the presence of a branched thalianol-derived biosynthetic pathway. The identities of these TFAEs were confirmed with chemical synthesis.

Identification of missing genes for the biosynthesis of thalianin, TFAEs, and arabidin

To identify the missing genes required for the biosynthesis of metabolites **T9** and **T10**, we carried out a genome-wide search for coexpressed candidate biosynthetic genes in the ATTED-II plant coexpression database (http://atted.jp) using the four thalianol cluster genes as baits (*29*) because no biosynthetic genes in or near the gene cluster region appeared to be coexpressed. We selected eight candidates from the top 20 coexpressed genes for functional analysis in *N. benthamiana* (table S2). We
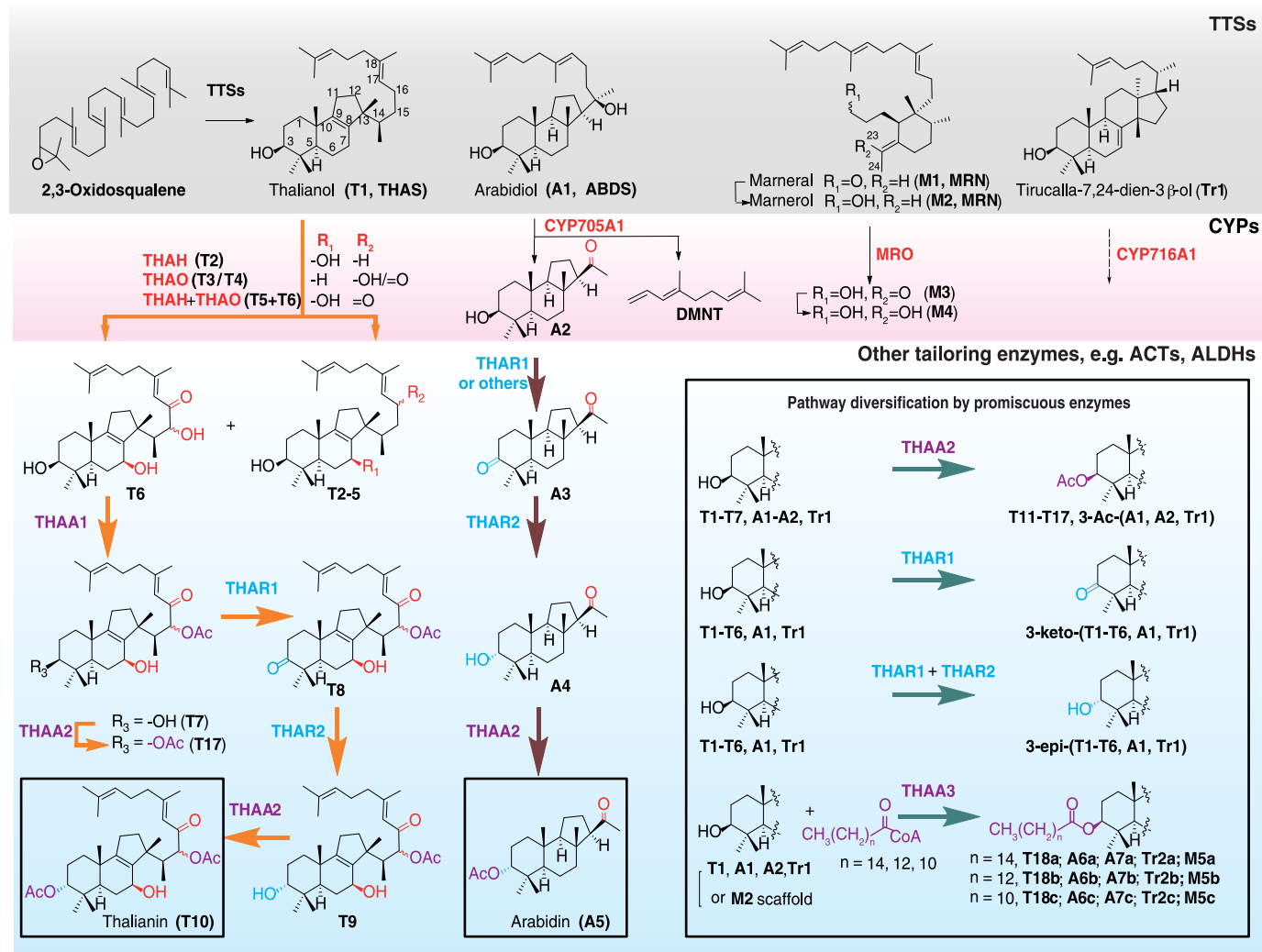
Fig. 2. The triterpene biosynthetic network in *A. thaliana* roots.
Transformations catalyzed by TTSs are shaded gray, clustered CYPs are shaded red, and other tailoring enzymes are shaded blue. Chemical diversification of pathway scaffolds and intermediates by promiscuous acyltransferases and alcohol dehydrogenases is highlighted in the black box, bottom right. DMNT, (*E*)-4,8-dimethyl-1,3,7-nonatriene. Black arrows, known transformations; orange and burgundy red arrows, newly characterized steps in the thalianin and arabidin pathways, respectively; cyan arrows, pathway diversification by promiscuous enzymes; dotted black arrow, unknown transformation. The enzymes for each step are color-coded to match the classes of enzyme shown in Fig. 1A, with the exception that all CYPs are in red.

identified two genes, *At3g29250* (*THAR1*) and *At1g66800* (*THAR2*), that encode a pair of promiscuous oxido-reductases capable of epimerizing the C3 hydroxy moiety of **T1** to **T7** (Figs. 1, A and B, and 2 and fig. S6). In this epimerization sequence, THAR1 converted the C3β hydroxy of **T1** to **T7** into the C3 ketones [3-keto-(**T1**-**T6**) and **T8**], whereas THAR2 reduced the C3-ketones into 3α alcohols [3-epi-(**T1**-**T6**) and **T9**] sequentially (Fig. 2, fig. S6, and table S9). Coexpression of *THAR1* and *THAR2* with the thalianol cluster genes and *THAA2* completed the biosynthesis of **T10** in *N. benthamiana* (fig. S7). The structure of **T10** was established as 7β-hydroxy-16-keto-thalian-3α-15-yl diacetate by means of nuclear magnetic resonance, and **T10** is named thalianin hereafter (table S10).

To identify genes responsible for the biosynthesis of TFAEs **T18a** to **T18c**, we screened seven *O*-acyltransferase genes (table S4) from *A. thaliana* identified based on annotation (*30*) and found that only one of these [*THAA3*, *At3g51970*; previously reported to function in sterol fatty acid ester biosynthesis (*31*)] could catalyze the formation of thalianyl palmitate **T18a** when coexpressed with *THAS* in *N. benthamiana* (fig. S8, A and B). We also generated a *THAA3* overexpression line (*thaa3-oe*) and found that THAA3 could catalyze the formation of **T18a** in planta because elevated levels of **T18a**, but not **T18b** or **T18c**, were detected in this line. *THAA3* is presumably partially redundant because **T18a** was still detected in the *THAA3* mutant (*thaa3-ko*) (fig. S8, C and D). We further showed that THAA3 could catalyze the formation of **T18b** when coexpressed with *THAS* and a characterized chain-length specific 14:0-acyl carrier protein (ACP) thioesterase gene from *Cuphea palustris*, *CpFatB2* in *N. benthamiana* (fig. S8A) (*32*), suggesting that there are as yet unidentified genes encoding chain-length–specific ACP thioesterases involved in TFAE (**T18a** to **T18c**) biosynthesis. Besides thalianol, THAA3 could also act on other triterpenes—including arabidiol **A1** and its derivative **A2**, the PEN3 product **Tr1**, and marnerol **M2**—to introduce a palmityl or myristyl group depending on the type of fatty acyl CoA available (fig. S9). However, these products were not detected in *A. thaliana* Col-0 or *thaa3-oe* roots, possibly because of their very low abundance.

The promiscuity of enzymes encoded by the nonclustered [referred to as peripheral (*14*)] genes—including *THAR1*, *THAR2*, and *THAA2* and their strong coexpression with other divergent triterpene gene clusters in roots (Figs. 1B and 2)—prompted us to perform combinatorial biosynthesis experiments in *N. benthamiana* using these genes and the arabidiol, marneral, and tirucalladienol cluster TTS and CYP genes.
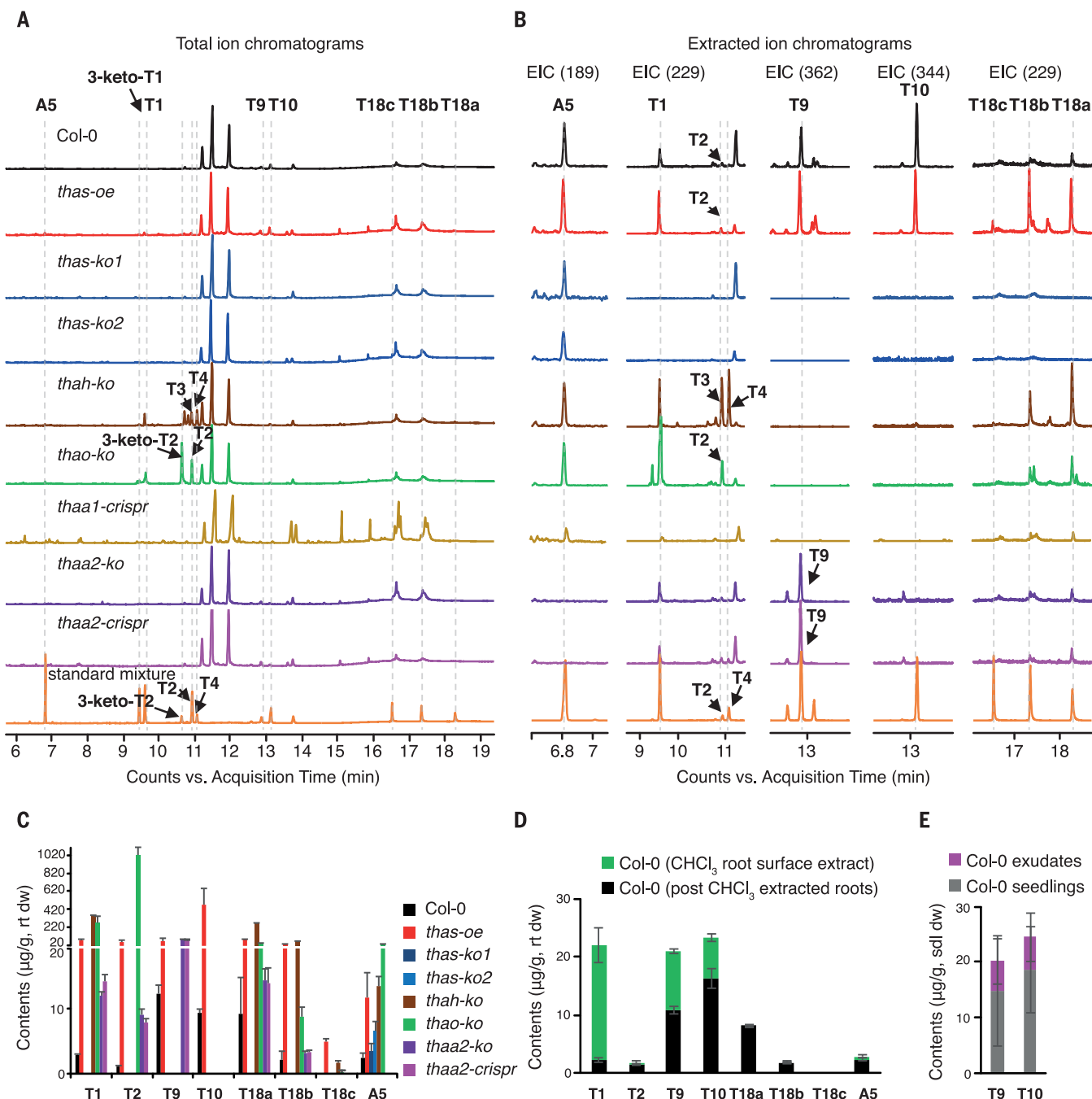
Fig. 3. Metabolite analysis of roots of different *Arabidopsis* lines.
(**A**) Comparative GC-MS total ion chromatograms (TICs) of root extracts of the *A. thaliana* wild-type Col-0 (black), *thas-oe* (red), *thas-ko1* (dark blue), *thas-ko2* (blue), *thah-ko* (brown), *thao-ko* (green), *thaa1-crispr* (dark yellow), *thaa2-ko* (purple), *thaa2-crispr* (pink), and an authentic standard mixture containing **T1**, **3-keto-T1**, **T2**, **3-keto-T2**, **T4**, **T9**, **T10**, **T18a** to **T18c**, and **A5** (orange). (**B**) Comparative extracted ion chromatograms (EICs) from Fig. 2A for characteristic fragments of the mass spectra of **A5** (189), **T1** to **T4** (229), **T9** (362), **T10** (344), and **T18a** to **T18c** (229). (**C**) Triterpene pathway metabolites detected in whole-root extracts from wild-type and mutant lines. Col-0, wild type. *thas-oe*, *thas-ko1*,

thas-ko2, *thah-ko*, *thao-ko*, *thaa2-ko*, and *thaa2-crispr* are mutants for *THAS*, *THAH*, *THAO*, and *THAA2* (supplementary materials, materials and methods). (**D**) Triterpenoids detected in chloroform (CHCl₃) extracts from the surfaces of fresh roots of *A. thaliana* wild-type Col-0 seedlings and subsequent ethyl acetate extracts of dry and powdered chloroform-extracted roots. All lines used in qualitative and quantitative GC-MS analysis (Fig. 2, A to D) were grown on ¼ Murashige-Skoog (MS) agar plates at 22°C (8 hours dark/16 hours light cycle) for 10 days. (**E**) Determination of compounds **T9** and **T10** by LC-MS in root exudates of Col-0 seedlings grown hydroponically in liquid ¼ MS after 10 days. Error bars represent standard deviation of three biological replicates.

Our results showed that THAR1, THAR2, and THAA2 could also act on arabidiol **A1**, 14-apo-arabidiol **A2**, and tirucalladienol **Tr1** (figs. S10 and S11), but not marnerol **M2**, to give the corresponding C3 ketones, 3α-alcohols, and acetates,

respectively. Arabidiol **A1** was fully converted to **A5** when *THAR1*, *THAR2*, and *THAA2* were coexpressed together with *ABDS* and *CYP705A1* (Fig. 2 and fig. S12). **A5** is an *A. thaliana* root metabolite identified through comparative metabolomics analysis of the *A. thaliana* wild type (Col-0) with *ABDS* and *CYP705A1* mutants (*33*). Our results show that *THAR1*, *THAR2*, and *THAA2* encode enzymes that can convert **A2** to **A3**, **A3** to **A4**, and **A4** to **A5**, respectively, reconstituting

the complete biosynthesis of **A5** in *N. benthamiana* (Fig. 2, fig. S12, and table S17).

Metabolite analyses of root extracts of *A. thaliana* T-DNA insertion mutants of *THAR1* (*thar1-ko*), *THAR2* (*thar2-ko*), and *THAA2* (*thaa2-ko*) confirm that these three genes are indeed required for the synthesis of thalianin **T10** in *A. thaliana*. **T10** was absent in root extracts from all three mutant genotypes, and the pathway intermediates **T7** and **T17**, **T8**, and **T9**, respectively, accumulated instead (fig. S13). Additionally, *THAR2* and *THAA2* are also responsible for the biosynthesis of 14-apo-arabi-3α-yl acetate

A5 (referred hereafter as arabidin) because mutants *thar2-ko* and *thaa2-ko* lacked **A5** and instead accumulated **A3** and **A4**, respectively (fig. S14). **A5** was still detected in the root extracts from the *thar1-ko* line, indicating that *THAR1* is partially redundant in 14-apo-arabi-3α-yl acetate **A5** biosynthesis. Thus, these enzymes are involved in multiple pathways.

Triterpene biosynthesis affects *Arabidopsis* root microbiota assembly

Having discovered and reconstituted the complete biosynthetic pathways of thalianin **T10**,

arabidin **A5**, and TFAEs **T18a** and **T18b** in *N. benthamiana*, we sought to investigate the potential biological role of this metabolic network. The thalianin **T10** and arabidin **A5** pathway genes are coexpressed in *A. thaliana* root epidermis and pericycle/stele, respectively (fig. S15), and thalianol pathway compounds **T1**, **T2**, **T9**, **T10**, and arabidin **A5** could be detected in root surface wax extracts and **T9** and **T10** also in exudates of hydroponically grown seedlings (Fig. 3, D and E, and fig. S16). Moreover, both the thalianin and arabidin pathway genes are up-regulated upon methyl jasmonate treatment
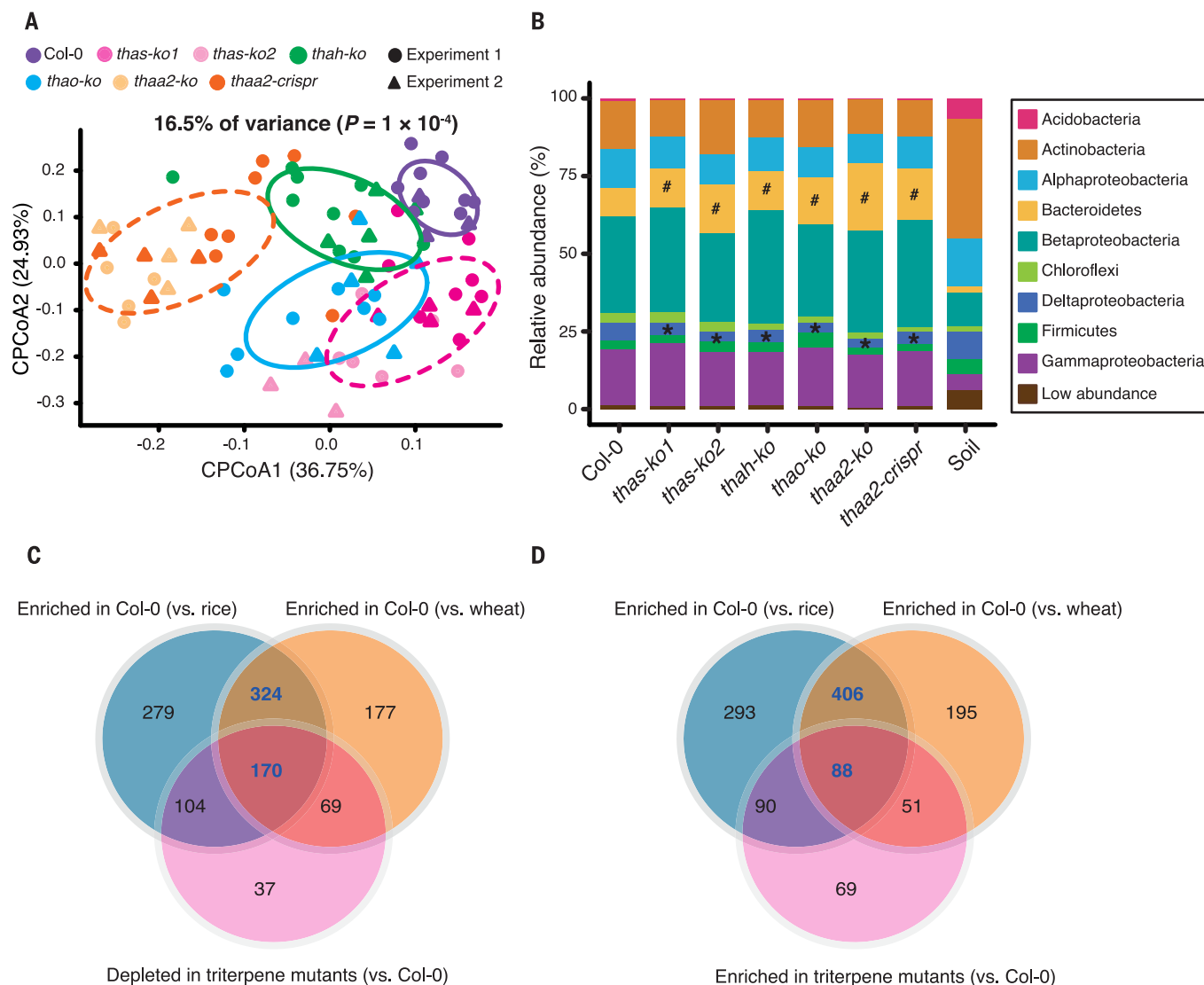

Fig. 4. Modulation of specific root bacterial taxa in triterpene pathway mutants. (**A**) Constrained principal coordinate analysis (CPCoA) of Bray-Curtis dissimilarity showing triterpene mutant effects. Total number of individual plants used for analyses: Col-0 ($n = 12$), *thas-ko1* ($n = 12$), *thas-ko2* ($n = 9$), *thah-ko* ($n = 14$), *thao-ko* ($n = 13$), *thaa2-ko* ($n = 9$), and *thaa2-crispr* ($n = 12$). Biological replicates (individual plants) from two independent experiments (experiment 1 and 2) are indicated with dots and triangles, respectively. Ellipses include 68% of samples from each genotype. (**B**) Phylum distribution of the root microbiota compositions of the tested *A. thaliana* genotypes. Because the relative abundance of Proteobacteria is greater than 50%, bacteria in this phylum are

shown at the class level. Pound sign (#) indicates Bacteroidetes significantly higher than that in Col-0 roots at $P < 0.05$; asterisk indicates Deltaproteobacteria significantly lower than that in Col-0 at $P < 0.05$. (**C** and **D**) Venn diagrams showing substantial overlap of OTUs (C) depleted or (D) enriched in the root microbiota of *A. thaliana* triterpene mutant lines as compared with the wild type (Col-0) (pink circles), compared with those depleted in the root microbiota of rice (blue circles) and wheat (orange circles) versus the *A. thaliana* Col-0 wild type. The OTU numbers specifically enriched in the root microbiota of *A. thaliana* Col-0 compared with rice and wheat are highlighted in blue and bold in the Venn diagram overlaps.

(fig. S17), suggesting that they may have a role in interactions with root microbes (*34*).

We selected mutants disrupted in the thalianin (**T10**), TFAE (**T18a** to **T18c**), and arabidin (**A5**) pathways (*thas-ko1, thas-ko2, thah-ko, thao-ko, thaa2-ko,* and *thaa2-crispr*) along with the wild type (Col-0) for root microbiota analyses (table S2). Comprehensive unbiased untargeted metabolomics analysis of whole-root extracts, root-surface extracts, and root exudates from these mutant lines versus Col-0 suggests that the thalianin pathway compounds are the most affected of all root metabolites analyzed, showing the largest fold changes across the respective metabolomes of the mutants (figs. S18 to S22). We grew these lines in natural soil from Changping Farm in Beijing for 6 weeks under controlled experimental conditions. The roots were harvested and washed with phosphate-buffered saline buffer to remove soil particles and loosely attached microbes before carrying out deep 16S

ribosomal RNA (rRNA) gene sequencing of the root microbiota by using previously established methodology (*3, 4, 35*). There were no obvious differences in root phenotypes between the wild-type (Col-0) and mutant lines when the plants were harvested. However, we found that mutants affected in these pathways assembled different root microbiota when compared with the wild type (Col-0). Constrained principal coordinate analysis (CPCoA) revealed differences in root microbiota between the wild-type (Col-0) and mutant lines (16.5% of total variance was explained by the plant genotypes, $P < 0.001$, permutational multivariate analysis of variance) (Fig. 4A and tables S18 to S22). Pairs of independent mutant lines for *THAS* (*thas-ko1* and *thas-ko2*) and *THAA2* (*thaa2-ko* and *thaa2-cripsr*) each display similar metabolite defects (figs. S18 to S22) and have similar microbiota profiles and microbial diversity (Fig. 4A, fig. S23, and tables S23 to S26). Furthermore, all the pathway mu-

tants tested showed similar root microbiota modulation patterns for Bacteroidetes (enrichment) and Deltaproteobacteria (depletion) compared with the *A. thaliana* Col-0 wild type at the phylum and operational taxonomic unit (OTU) levels (Fig. 4B, figs. S24 and S25, and tables S27 to S44), which is consistent with these genes operating in the same pathway for thalianin biosynthesis. Of the OTUs that were coenriched/codepleted in the mutants, 93% (28 out of 30) showed higher abundance in Col-0 roots than bulk soil, suggesting active selection of root bacteria by the *A. thaliana* plants (fig. S26 and tables S45 and S46). These data indicate that the triterpene biosynthetic network that we have unveiled contributes to root microbiota assembly and establishment.

To understand whether and how this specialized metabolic network might modulate *A. thaliana*–specific root bacteria, we also compared the root bacterial profiles of the *A. thaliana* Col-0 and mutant lines with those from the taxonomically
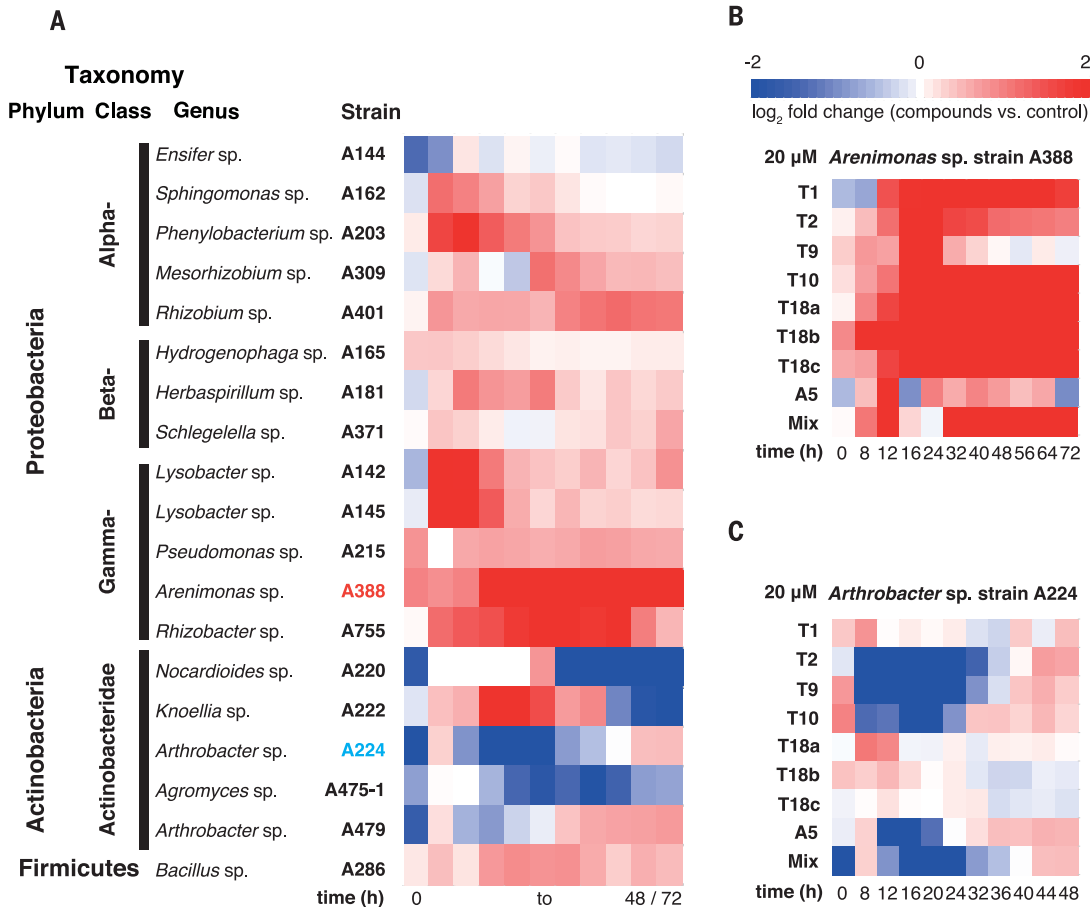
Fig. 5. Effects of pathway metabolites on the growth of isolated *A. thaliana* root–associated bacteria and bacterium-mediated chemical transformations. (**A**) Growth modulation activity of compound mixture **Mix** (10 μM **T1**, 5 μM **T2**, 20 μM **T9**, 20 μM **T10**, 10 μM **T18a**, 5 μM **T18b**, 1 μM **T18c**, and 10 μM **A5**) against the 19 strains of *A. thaliana* root bacteria from different taxa. The heatmap shows log₂ fold change of individual strains treated with **Mix** versus control (ethanol only) over 48 or 72 hours. The corresponding graphical growth curves are depicted in figs. S30 to S34.

(**B**) Heatmap showing the log₂ fold change in cell density (OD₆₀₀) of *Arenimonas* sp. strain A388 treated with eight different purified compounds (**T1, T2, T9, T10, T18a, T18b, T18c,** and **A5,** respectively) at 20 μM and **Mix** over 72 hours. All compounds were dissolved in ethanol. Control, 0 mM, ethanol. (**C**) Heatmap showing the log₂ fold change in cell density (OD₆₀₀) of *Arthrobacter* sp. strain A224 treated with eight different purified compounds (**T1, T2, T9, T10, T18a, T18b, T18c,** and **A5,** respectively) at 20 μM and **Mix** over 48 hours. All compounds were dissolved in ethanol. Control, 0 mM, ethanol.

distant species rice (*36*) and wheat, also previously grown in the soil from Changping Farm on different occasions. Although the *A. thaliana*, rice, and wheat samples differ from each other in many aspects—including growth conditions, germination periods, and climate conditions (supplementary materials, materials and methods)—the starting inocula (soils) are very similar, sharing substantial overlap in total OTUs (67%, 2377 out of 3531) (fig. S27 and tables S47 to S49). We found that of the 494 OTUs specifically enriched in the root microbiota of *A. thaliana* Col-0 compared with rice and wheat (represented in the overlap between the blue and orange circles in Fig. 4, C and D), 34% (170 out of 494) (Fig. 4C and tables S50 to S55) were depleted, and 18% (88 out of 494) (Fig. 4D and tables S50 to S55) enriched in the root microbiota of the triterpene mutant lines compared with those of *A. thaliana* Col-0. Unlike *A. thaliana*, rice and wheat do not make thalianin, TFAEs, or arabidin (fig. S28). Our results suggest that the specialized triterpene biosynthetic network that we have uncovered may contribute to enrichment of around a third of the *A. thaliana*–specific root bacteria present in the roots of the wild-type line (Fig. 4C) while deterring another 18% of this bacterial population (Fig. 4D). The relatively larger number of OTUs depleted in the triterpene mutants (versus Col-0) (a total of 380) (Fig. 4C, pink circle) in comparison with the total number of OTUs that are enriched in the mutant lines versus Col-0 (298) (Fig. 4D, pink circle) further suggests that this triterpene biosynthetic network may play a more

important role in enriching *Arabidopsis*–specific root bacteria rather than repelling other bacteria.

Purified triterpenes selectively modulate root bacteria

To test whether triterpene pathway metabolites directly regulate root microbiota members, we isolated and identified bacteria from the roots of *A. thaliana* Col-0 plants grown in soil from the aforementioned Changping Farm by limiting dilution and barcoded sequencing (*35*). We selected a total of 19 bacterial strains that belong to 17 genera within three major bacterial phyla (Proteobacteria, Actinobacteria, and Firmicutes). These strains shared >97% 16*S* rRNA gene similarity to the OTUs that showed differential abundances in the microbiota of *A. thaliana* wild type (Col-0), mutants, and soil (fig. S29 and table S56). These bacterial strains were grown in liquid culture with a formulated cocktail of purified compounds that reflected the content and composition of the pathway metabolites in the roots of *A. thaliana* Col-0 (figs. S30 to S34). We found that most of the Proteobacteria strains tested proliferated faster in the presence of the *A. thaliana* triterpene mixture, whereas all five Actinobacteria strains were inhibited (Fig. 5A and figs. S30 to S34). The OTUs corresponding to these bacterial isolates (16 out of 19) showed consistent enrichment or depletion patterns in plant roots versus soil that corresponded with the growth promotion and inhibitory effects of the triterpene mixture, suggesting that the compounds tested contribute to the active selection of plant root bacteria (figs. S30 to S34). Moreover, the corresponding enrichment or depletion patterns for 10 bacterial genera (59% of the 17 tested) to which the metabolite-sensitive bacterial isolates belong were detected in the microbiota of at least one pathway mutant included in our microbiota analysis; the genera of bacteria for which growth is either promoted or inhibited by the triterpene cocktail mixture are depleted or enriched, respectively, in the pathway mutants compared with the wild type (Col-0) (fig. S35 and tables S57 to S63). Further tests of the sensitive strains with purified individual compounds revealed that pathway metabolites can selectively modulate the growth of bacteria and that small structural differences between compounds can affect activities (fig. S36). For example, we found that Actinobacteria *Arthrobacter* sp. strain A224 was inhibited by compounds **T2**, **T9**, **T10**, and **A5** at 20 μM but not by the other triterpenes tested, whereas all compounds tested showed growth promoting effects on Gammaproteobacteira *Arenimonas* sp. strain A388 (Fig. 5, B and C). We also found that strain A475-1 (*Agromyces* sp.) has alcohol dehydrogenase activity and could selectively convert **T2** into **3-keto-T2** but not **T9/T10** (Fig. 6, A and B, and fig. S37A), whereas strain A215 (*Pseudomonas* sp.) has lipase activity and is able to cleave the TFAEs **T18a** to **T18c** to give **T1** and the corresponding fatty acids but not the acetate **T11** (Fig. 6, C and D, and fig. S37, B to D). Moreover, strain A215 could use the cleavage product palmitic acid as a carbon source for proliferation (Fig. 6E). Such diverse and substantial
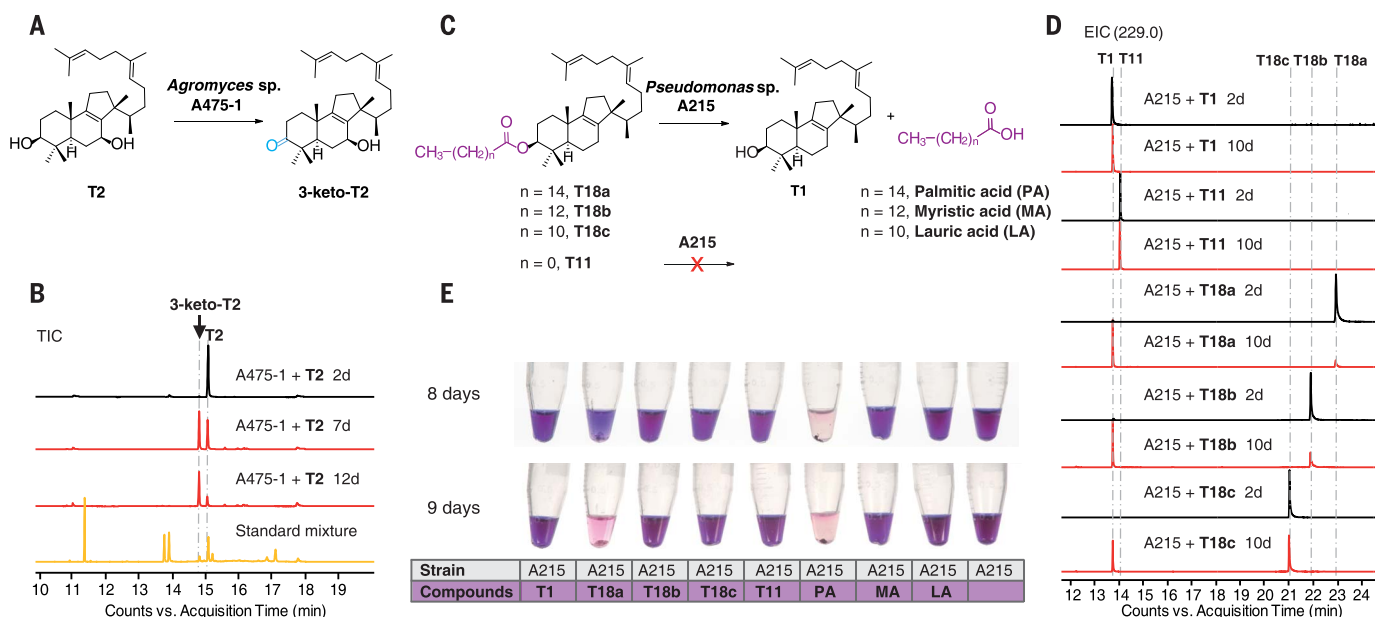
Fig. 6. Bacterium-mediated transformations of triterpenes. (**A**) Conversion of thaliandiol **T2** to **3-keto-T2** by *Agromyces* sp. strain A475-1 as shown by means of (**B**) comparative GC-MS TICs of EtOAc extracts of bacterial cultures supplemented with **T2** on the 2nd, 7th and, 10th day, respectively, with the standard mixture described in Fig. 3A. (**C**) Selective conversion of TFAEs **T18a** to **T18c** but not **T11** to **T1** by *Pseudomonas* sp. strain A215 as shown by means of (**D**) comparative GC-MS EICs [mass/charge ratio (*m/z*) = 229] of EtOAc extracts of bacterial strain A215 cultures supplemented with **T1**, **T11**, and **T18a** to **T18c** on the 2nd and 10th day, respectively. (**E**) *Pseudomonas* sp. strain A215 could use palmitic acid as carbon source, as shown by the proliferation of A215 in minimal salt media supplemented with 0.5 mM (in ethanol) of **T18a** or palmitic acid (**PA**) but not in those with **T1**, **T18b**, **T18c**, **T11**, myristic acid (**MA**), lauric acid (**LA**), and control. The minimal salt media contain 0.3 mg/ml resazurin as indicator of bacterial growth (from purple blue to pink).

interaction patterns of root metabolites with taxonomically distinct root microbiota members suggest that the metabolites from this biosynthetic network alter the assembly of *A. thaliana* root microbiota. Taking the results of the root microbiota sequencing and in vitro bioassays together, we conclude that this triterpene biosynthetic network tunes the ecological niche for the assembly and maintenance of *A. thaliana* root microbiota.

Conclusion

The triterpene biosynthetic network described here has a latent capacity for the synthesis of more than 50 root metabolites (Fig. 2). This is a relatively large number considering the total of ~300 nonvolatile root metabolites that we detected in the polar methanolic (~220) and non-polar ethyl acetate extracts (~85) of *A. thaliana* Col-0 roots under our experimental conditions (tables S64 and 65). This network originates from evolutionary divergent biosynthetic gene clusters coupled with cross-talk that involves enzymes encoded by peripheral genes that service multiple biosynthetic pathways. These root metabolites can selectively regulate the growth of *Arabidopsis* root bacteria from different taxa by acting as antibiotics or proliferating agents. Biosynthesis of these specialized root triterpenes dynamically modulate a large portion [52%, 258 (170+88) out of 498 OTUs] (Fig. 4, C and D) of the *A. thaliana*–specific bacteria, shaping the *A. thaliana*–specific root microbiota. Triterpenes, a large and diverse groups of plant natural products (>20,000 reported so far) (*12*), likely also sculpt the microbiota of other plant species, tailoring them to generate plant species–specific microbial communities, and may be useful for engineering root microbiota for sustainable agriculture (*37–39*). It is tempting to speculate that metabolic diversification in the plant kingdom may provide a basis for communication and recognition that enables the shaping of microbial communities tailored to the needs of the host, and that this may in part explain the existence of plant-specialized metabolism.

Materials and methods

Detailed materials and methods can be found in the supplementary materials.

Plant materials

Arabidopsis thaliana accession Columbia (Col-0) was used as wild type in this study. All *A. thaliana* T-DNA insertion mutants were obtained from Nottingham *Arabidopsis* Stock Centre (NASC) unless otherwise stated. Homozygous mutants were identified by PCR-based genotyping using the primers listed in the table S5A. A 35S promoter-driven overexpression line of thalianol synthase (*thas-oe*) was generated previously (*15*). Overexpression lines for *THAA3* were generated by transforming *A. thaliana* Col-0 plants with *Agrobacterium tumefaciens* strain LBA4404 harboring the expression vector pMDC32 which contains the coding sequence of *THAA3* (*At3g51970*) under the control of the 35S promoter

via floral dipping (*40*). Homozygous CRISPR knockout lines of *THAA1* (*At5g47980*) and *THAA2* (*At5g47950*) were generated by transforming *A. thaliana* Col-0 wild type with *A. tumefaciens* C58C1 harboring CRISPR/Cas9 constructs with specific single guide RNAs as previously described (*41*).

Cloning and transient expression

The coding sequences for *THAS* (*At5g48010*), *THAH* (*At5g48000*), *THAO* (*At5g47990*), *THAA1* (*At5g47980*) and *THAA2* (*At5g47950*), *THAR1* (*At3g29250*), *THAR2* (*At1g66800*), *THAA3* (*At3g51970*), *ABDS* (*At4g15340*), *CYP705A1* (*At4g15330*), *At1g14960*, *At5g23840*, *At2g16005*, *At5g38030*, *At1g50560*, *At5g38020*, *MRO* (*At5g42580*), *CYP705A12* (*At5g42590*), *MRN* (*At5g42600*), *At5g12420*, *At5g55380*, *At3g49210*, *At1g54570*, *At3g49190*, *At3g26840* were amplified from a root cDNA library of the *A. thaliana* Col-0 accession (*26*). The coding sequences for *PEN3* (*At5g36150*), *CYP716A1* (*At5g36110*) and the truncated HMG CoA reductase (tHMGR) gene from oat had been cloned previously and were included in this study (*18, 27*). The coding sequence of *CpFatB2* from *Cuphea palustris* (accession no. U38189) was retrieved from NCBI and synthesized by Integrated DNA Technologies. These sequences were cloned into the pEAQ-HT expression vector for transient expression in *N. benthamiana* leaves as detailed in the supplementary materials.

Metabolite extraction and analysis

N. benthamiana leaves from transient expression experiments were harvested 5 days post infiltration, lyophilized, extracted with EtOAc and analyzed by means of gas chromatography–mass spectrometry (GC-MS). Seedlings of *A. thaliana* Col-0 and mutants, rice and wheat were grown on Murashige-Skoog (¼ MS) agar plates or liquid media at 22°C under short day conditions (8 hours light/16 hours dark) for 10 days. Roots were harvested from 10-day-old seedlings grown on agar plates, lyophilized, extracted with EtOAc and analyzed by GC-MS or extracted with MeOH and analyzed by means of liquid chromatography–MS (LC-MS). Spent media and whole seedlings of 10-day-old *A. thaliana* grown in ¼ MS liquid media were harvested separately, lyophilized, extracted with MeOH and analyzed by LC-MS. Targeted and untargeted metabolomics analysis of GC-MS and LC-MS data were performed using Agilent MassHunter and XC-MS, respectively.

Production and isolation of thalianol and arabidiol derived metabolites

Compounds **T1-T10**, **3-keto-T2**, **T17**, *cis*-**T10**, *cis*-**T17**, **A1**, **A2**, and **A4** were obtained by large-scale vacuum infiltration of *N. benthamiana* leaves with *A. tumefaciens* LBA4404 strains harboring the corresponding expression constructs followed by extraction and purification. Compounds **3-keto-T1**, **T11**, **T18a-c**, **A3**, and **A5** were chemically synthesized from the corresponding precursors.

Microbiota analysis

A. thaliana plants (Col-0 and triterpene mutants) were grown under controlled short-day conditions in the natural soil from Changping Farm (40°5′49″N, 116°24′44″ E, Beijing, China). Two wheat (Xiaoyan54 and Jing411) and rice (IR24 and Nipponbare) varieties were grown in Changping Farm under field conditions in 2016 and 2017, respectively. Roots from *A. thaliana*, wheat and rice were harvested six weeks post plantation in natural soil for 16S rRNA gene profiling. Data analysis was performed using QIIME 1.9.1 (*42*), USEARCH 10.0 (*43*) and in-house scripts. OTUs with differential abundance were identified with a negative binomial generalized linear model in the edgeR package with a fold change threshold >1.2 (*20*). Venn diagrams were generated using the Venndiagram package (*22*).

In vitro bioassays

Root-associated bacteria were isolated and identified from *A. thaliana* Col-0 plants grown in the aforementioned natural soil from Changping Farm with limiting dilution and barcoded sequencing (*35*). 19 bacterial isolates from diverse taxa that share greater than 97% 16*S* rRNA gene similarity with representative OTU sequences were selected for bioassay. The bacteria were cocultured with different formulated cocktails of triterpene mixtures or individual triterpenes in TSB media and their growth (OD$_{600}$) monitored over 48 to 72 hours. Bacteria-mediated transformation of triterpenes **T1**, **T11**, and **T18a** to **T18c** was tested against all 19 bacteria, whereas **T2**, **T9**, and **T10** against sensitive bacterial strains A224, A475-1, and A479.

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SUPPLEMENTARY MATERIALS

Science

A specialized metabolic network selectively modulates *Arabidopsis* root microbiota

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Mix of metabolites tunes root microbiota

Uncharacterized biosynthetic genes in plant genomes suggest that plants make a plethora of specialized metabolites. Huang *et al.* reconstructed three biosynthetic networks from the small mustard plant *Arabidopsis thaliana*. Promiscuous acyltransferases and dehydrogenases contributed to metabolite diversification. The plant may use these specialized metabolites to modulate the microbiota surrounding its roots. Disruption of the pathways and intervention with purified compounds caused changes in the root microbiota.
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