The impact of defense hormones on the interaction between plants and the soil microbial community

Zhang, J.

Chapter 3

Activation of the SA-associated plant defense pathway alters the composition of soil bacterial communities

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Abstract

Many plant species grow better in sterilized than in live soil. Foliar application of SA mitigated this negative effect of live soil on the growth of the plant *Jacobaea vulgaris*, as described in Chapter 2. This "SA-effect" on plant growth in live soils did not change over further cycles (generations), neither did the negative effect of live soils. To examine what causes the positive effect of SA application on plant growth in live soils, in this chapter we analyzed the effects of SA application on the composition of active rhizosphere bacteria in the live soil and how this change over time using RNA sequencing of the microbial communities in the rhizosphere of *Jacobaea vulgaris*. Our study shows that the composition of the rhizosphere bacterial communities of *J. vulgaris* greatly differed among generations. Application of SA resulted in both increases and decreases in a number of bacterial genera in the rhizosphere soil, but the genera that were affected by the treatment differed among generations. In the first generation, there were no genera that were significantly affected by the SA treatment, indicating that induction of the SA defense pathway in plants does not lead to immediate changes in the soil microbial community. 89 species out of the total 270 (32.4%) were present in all generations in all soils of SA-treated and control plants suggesting that these make up the "core" microbiome. On average in each generation, 72.9% of all genera were present in both soils. Application of SA to plants significantly up-regulated genera of *Caballeronia*, unclassified *Cytophagaceae, Crinalium* and *Candidatus Thermofonsia Clade* 2, and down-regulated genera of *Thermomicrobiales*, unclassified *Rhodobacterales*, *Paracoccus* and *Flavihumibacter*. While the functions of many of these bacteria are poorly understood, bacteria of the genus *Caballeronia* play an important role in fixing nitrogen and promoting plant growth, and hence this suggests that activation of the SA signaling pathway in *J. vulgaris* plants may select for bacterial genera that are beneficial to the plant. Further studies should examine how activation of the SA signaling pathway in the plant changes the functional genes of the rhizosphere soil bacterial community. Overall, our study shows that aboveground activation of defenses in the plant affects soil

microbial communities and as soil microbes can greatly influence plant performance, this implies that induction of plant defenses, can lead to complex above-belowground feedbacks.

Keywords

Metatranscriptomics, Soil microbial community, Taxonomy, Plant-soil interactions, Microbial diversity, Rhizosphere soil, Salicylic acid

Introduction

Plants encounter a myriad of threats from the surrounding environment, including both abiotic and biotic stresses (Suzuki et al., 2014). Biotic stresses are mostly due to herbivory and pathogen infestation both below- and above-ground (Pieterse and Dicke, 2007; Adair and Douglas, 2017). Microbes in the soil can have a beneficial, pathogenic or neutral effect on the host plant. For example, soil bacteria such as *Rhizoctonia species*, often strongly negative affect plant growth and survival (Issac et al., 1971). On the other hand, plant growth-promoting rhizobacteria (PGPR), such as *Pseudomonas* and *Burkholderia* species are beneficial for the plant, e.g. via suppressing the growth of soil-borne pathogens or increasing nutrient availability (Bhattacharyya and Jha, 2012). However, the overall net effect of soil microbial communities on plant growth is often negative (Nijjer et al., 2007). Most plant species grow less well in soils that contain a natural microbial community than in sterilized soils. This might be due e.g. competition between plants and microbes for available nutrients or due to soil-borne plant pathogens (Callaway et al., 2004; Berendsen et al., 2012; Mazzoleni et al., 2015; Cesarano et al., 2017).

Systemic acquired resistance (SAR) is one of the most common defensive strategies of plants against biotrophic pathogenic microbes. Foliar application of salicylic acid to plant tissues can activate SAR and boost the innate immune system of a plant (Reymond and Farmer, 1998). Cultivars with a higher sensitivity to SA are often better defended against the pathogens. For example, in tomato, exogenous application of SA can be effective against the pathogens *Oidium neolycopersici* and *Botrytis cinerea*, which cause powdery mildew and grey mould diseases (Seskar et al., 1998; Achuo et al., 2004). In agriculture, application of SA is now used to suppress pathogenic microbial effects in e.g. tomato, pepper and pea crops (Esmailzadeh et al., 2008; Barilli et al., 2010; Choi and Hwang, 2011). How SA application to the plant affects the microbial community in the soil is not fully uncovered.

Because plants alter the composition of the microbial community in the soil in which they grow, and SAR protects plants against pathogens, an important question is how activation of SAR alters the plant's effect on the soil microbial community. Several studies have demonstrated that the activation of SAR indeed altered the composition

of soil microbial community and that SA can play a key role as a regulator in shaping root bacterial communities (Kniskern et al., 2007; Hein et al., 2008; Lebeis et al., 2015). However, several other studies reported that foliar application of SA did not affect the bacterial composition in the soil (Doornbos et al., 2011; Wang et al., 2015). These studies on the effects of SAR on soil bacterial composition were mostly limited to the model plant species *Arabidopsis.* As plant species differ greatly in the way and magnitude in which they influence the soil bacterial community (Wubs and Bezemer, 2018; Hannula et al., 2019; Pineda et al., 2020), we may expect that the effects of SA application on the soil microbial community also differ among plant species.

Several studies have shown that the composition of the soil bacterial microbial community varies greatly over time (e.g. Hannula et al., 2019). In a study on temporal variation in three land-use types, the number of taxa present in the soil showed strong temporal variability, and these changes over time were considerably larger than the variation associated with land-use types (Lauder et al., 2013). In contrast, Shade et al. (2012) demonstrated that soil microbial communities have clear successional trajectories. If generally true this would imply that application of SA to plants could also cause directed changes in the soil microbial community over time. An important question is therefore how activation of SAR will alter the soil microbial community over time.

In Chapter 2, we showed that inoculation of a sterilized soil with natural, live soil, reduced plant growth in comparison with that in sterilized soil for the plant species *Jacobaea vulgaris*. Interestingly, applying SA to the leaves mitigated these negative effects. This implies that activation of SA-induced resistance may potentially suppress microbial pathogens present in live soil. If this is the case, an important question is whether the repeated foliar application of SA during consecutive generations of plant growth will increase this effect and hence, whether there is a selection for a more beneficial bacterial community. Conceptually, the temporal dynamics of foliar application of SA can follow different trajectories (Fig. 1). First, it is possible that both foliar application of SA and the effect of different generations do not alter the soil bacterial composition (Fig. 1-i). Second, foliar application of SA may lead to different bacterial communities independent of time (Fig. 1-ii). Third, bacterial communities may differ among generations but are not influenced by the SA

application (Fig. 1-iii). Fourth, foliar application of SA may influence bacterial communities but these effects may differ among generations (Fig. 1-iv).

In this study, we sequenced the mRNA from rhizosphere soil samples of both SA-treated and control plants during four consecutive generations of growth of *J. vulgaris*. In each consecutive generation soil from the previous plant growth period was used. Using mRNA instead of DNA or rRNA enabled us to focus on the active soil microbial community (Gilbert et al., 2008). In this study, we focus on the bacterial community. Twenty-four rhizosphere soils were sequenced with an Illumina sequencing platform. The goal of this study is to answer the following questions: (1) How does the foliar application of SA in *J. vulgaris* affect the bacterial composition in the rhizosphere and is there a time effect or an interactive time x SA effect on the bacterial community? (2) What is the "core" bacterial community in the soils of plants exposed to the SA treatment and of control plants? (3) How does the application of SA influence the bacterial community in each generation? Are the SA effects consistent over time?

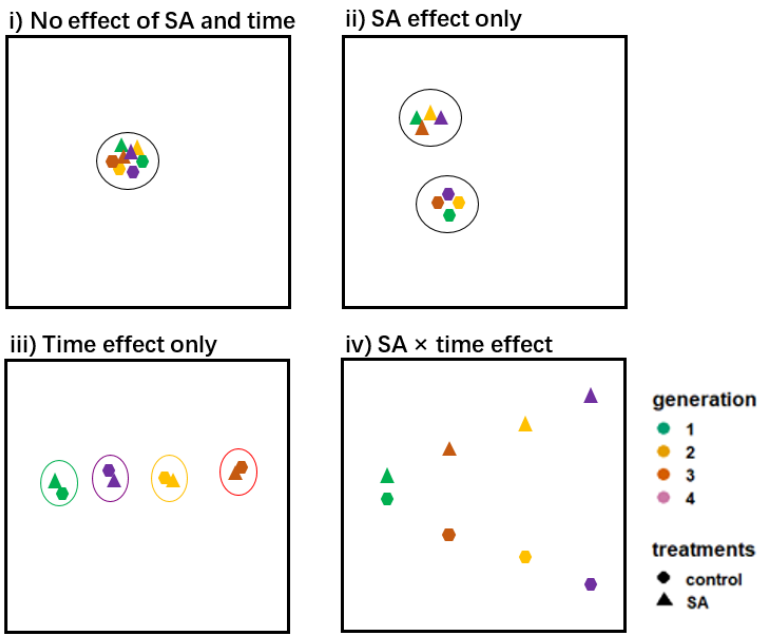

Fig. 1 Conceptual figure showing the potential effects of SA application on *J. vulgaris* over four generations.
i) No effect of SA and time. The bacterial community does not differ between SA-treated plants and control, and does not differ over time. ii) SA effect only. The bacterial community is affected by SA application but the effect does not differ over time. iii) Time effect only. The bacterial community changes over time, but is not affected by the SA treatment. iv) SA × time effect. The bacterial community is affected by SA-application but these effects differ among generations.

Materials and methods

Multi-generational plant growth experiment

In Chapter 2, we report the effects of foliar application of SA on plant growth in inoculated and sterilized soils. The current chapter focuses on the effect of foliar SA application on the composition of the bacterial community in the rhizosphere in the inoculated soil. Details of the experiment are described below.

J. vulgaris (common ragwort) seeds were collected at the dunes of Meijendel (a calcareous sandy area from a coastal dune area north of The Hague, The Netherlands, 52°11´N, 4°31´E). Prior to germination, all seeds were surface sterilized (shaken for 2 min in 70% ethanol, then rinsed with sterilized water, put for 12 min in 2% bleach, and then rinsed again four times with sterilized water to minimize influences of seed-borne microbes (Bakker et al., 2015). The soil was also collected at Meijendel. The topsoil was collected to a depth of 15 cm after removing the grassland vegetation and the organic layer of the surface. The soil was sieved using a 5 mm sized mesh, homogenized with a concrete mixer, and then stored into 20-liter plastic bags (Nasco Whirl-Pak Sample Bag). Bags were either sterilized by 35-K Gray gamma-irradiation (Synergy Health Company, Ede, The Netherlands) or kept at 4°C for inoculation.

Surface sterilized seeds were germinated in sterile Petri dishes on filter paper. After one week, seedlings were randomly transferred individually to 500 ml pots consisting of a mixture of 90% sterilized soil and 10% live soil. Prior to potting but after mixing, the soil was kept in bags and left in the climate room for 14 days so that the mixed

soil could settle and microbial communities could colonize the sterilized soil. After potting the seedlings, pots were randomly distributed over a climate room (relative humidity 70%, light 16h at 20°C, dark 8h at 20°C). Plants were watered regularly with Milli-Q and 5 ml Steiner nutrient solution was added per plant on day 7 after planting, 10 ml Steiner nutrient solution (Steiner, 1979) was added on day 13, and 20 ml Steiner nutrient solution was added on days 19, 28, 37, 42. The Steiner nutrient solution was prepared from 7 different stock solutions (106.2 g $Ca(NO_3)_2 \cdot 4H_2O$, 29.3 g KNO_3, 13.6 g KH_2PO_4, 49.2 g $MgSO_4 \cdot 7H_2O$, 25.2 g K_2SO_4 and 2.24 g KOH, 3.29 g Fe-EDTA added to 1 liter demineralized water, and a stock solution with micro elements (a mixed solution of 0.181 g $MnCl_2 \cdot 4H_2O$, 0.286 g H_3BO_3, 0.022 g $ZnSO_4 \cdot 7H_2O$, 0.0078 g $CuSO_4 \cdot 5H_2O$ and 0.0126 g $NaMoO_4 \cdot 2H_2O$ added to 1 liter demineralized water). Ten ml of each stock solution was diluted in 1 liter of demineralized water before use.

Plants were allocated to either a hormonal treatment (SA) or served as control (only solvent). Both treatments were replicated 10 times. During plant growth, the phytohormone SA was applied through foliar application three times a week for four consecutive weeks. The first application was given when plants were 14 days old. About 0.75 ml of 100 µM SA was sprayed on the leaves while carefully avoiding spillover to the soil. One week later the treatment was repeated with 1.50 ml of SA. In the next week, the treatment was repeated with 2.25 ml of SA. SA solvent (purchased from Sigma-Aldrich, ≥99.0%) was made by dissolving 6.91 mg in 69.10 µl of ethanol. Milli-Q water was then added until a final volume of 500 ml. Control plants were sprayed with sterile water with the same solvent (ethanol in Milli-Q water).

After six weeks, plants were gently removed from the pots. The rhizosphere soil for each treatment was harvested for each pot individually by gently shaking three times to remove the loosely adhering soil, after which rhizosphere soil samples were collected onto a sterile filter paper by removing the remnant soil with a fine sterilized brush. Rhizosphere soil samples were put in a 2 ml Eppendorf tube and stored at -80°C for further RNA extraction. The remaining rhizosphere soil and adhering soil of the ten pots were mixed and used as inoculum (live soil) for the next generational of plant growth. The inoculum soil (10%) was mixed with 90% sterilized soil.

The set-up was repeated for another three generations under the same conditions as described above so that there were four generations of plant growth. For the second, third, and fourth generation, the soil inoculum was derived from the previous generation from the same treatment and was a mixture of rhizosphere soil and the loosen adhering soil surrounding the roots. Again, after mixing, the soil was kept in bags and left in the climate room for 14 days. Hereafter, pots were filled with soil and a *J. vulgaris* seedling was planted into each pot. All replicate soils from the SA or control treatment were mixed before the inoculation. The SA treatment was carried out as described above in each generation. Fifty-four days after planting, all plants were harvested each time.

RNA extraction and metatranscriptomic sequencing

For each treatment, the three successively labeled samples (No. 1, 2, 3, No. 4, 5, 6 and No. 7, 8, 9) were mixed and used as one composed replicate, Hence, three replicates were generated and used for RNA extraction for each treatment in each generation and a total of 24 soil samples were used for RNA extraction (3 replicates x 2 treatments x 4 generations). Total RNA was extracted with the RNeasy PowerSoil Total RNA kit (Qiagen). RNA concentration and quality were assessed by running 1µl of the extracted raw RNA on the 4200 TapeStation (Agilent). Subsequently, unwanted DNA, salts and buffers were removed with the RNeasy minElute Cleanup Kit (Qiagen). Later, the Ribo-Zero Magnetic kit for bacteria (Illumina) was used for mRNA enrichment. In the end, a RNA Clean & Concentrator kit (Zymoresearch) was used to clean additional buffers and proteins of the rRNA-depleted RNA. All the steps in extracting and cleaning RNA were according to the supplier's instructions. Double-stranded cDNA was generated from the cleaned RNA obtained in the final step. Library preparation (Illumina Nextera XT DNA library), processing and sequencing were performed by FG Technologies (Leiden, The Netherlands) with paired-end (PE) 150 bp templates. Twenty-four metatranscriptomic libraries were generated, the size of each library was indicated in Table S1 and Fig. S1.

Bioinformatics processing

Trimmomatic 0.39 was used for the removal of adapters of paired-end raw reads (Bolger et al., 2014). FastQC was applied to check the qualities, the bases with a threshold lower than 30 were cut off with Trimmomatic (Andrews, 2010). Ribosomal RNAs of all 24 metatranscriptomic libraries were filtered with SortMeRNA (Sorting ribosomal RNA) (Kopylova et al., 2012). Eight rRNA representative databases (silva-bac-16s-id90, silva-arc-16s-id95, silva-euk-18s-id95, silva-bac-23s-id98, silva-arc-23s-id98, silva-euk-28s-id98 rfam-5s-id98, rfam-5.8s-id98) were derived from the SILVA SSU and LSU databases (release 119) and the RFAM databases with HMMER 3.1b1 and SumaClust v1.0.00 were used for fast filtering of rRNA from eukaryote, prokaryote and archaea. Then, all reads of the 24 metatranscriptomic libraries were combined into one set, which was the input of a de novo assembly. Trinity with default parameters was used for the metatranscriptomic assembly (Haas et al., 2013). Later, the quality of assembled contigs was assessed with Trinity scripts. The CD-HIT-EST algorithm was used to remove the duplicates of each transcript and reads with shorter than 300 bps were removed with a homemade script (Li and Godzik, 2006), after which reads of each library were mapped back to transcriptome with Bowtie2 (Langmead and Salzberg, 2012). The isoform IDs per sample were extracted with Seqkit (Shen et al., 2016). Contigs of each sample were generated and then aligned against the NCBI NR (non-redundant) database by DIAMOND with a cut off e-value at 1e-5 (Buchfink et al., 2015). The closest match with an identity higher than 80% was kept for mapping. The output file of Blastx was further analyzed with the lowest common ancestor (LCA) algorithm in MEGAN (version 6.0) with all default parameters (Camon et al., 2005; Huson et al., 2016). MEGAN helped to compute and explore our data at different taxonomic levels and in this process NCBI taxonomy was employed for summarizing and outputting results, the detailed workflow is referred to Huson et al. (2007). A count table of microbial species was obtained with read counts assigned directly to taxon for the 24 samples. The number of assigned reads per taxa was extracted at species, genus, family and phylum levels respectively. The number of identified phyla, families, genera and species were counted, and the composition and the percentage of reads used for each classification level were calculated.

Statistical analyses

Differences in the numbers of the total reads and the numbers of the non-rRNA reads over four generations were presented as mean ± SD. A Heinrich's triangle figure was generated to visualize microbial composition at different phylogenetic levels of all the identified microbes from the 24 rhizosphere soil samples. Log10 transformed hit numbers of each genus were plotted as a function of ranked genus abundance numbers including all species and a cut-off was performed with an abundance larger than 0.01% of the total reads. A Shapiro-Wilk test was used to test for differences between the distribution of abundance between the SA and control treatment.

The Shannon-diversity index was calculated for the 24 samples and differences between the Shannon-diversity of soils of SA treated plants and soils of control plants were tested with a student t-test. Subsequently, abundance at genus level was used for to construct NMDS (nonmetric multidimensional scaling), PCA (Principal component analysis), OPLS-DA (orthogonal projection to latent structures discriminant analysis), and Venn diagrams, and Pearson distance and the Ward clustering algorithm statistical analysis was calculated since most of the reads were identified at the genus level.

Two-factor Venn diagrams were constructed to illustrate the numbers of unique and common genera in soil samples within each generation for the SA and control treatments, and a four-factor Venn diagram including all generations was performed for the SA and the control treatment separately (Heberle et al., 2015).

PCA and OPLS-DA were performed with SIMACA 15.0 using relative abundance at genus level. The relative abundance was calculated using the absolute abundance number of one genus divided by the total abundance of all bacterial genera in the sample. Before performing OPLS-DA analysis, we checked that our data fitted the model with a cross-validated residual (CV)-ANOVA significance testing ($n = 270$, $P < 0.02$).

To visualize the compositional changes among different treatment and time categories, a NMDS using the Bray-Curtis index as a measure of dissimilarity was generated

using relative abundances. To verify changes in composition due to the SA treatment and time effect, a PERMANOVA test was performed using the *Adonis* function (number of permutations = 999) in R within the "vegan" package.

Local "immigration" and "extinction" in the rhizosphere soil of SA-treated or control plants over generations at genus level was calculated and the numbers were presented in Venn diagram. A Student's t-test was used to identify bacterial genera that were significantly enriched in soil samples of SA-treated or control plants. *P* values were adjusted for false discovery rates (FDR).

Spearman's rank correlation without multiple comparison tests were performed to identify the genera that were significantly positively or negatively correlated with generation within the SA or control treatment. Genera with *P* values smaller than 0.05 were selected to create a heatmap for all the 24 samples. Hierarchical clustering analysis was done for the 24 samples together, based on the relative abundance to show the similarity. The row-centered relative abundance of each genus was used to construct the color key (Chong et al., 2018). Heatmaps for only SA and only control treatments were generated in addition.

Results

Metatranscriptomic sequence data

A total of 898.4 million raw sequence reads were obtained from the 24 metatranscriptomic libraries, the smallest and largest library contained 25.0 and 52.0 million raw sequence reads, respectively (supplementary data Table S1). 846.9 million reads were kept after removing adapters and quality filtering control with FastQC. In total, 775.3 million reads were removed with the SortMeRNA program as ribosomal RNA (rRNA) reads when aligning them against eight rRNA representative databases (silva-bac-16s-id90, silva-arc-16s-id95, silva-euk-18s-id95, silva-bac-23s-id98, silva-arc-23s-id98, silva-euk-28s-id98 rfam-5s-id98, rfam-5.8s-id98), and 71.6 million reads were used as non-rRNA reads for further de novo assembly with Trinity (Fig. S1), of which the smallest library contained 1.5 million reads and the largest library 5.9 million reads. Reads for de novo assembly were normalized with Trinity

in silico normalization algorithm. The average guanine-cytosine (GC) content for the 24 libraries was 60.10%. After assembly, 0.99 million contigs were removed because their length was shorter than 300 bps. A total of 1.3 million unique contigs were identified after removing duplicates with CD-HIT-EST. In total, 392.4 million bases were assembled. After we checked the quality of the contigs in all samples by realigning all contigs back to the assemblies using Bowtie2, the average mapping rate for proper pairs was 45.41%.

Overview of the assigned reads at differential microbial classification levels

When we aligned the 1.3 million unique contigs against the NR (non-redundant) database with DIAMOND and MEGAN 6.0, 0.39 million contigs were taxonomically classified, while the others did provide a match with the available taxonomic information. Based on the analysis in MEGAN, the identified contigs were assigned at different classification levels. 22 different bacterial phyla were identified, 283 families and 382 bacterial genera and 1081 bacterial species (Fig. S2). At the phylum, family, genus and species level 23.4%, 23.4%, 20.4% and 14.9% of the total number of contigs were assigned, respectively. Bacteria were the most prevalent in the microbial community taking up 98.3 % of the total number of reads (Fig. S3a). Eukaryotes, with algae taking the largest proportion, were the second dominant, but Eukaryotes only covered 1.5% of the total number of reads (Fig. S3b).

SA application and time effects on bacterial community diversity and composition

From the total of 408 bacterial genera, 270 genera were included in the analysis (contigs with more than 0.01% of the total number of reads Fig. S5). The genera in both soils showed significantly different abundance curves (Shapiro-Wilk test, df = 407, $P < 0.0001$; Fig. S5), the abundance curve in the SA soil is lower than that in the control soil. Application of SA did not significantly increase or decrease the Shannon diversity at genus level within each generation (t-test for the 1st generation: t = -0.63, df = 5, P = 0.27; 2nd generation: t = 0.07, df = 5, P = 0.47; 3rd generation: t = 0.67, df = 5, P = 0.26; 4th generation: t = 0.50, df = 5, P = 0.31).

The NMDS plot showed that the bacterial communities of the same generation clustered together (Fig. 2a), PERMANOVA R^2 = 0.30, P = 0.001). The SA and control separated in the NMDS plot (Fig. S6) but this was not significant (PERMANOVA R^2 = 0.05, P = 0.18). Similar patterns were observed in a principal component analysis (PCA; Fig. S7). The OPLS-DA analysis showed clusters for replicates within each generation, and clear separation for the SA effect but only in the 2nd, 3rd and 4th generation (Fig. 2b). However, the generation effect was more evident than the SA effect.

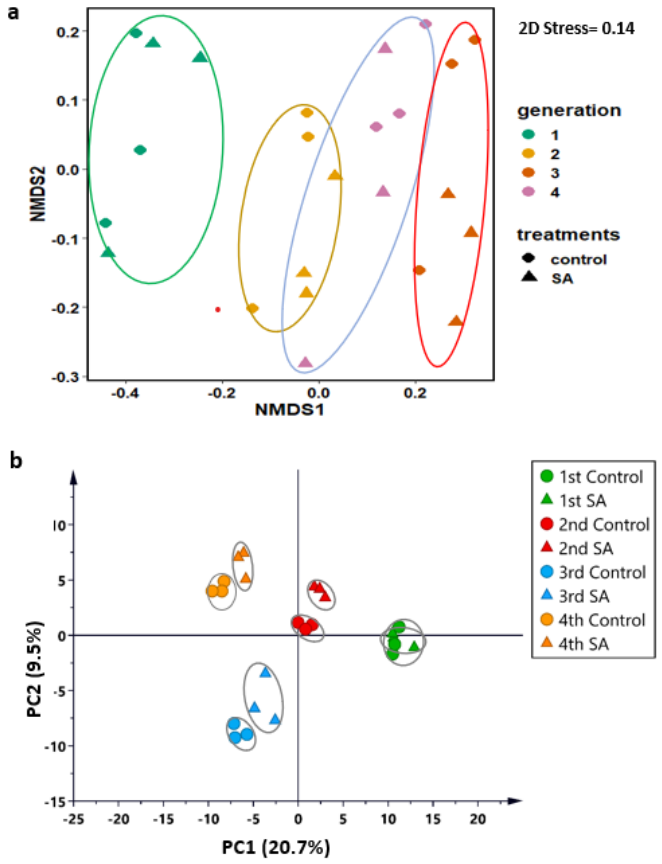

Fig. 2 Multivariate analysis of the bacterial community in soil samples from SA-treated and control plants grown in four generations. Shown are sample scores from a Nonmetric Multi-Dimensional Scaling (NMDS) plot (a) and an Orthogonal

Projections to Latent Structures Discriminant analysis (OPLS-DA) plot (b) from the 24 rhizosphere soil samples.

Core bacterial community

89 species out of the total of 270 (32.4%) were present in all generations in at least two out of the three replicates of the soils of SA-treated and control plants suggesting that these make up the "core" microbiome (Fig. 3a). On average in each generation, 72.9% of all the genera were present in both soils (Fig. 3b). In the first generation, both soils shared about 74.2% of the genera while 7.7% only occurred in the SA-treatment and 18.0% only in the control (Fig. 3b-1). The percentage of shared genera by the two soils in the 2nd, 3rd and 4th generation was 67.6%, 72.9% and 76.8% (Fig. 3b-2, 3, 4). For soils of the control treatment, 49.5% of the genera were shared over all four generations; while 45.1% of genera were shared in soils of the SA treated plants over four generations (Fig. S2c; Table S2). Immigration was somewhat higher in the SA treatment (on average 42 new genera) than in the control (on average 34 new genera) while the opposite was true for extinction rates (on average 31 genera in the SA treatment and 33 in the control treatment; Fig. 4). The information of Archaea, virus and eukaryote is listed in supplementary Fig. S4.

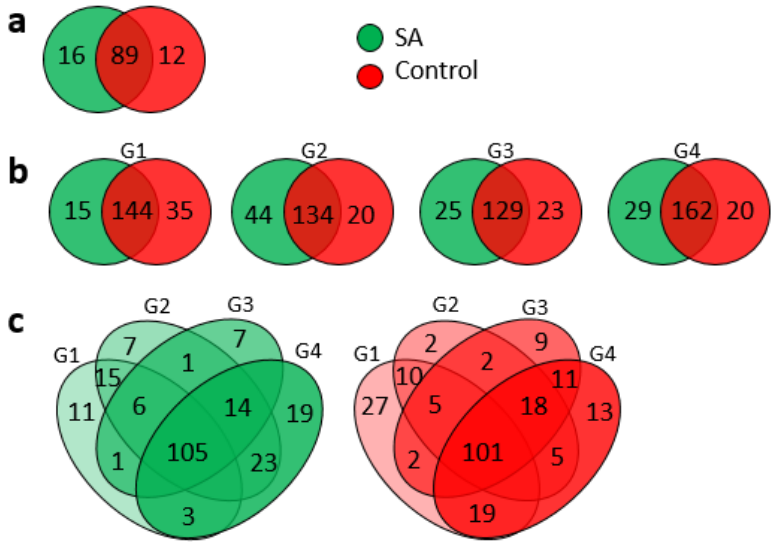

Fig. 3 Venn diagrams showing the unique and shared genera of bacteria in the rhizosphere soil samples of SA-treated and control *J. vulgaris* plants. The diagram in (a) is based on an analysis of genera that occur in all growth generations of the SA treatment, in (b) each generation is analyzed separately, (c) shows the diagram for all generations combined for the SA and control treatment.

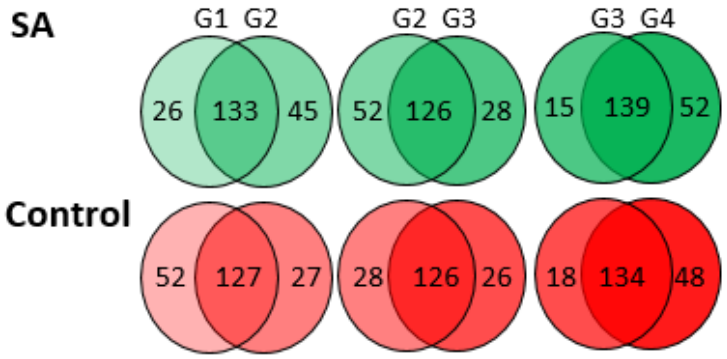

Fig. 4 Local "immigration" and "extinction" of bacterial genera in the rhizosphere soil of SA-treated and control plants over time. For each two consecutive generations, shown are the number of genera present only in the first of those generations (i.e., representing genera that go extinct), present in both generations, and present only in the second of those generations (i.e., representing generate that immigrate). Genera were considered present in a treatment when present in at least two of the tree replicates. 1, 2, 3 and 4 represent the 1st, 2nd, 3rd and 4th generation.

SA selection of soil bacteria

When analyzed per generation, in total eight genera differed among the SA treatment and control (Fig. 5). No genus was significantly affected in more than one generation and no genera were significantly affected in the first generation. Most of the significant genera were only present in either the control or SA treatment. A Spearman's rank correlation showed that 41 (out of 240) genera in the rhizosphere soil of SA-treated plants were significantly increasing or 31 genera were decreasing over generations. For the control soils these numbers were 47 and 27, respectively out of a total of 239 genera (Table S3). The heatmap including all 24 samples showed a clear generation effect, but no clear SA effect (Fig. 6). A heatmap representing the patterns of all identified genera in the 12 rhizosphere soils of SA-treated plants showed that replicates within a generation clustered and that the 2nd, 3rd and 4th generation showed a higher similarity than the 1st generation (Fig S8a). For the control plants, the samples from the 1st generation differed from the three other generations (Fig S8b).

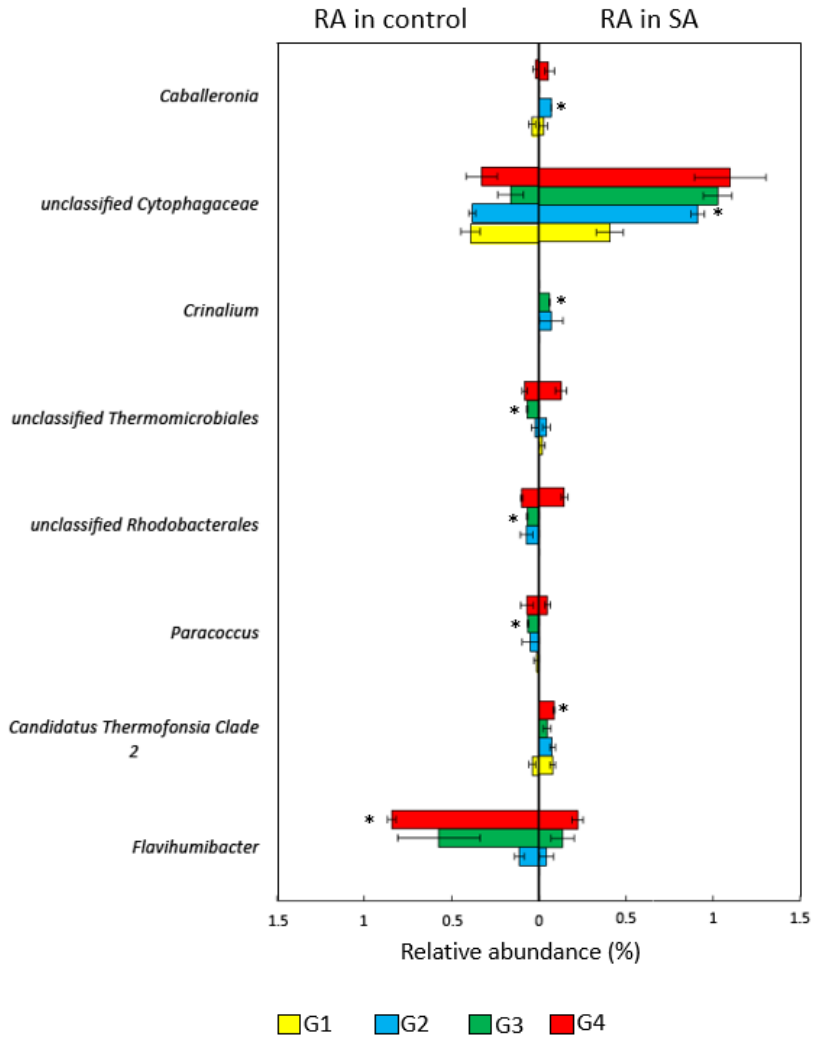

Fig. 5 Bar chart showing relative abundance (%) (mean ± SE) of the significant up or down regulated genera in the rhizosphere soils by SA-treated *J. vulgaris* plants. The significance is based on a student t-test with a false discovery rate (FDR) adjusted *P* values (< 0.05).

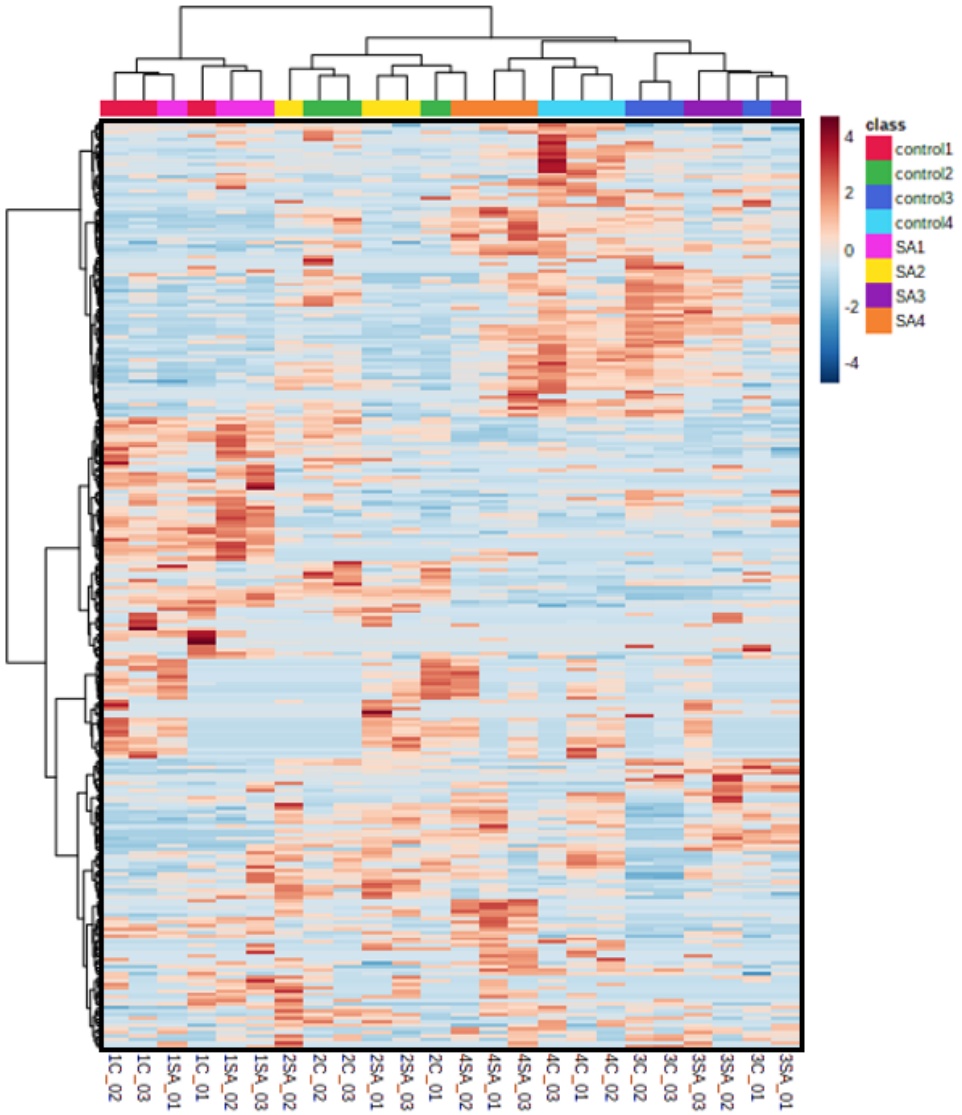

Fig. 6 Heatmap with a hierarchical clustering analysis of all the bacterial genera of rhizosphere soil of SA-treated *J. vulgaris* plants and control plants in the 24 samples. The hierarchical clustering was calculated with Pearson distance and the Ward clustering algorithm based on the relative abundance of the reads of each genus. The color code represents the row-centered relative abundance. SA1, SA2, SA3 and SA4 represent SA treatments from the 1st generation, 2nd generation of plant growth, 3rd generation and 4th generation. Control 1, control2, control3 and control4 represent

control treatments from the 1st generation, 2nd generation, 3rd generation and 4th generation.

Discussion

In this study, we examined how the activation of SA-induced resistance in the plant impacts the microbial composition in the rhizosphere, and how this change over generations of plant growth. Our study shows that the composition of rhizosphere bacteria communities of *J. vulgaris* changed significantly over generations, *but* that neither the effects of activation of SA-associated plant defense pathways nor the interaction between generation number and SA on the bacterial composition was significant. Within generations the application of SA selected for different bacterial genera in the rhizosphere soil, but these selected genera differed from generation to generation. There were no SA-mediated changes in active bacterial genera in the first generation, suggesting that there are no immediate effects of activation of the SA defense pathway on the soil microbial composition. The majority (76.1%) of the bacterial genera that we detected was present in all soils and represents the "core" bacterial microbiome.

Our study showed that aboveground activation of SA-associated plant defense pathways influenced different bacterial genera in the second, third and fourth generations. Effects of SA-induced resistance on the soil microbial community have been reported in several other studies. For example, Hein et al. (2008) compared the effect of SA application on the composition of rhizosphere bacterial communities in several *Arabidopsis* mutants with terminal restriction fragment length polymorphism (T-RFLP) analysis. They found that SA-induced resistance changed the structure of bacterial communities in the rhizosphere. In addition, Lebeis et al. (2015) demonstrated that SA application modulates colonization of the root microbiome by specific bacterial taxa. SA in plants is associated with the expression of pathogenesis-related proteins (PRPs). These PRPs possess antimicrobial activities resulting in suppression of microbial pathogens, consequently changing the microbial composition (Yalpani et al., 1991; Van Loon and Van Strien, 1999). Alternatively, hormonal-induced resistance in the plant may promote beneficial bacteria and fungi.

However, the impact of SA-induced resistance on soil microbial communities is still debated. For instance, Wang et al. (2015) and Doornbos et al. (2011) both demonstrated that activation of SA-induced resistance did not significantly affect the composition and diversity of the rhizosphere bacterial community.

Even though the experimental conditions and plant genotypes remained the same throughout the experiment, the effects of SA application on the bacterial community differed among generations. In this context, it is important to note that for each generation we used an inoculum, which means that we placed a subset of the microbial community in a sterile background. This may explain why we saw so much variation temporally as in each generation a different subset of the microbial community may have been activated. It is also possible that the composition of the bacterial community is variable over time within each generation and as a consequence also among generations (Gilbert et al., 2009; Hickey et al., 2013; Lauber et al., 2013; Hannula et al., 2019).

Of the four potential models, our data confirmed the third hypothesis (Fig. 1-iii), showing that the bacterial communities did differed among generations but were not strongly influenced by SA application. This is line with studies showing that the composition of the soil bacterial microbial community exhibits large fluctuations over time (Hannula et al., 2019; Lauder et al., 2013). Moreover, our data also shows that the application of SA selects for different bacterial genera in the rhizosphere soil but that these selected genera differ from generation to generation. This suggests that the effects of SA application to plants on the soil microbial community are not consistent over time and that it will be difficult to predict the effects of activation of plant defenses on soil microbes, and ultimately how this will influence the interactions between plants and microbes in the rhizosphere.

Interestingly, in soils of SA-treated plants, we found an increase of *Caballeronia*, unclassified *Cytophagaceae*, *Crinalium* and *Candidatus Thermofonsia Clade* 2. The *Caballeronia* genus is often reported as playing an important role in fixing nitrogen and promoting plant growth. Species in this genus are predominantly endophytic diazotrophic bacteria and N-fixing bacteria (Padda et al., 2018; Puri et al., 2018; Puri et al., 2020). This suggests that activation of SA signaling pathways in *J. vulgaris*

plants benefited bacteria that were more beneficial to plant growth, but further studies are needed to confirm this. The functions of the other species of which their abundance differentially increases are poorly understood. It is noteworthy though that *Crinalium* is a genus that is often isolated from sandy dune soils so it not surprising that we detected this genus as we used dune soils in our experiment. Further studies should extract the information of these detected genera at the species level.

In conclusion, we provide evidence that the composition of bacterial communities in the rhizosphere significantly differed between plant cycles (generation), but we found no evidence that application of SA altered this pattern. However, application of SA influenced different bacterial genera in the rhizosphere, but the responsive genera varied between generations. No bacterial genera were detected that responded to SA application in the first generation suggesting that there are no immediate responses of bacteria in the rhizosphere to SA application to plants. This would question the so-called 'cry for help" hypothesis (Biere and Bennett, 2013; Rasmann et al., 2017; Pineda et al., 2013), but further studies are required before we can make firm conclusions about this. Our results provide a new perspective on the effects of plant hormones on temporal changes in the soil microbial community.

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Supplementary data

Table S1. Summary of Illumina sequencing of the 24 libraries. Total RNA was extracted from 24 soil samples collected from *J. vulgaris* rhizospheres (2 treatments (control/SA application) × 4 generations × 3 replicates). The mapped reads and the percentage of properly paired reads that mapped back to the assembled metatranscriptome by Bowie2 are presented.

Gene.	Treatment (replicate)	No. Raw reads	No. filtered reads (percentage)	No. rRNA reads (percentage)	No. Non rRNA reads (percentage)	Mapped reads (percentage)
1st	Con-1	33,319,926	31,883,498 (95.6)	29,931,407 (93.9)	1,952,091 (6.1)	961,492 (49.3)
	Con-2	47,464,976	45,550,842 (96.0)	43,003,695 (94.4)	2,547,147 (5.6)	1,260,216 (49.5)
	Con-3	38,074,824	35,977,802 (94.5)	33,611,532 (93.4)	2,366,270 (6.6)	1,072,164 (45.3)
	Sa-1	36,891,926	35,557,542 (96.4)	33,148,711 (93.2)	2,408,831 (6.8)	1,046,220 (43.4)
	Sa-2	29,685,378	28,640,038 (96.5)	27,053,138 (94.5)	1,586,900 (5.5)	644,792 (40.6)
	Sa-3	41,699,758	39,325,110 (94.3)	36,398,366 (92.6)	2,926,744 (7.4)	1,445,924 (49.4)
2nd	Con-1	51,523,968	46,908,512 (91.0)	43,467,604 (92.7)	3,440,908 (7.3)	1,682,552 (48.9)
	Con-2	36,922,306	33,800,864 (91.6)	30,217,009 (89.4)	3,583,855 (10.6)	1,198,916 (33.5)
	Con-3	36,098,466	34,487,094 (95.5)	31,687,996 (91.9)	2,799,098 (8.1)	1,360,320 (48.6)
	Sa-1	34,498,252	33,458,636 (97.0)	30,528,949 (91.2)	2,929,687 (8.8)	1,122,652 (38.3)
	Sa-2	30,828,960	29,287,310 (95.0)	25,812,484 (88.1)	3,474,826 (11.9)	1,215,556 (35.0)
	Sa-3	30,241,370	29,244,564 (96.7)	27,187,397 (92.9)	2,057,167 (7.0)	724,424 (35.2)
3rd	Con-1	32,336,630	31,153,526 (96.3)	28,690,779 (92.1)	2,462,747 (7.9)	1,652,708 (67.1)
	Con-2	32,877,082	31,698,262 (96.4)	25,792,802 (81.4)	5,905,460 (18.6)	2,090,760 (35.4)
	Con-3	31,458,902	30,142,612 (95.8)	24,823,022 (82.4)	5,319,590 (17.7)	2,182,264 (41.0)
	Sa-1	25,035,684	24,001,844 (95.9)	22,402,014 (93.3)	1,599,830 (6.7)	866,692 (54.2)
	Sa-2	29,313,124	27,093,298 (92.4)	24,721,628 (91.2)	2,371,670 (8.8)	1,293,100 (54.5)
	Sa-3	52,080,670	48,472,384	43,560,075	4,912,309	2,907,372

			(93.1)	(89.9)	(10.1)	(59.2)
4th	Con-1	40,952,998	37,197,048 (90.8)	33,471,054 (90.0)	3,725,994 (10.0)	1,727,084 (46.4)
	Con-2	40,803,576	37,914,816 (92.9)	34,101,981 (89.9)	3,812,835 (10.1)	1,641,020 (43.0)
	Con-3	46,249,750	42,340,292 (91.6)	39,136,920 (92.4)	3,203,372 (7.6)	1,100,132 (34.3)
	Sa-1	39,495,864	36,597,750 (92.7)	34,868,480 (95.3)	1,729,270 (4.8)	814,560 (47.1)
	Sa-2	41,678,588	39,339,896 (94.4)	36,363,915 (92.4)	2,975,981 (7.6)	1,552,592 (52.2)
	Sa-3	38,834,084	36,852,408 (94.9)	35,344,564 (95.9)	1,507,844 (4.1)	580,832 (38.5)

Table S2. A list of all bacterial genera presents in different generations in the SA and control treatment. "+" represents present and "-" absent. When a genus is present in at least two out of three replicates within a treatment it is recorded as "+".

	1SA	2SA	3SA	4SA	1C	2C	3C	4C
Acidovorax	+	+	+	+	+	+	+	+
Acinetobacter	+	+	+	+	+	+	+	+
Aeromicrobium	+	+	+	+	+	+	+	+
Afipia	+	+	+	+	+	+	+	+
Aminobacter	+	+	+	+	+	+	+	+
Aquabacterium	+	+	+	+	+	+	+	+
Arenimonas	+	+	+	+	+	+	+	+
Arthrobacter	+	+	+	+	+	+	+	+
Azohydromonas	+	+	+	+	+	+	+	+
Bosea	+	+	+	+	+	+	+	+
Bradyrhizobium	+	+	+	+	+	+	+	+
Caenimonas	+	+	+	+	+	+	+	+
Cellvibrio	+	+	+	+	+	+	+	+
Chryseolinea	+	+	+	+	+	+	+	+
Cupriavidus	+	+	+	+	+	+	+	+
Curvibacter	+	+	+	+	+	+	+	+
Devosia	+	+	+	+	+	+	+	+
Dongia	+	+	+	+	+	+	+	+
Ensifer	+	+	+	+	+	+	+	+
environmental Bacteria	+	+	+	+	+	+	+	+
Flavobacterium	+	+	+	+	+	+	+	+
Fluviicola	+	+	+	+	+	+	+	+
Gemmatimonas	+	+	+	+	+	+	+	+
Herbaspirillum	+	+	+	+	+	+	+	+
Herminiimonas	+	+	+	+	+	+	+	+
Hydrogenophaga	+	+	+	+	+	+	+	+
Ideonella	+	+	+	+	+	+	+	+
Janthinobacterium	+	+	+	+	+	+	+	+
Lacibacter	+	+	+	+	+	+	+	+
Lacunisphaera	+	+	+	+	+	+	+	+
Luteimonas	+	+	+	+	+	+	+	+
Lysobacter	+	+	+	+	+	+	+	+
Marmoricola	+	+	+	+	+	+	+	+
Massilia	+	+	+	+	+	+	+	+
Mesorhizobium	+	+	+	+	+	+	+	+
Methylibium	+	+	+	+	+	+	+	+
Methylotenera	+	+	+	+	+	+	+	+
Microbacterium	+	+	+	+	+	+	+	+
Mycobacterium	+	+	+	+	+	+	+	+
Niastella	+	+	+	+	+	+	+	+
Nitrospira	+	+	+	+	+	+	+	+
Nocardioides	+	+	+	+	+	+	+	+
Noviherbaspirillum	+	+	+	+	+	+	+	+
Opitutus	+	+	+	+	+	+	+	+
Pelomonas	+	+	+	+	+	+	+	+
Phenylobacterium	+	+	+	+	+	+	+	+

Phycicoccus	+	+	+	+	+	+	+	+
Polaromonas	+	+	+	+	+	+	+	+
Pseudarthrobacter	+	+	+	+	+	+	+	+
Pseudomonas	+	+	+	+	+	+	+	+
Pseudoxanthomonas	+	+	+	+	+	+	+	+
Ramlibacter	+	+	+	+	+	+	+	+
Reyranella	+	+	+	+	+	+	+	+
Rhizobacter	+	+	+	+	+	+	+	+
Rhizobium	+	+	+	+	+	+	+	+
Rhodoferax	+	+	+	+	+	+	+	+
Rivibacter	+	+	+	+	+	+	+	+
Rubrivivax	+	+	+	+	+	+	+	+
Solimonas	+	+	+	+	+	+	+	+
Sphingomonas	+	+	+	+	+	+	+	+
Sphingopyxis	+	+	+	+	+	+	+	+
Sporichthya	+	+	+	+	+	+	+	+
Staphylococcus	+	+	+	+	+	+	+	+
Streptomyces	+	+	+	+	+	+	+	+
unclassified *Acidobacteria*	+	+	+	+	+	+	+	+
unclassified *Actinobacteria (class) (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Alphaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Bacteroidetes (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Betaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Burkholderiaceae*	+	+	+	+	+	+	+	+
unclassified *Burkholderiales (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Chitinophagaceae*	+	+	+	+	+	+	+	+
unclassified *Chloroflexi (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Comamonadaceae*	+	+	+	+	+	+	+	+
unclassified *Cytophagaceae*	+	+	+	+	+	+	+	+
unclassified *Deltaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Gammaproteobacteria (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Gemmatimonadales*	+	+	+	+	+	+	+	+
unclassified *Gemmatimonadetes*	+	+	+	+	+	+	+	+
unclassified *Myxococcales (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Planctomycetes*	+	+	+	+	+	+	+	+
unclassified *Proteobacteria*	+	+	+	+	+	+	+	+
unclassified *Rhizobiales (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Sphingobacteriaceae*	+	+	+	+	+	+	+	+
unclassified *Sphingobacteriales*	+	+	+	+	+	+	+	+
unclassified *Verrucomicrobia (miscellaneous)*	+	+	+	+	+	+	+	+
unclassified *Xanthomonadaceae*	+	+	+	+	+	+	+	+
Variovorax	+	+	+	+	+	+	+	+
Vulcaniibacterium	+	+	+	+	+	+	+	+
Azospira	+	+	+	+	-	+	+	+
Candidatus Thermofonsia Clade 2	+	+	+	+	-	+	+	+
Clostridioides	+	+	+	+	-	+	+	+
Collimonas	+	+	+	+	-	+	+	+
Escherichia	+	+	+	+	-	-	+	+
Hassallia	+	+	+	+	+	+	-	+
Ohtaekwangia	+	+	+	+	+	+	-	+
Pedobacter	+	+	+	+	+	+	-	+
Pedosphaera	+	+	+	+	+	+	-	+
Ralstonia	+	+	+	+	+	+	-	+
Rhodobacter	+	+	+	+	-	+	-	+

Shigella	+	+	+	+	+	-	-	+
Sinorhizobium	+	+	+	+	+	-	-	+
Tabrizicola	+	+	+	+	+	+	+	-
unclassified *Acidimicrobiaceae*	+	+	+	+	+	+	+	-
unclassified *Flavobacteriales (miscellaneous)*	+	+	+	+	+	-	+	-
unclassified *Oxalobacteraceae*	+	+	+	+	+	-	+	-
unclassified *Sphingomonadales*	+	+	+	+	+	-	-	-
Acidobacterium	-	+	+	+	+	+	+	+
Algoriphagus	-	+	+	+	+	+	+	+
Altererythrobacter	-	+	+	+	+	+	+	+
Paeniglutamicibacter	+	-	+	+	+	+	+	+
Piscinibacter	+	-	+	+	+	+	+	+
Rhodococcus	+	-	+	+	+	+	+	+
Rhodoplanes	+	+	-	+	+	+	+	+
Sphingobium	+	+	+	-	+	+	+	+
unclassified *Anaerolineaceae*	+	+	+	-	+	+	+	+
unclassified *Opitutae*	+	+	-	-	+	+	+	+
unclassified *Sinobacteraceae*	+	+	-	-	+	+	+	+
unclassified *Xanthomonadales*	+	-	-	-	+	+	+	+
Actinomycetales	+	-	+	+	-	+	+	+
Actinoplanes	+	-	+	+	-	+	+	+
Agromyces	+	-	+	+	+	-	+	+
Alistipes	+	-	+	+	+	-	+	-
Anaerobutyricum	+	-	+	+	+	-	-	-
Anaerostipes	+	-	+	+	+	-	-	-
Aquimonas	+	-	+	+	+	-	-	-
Aquincola	+	-	+	+	+	-	-	-
Azotobacter	+	+	-	+	+	+	-	+
Bacteriovorax	+	+	-	+	+	+	-	+
bacterium	+	+	-	+	+	+	-	+
Bacteroides	+	+	-	+	+	+	-	-
Batrachochytrium	+	+	-	+	-	-	-	-
Bdellovibrio	+	+	-	+	-	-	-	-
Bifidobacterium	+	-	-	+	+	+	-	+
Blautia	+	-	-	+	+	-	-	+
Brevundimonas	+	-	-	+	+	-	-	-
Bryobacter	+	+	+	-	-	-	+	+
Burkholderia	+	+	+	-	-	+	-	+
Caballeronia	+	+	+	-	+	+	+	-
Candidatus Kaiserbacteria	+	+	+	-	+	+	-	-
Candidatus Kapabacteria	+	-	+	-	+	+	-	-
Candidatus Nitrosocosmicus	+	-	+	-	-	-	-	-
Candidatus Rokubacteria	+	+	-	-	+	+	-	+
Catellatospora	+	+	-	-	+	+	-	+
Chitinophaga	+	+	-	-	+	+	-	+
Clostridium	+	+	-	-	+	+	+	-
Collinsella	+	+	-	-	+	+	-	-
Comamonas	+	+	-	-	+	-	-	-
Coprococcus	+	+	-	-	+	-	-	-
Crinalium	+	+	-	-	+	-	-	-
Crocinitomix	+	+	-	-	+	-	-	-
Cutibacterium	+	+	-	-	-	-	-	-
Dorea	+	+	-	-	-	-	-	-
Duganella	+	+	-	-	+	-	-	-

Dyadobacter	+	-	-	-	-	-	+	-
Dyella	+	-	-	-	+	+	-	-
environmental samples <bacteria,*phylum*								
Gemmatimonadetes>	+	-	-	-	+	+	-	-
environmental samples <*crenarchaeotes,phylum*								
Crenarchaeota>	+	-	-	-	+	+	-	-
environmental samples <*firmicutes,phylum*								
Firmicutes>	+	-	-	-	+	-	-	-
environmental samples <*GNS bacteria,phylum*								
Chloroflexi>	+	-	-	-	+	-	-	-
Erythrobacter	+	-	-	-	+	-	-	-
Eubacterium	+	-	-	-	+	-	-	-
Faecalibacterium	+	-	-	-	+	-	-	-
Flavihumibacter	+	-	-	-	-	-	-	-
Fontimonas	+	-	-	-	-	-	-	-
Gemmatirosa	+	-	-	-	-	-	-	-
Gemmobacter	-	+	+	+	-	+	+	+
Glaciecola	-	+	+	+	-	+	+	+
Herpetosiphon	-	+	+	+	-	+	+	+
Holdemanella	-	+	+	+	-	+	+	+
Hylemonella	-	+	+	+	-	+	+	+
Hyphomicrobium	-	+	+	+	-	-	+	+
Ilumatobacter	-	+	+	+	-	+	-	+
Inhella	-	+	+	+	-	+	-	+
Klebsiella	-	+	+	+	-	-	-	+
Knoellia	-	-	+	+	-	+	+	+
Kouleothrix	-	-	+	+	-	+	+	+
Kribbella	-	-	+	+	-	+	+	+
Lachnoclostridium	-	-	+	+	+	-	+	+
Leptolyngbya	-	-	+	+	-	-	+	+
Leptothrix	-	-	+	+	-	-	+	+
Leptothrix	-	-	+	+	-	-	-	+
Limnobacter	-	-	+	+	-	+	+	-
Limnohabitans	-	-	+	+	-	-	+	-
Listeria	-	+	-	+	-	-	+	+
Longispora	-	+	-	+	-	-	+	+
Methylobacteriaceae	-	+	-	+	+	+	-	+
Microcoleus	-	+	-	+	-	+	-	+
Micromonospora	-	+	-	+	+	-	-	+
Nitrobacter	-	+	-	+	+	-	-	+
Nitrosomonas	-	+	-	+	+	-	-	+
Nitrososphaera	-	+	-	+	+	-	-	+
Novosphingobium	-	+	-	+	+	-	-	+
Oscillochloris	-	+	-	+	+	-	-	+
Paenarthrobacter	-	+	-	+	+	-	-	+
Panacagrimonas	-	+	-	+	+	-	-	+
Parabacteroides	-	+	-	+	+	-	-	+
Paracoccus	-	+	-	+	+	-	-	+
Paucibacter	-	+	-	+	+	-	-	+
Paucimonas	-	+	-	+	+	-	-	+
Phormidium	-	+	-	+	+	-	-	+
Phyllobacterium	-	+	-	+	+	-	-	+
Pirellula	-	+	-	+	+	-	-	+
Planomicrobium	-	+	-	+	-	-	-	+

Prevotella	-	+	-	+	-	-	-	+
Pseudolabrys	-	+	-	+	-	+	+	-
Pseudonocardia	-	+	-	+	+	-	-	-
Pseudorhodobacter	-	+	-	+	+	-	-	-
Rheinheimera	-	+	-	+	-	-	-	-
Rickettsia	-	-	-	+	-	+	+	+
Roseateles	-	-	-	+	-	+	+	+
Roseburia	-	-	-	+	+	-	+	+
Roseiflexus	-	-	-	+	-	-	+	+
Ruminococcus	-	-	-	+	-	-	+	+
Sandaracinus	-	-	-	+	-	-	+	+
Sediminibacterium	-	-	-	+	-	-	-	+
Simplicispira	-	-	-	+	-	-	-	+
Sinimarinibacterium	-	-	-	+	-	-	-	+
Sphingorhabdus	-	-	-	+	-	-	-	+
Sporocytophaga	-	-	-	+	-	-	+	-
Stella	-	-	-	+	-	-	+	-
Stenotrophomonas	-	-	-	+	+	-	-	-
Streptococcus	-	-	-	+	-	-	-	-
Streptosporangiaceae	-	-	-	+	-	-	-	-
Subdoligranulum	-	-	-	+	-	-	-	-
Thermomonas	-	-	-	+	-	-	-	-
Thermomonosporaceae	-	-	-	+	-	-	-	-
unclassified *Acidobacteriia*	-	-	-	+	-	-	-	-
unclassified *Actinobacteria*	-	+	+	-	-	-	-	-
unclassified *Anaerolineae*	-	+	+	-	-	-	-	-
unclassified *Bradyrhizobiaceae*	-	-	+	-	-	+	+	+
unclassified *Caulobacteraceae*	-	-	+	-	+	-	+	+
unclassified *Clostridiales (miscellaneous)*	-	-	+	-	-	-	+	+
unclassified *Crocinitomicaceae*	-	-	+	-	+	+	+	-
unclassified *Cyanobacteria (miscellaneous)*	-	-	+	-	-	-	-	-
unclassified *Cyclobacteriaceae*	-	-	+	-	-	-	-	-
unclassified *Firmicutes sensu stricto (miscellaneous)*	-	+	-	-	-	-	-	+
unclassified *Frankiales (miscellaneous)*	-	+	-	-	+	+	-	-
unclassified *Hyphomicrobiaceae*	-	+	-	-	-	+	-	-
unclassified *Ignavibacteriae*	-	+	-	-	-	-	-	-
unclassified *Lachnospiraceae*	-	-	-	-	-	-	-	+
unclassified *Nitrosomonadales*	-	-	-	-	-	-	-	+
unclassified *Nitrosopumilales*	-	-	-	-	-	-	-	+
unclassified *Parcubacteria group*	-	-	-	-	-	-	-	+
unclassified *Phyllobacteriaceae*	-	-	-	-	-	--	+	-
unclassified *Pseudomonadales*	-	-	-	-	-	-	+	-
unclassified *Rhodobacteraceae*	-	-	-	-	-	-	+	-
unclassified *Rhodobacterales*	-	-	-	-	-	-	+	-
unclassified *Rhodospirillaceae*	-	-	-	-	-	-	+	-
unclassified *Rhodospirillales (miscellaneous)*	-	-	-	-	-	-	+	-
unclassified *Ruminococcaceae*	-	-	-	-	-	+	-	-
unclassified *Sphingomonadaceae*	-	-	-	-	-	+	-	-
unclassified *Thaumarchaeota (miscellaneous)*	-	-	-	-	+	-	-	-
unclassified *Thermomicrobiales*	-	-	-	-	+	-	-	-
unclassified *Verrucomicrobia subdivision 3*	-	-	-	-	+	-	-	-
unclassified *Verrucomicrobiaceae*	-	-	-	-	+	-	-	-
Undibacterium	-	-	-	-	+	-	-	-

Xanthomonas	-	-	-	-	+	-	-	-
Xenophilus	-	-	-	-	+	-	-	-

Table S3. Genera, of which the relative abundance was significantly positively or negatively correlated with generation number within the SA or control treatment. The correlation is based on a Spearman's rank correlation test. R_s represents Spearman's rank correlation coefficient. *P* values less than 0.05 were selected.

Genera Name	SA		Control	
	R_s value	*P*	R_s value	*P*
Agromyces	0.85	***	-0.68	*
Arthrobacter	0.93	***	-0.84	**
Candidatus Kaiserbacteria	-0.74	**	0.59	*
Crocinitomix	0.81	**	-0.83	**
Ensifer	0.89	***	-0.62	*
environmental samples <*crenarchaeotes, Crenarchaeota*>	0.77	**	-0.82	*
Erythrobacter	-0.83	**	0.70	*
Flavihumibacter	-0.81	**	0.86	***
Flavobacterium	0.73	**	-0.64	*
Gemmobacter	-0.83	**	0.69	*
Lacibacter	-0.60	*	0.68	*
Leptothrix	0.77	**	-0.72	*
Listeria	0.60	*	-0.82	**
Lysobacter	-0.76	**	0.64	*
Mycobacterium	-0.74	**	0.74	**
Opitutus	0.84	**	-0.82	**
Phyllobacterium	0.75	**	-0.62	*
Piscinibacter	0.95	***	-0.81	**
Polaromonas	-0.76	**	0.62	*
Pseudorhodobacter	-0.63	*	0.90	***
Pseudoxanthomonas	-0.73	**	0.67	*
Ramlibacter	0.81	**	-0.77	**
Rheinheimera	-0.87	***	0.62	*
Rhodobacter	0.82	**	-0.85	***
Rhodococcus	0.78	**	-0.69	*
Rhodoferax	0.60	*	-0.82	**
Rickettsia	-0.70	*	0.76	**
Sporichthya	-0.89	***	0.66	*

Tabrizicola	-0.60	*	0.59	*
unclassified *Acidobacteria*	-0.73	**	0.80	***
unclassified *Alphaproteobacteria (miscellaneous)*	-0.78	**	0.72	*
unclassified *Betaproteobacteria (miscellaneous)*	-0.71	*	0.73	*
unclassified *Chloroflexi (miscellaneous)*	-0.95	***	0.76	***
unclassified *Deltaproteobacteria (miscellaneous)*	-0.63	*	0.79	**
unclassified *Gemmatimonadales*	-0.70	*	0.64	*
unclassified *Myxococcales (miscellaneous)*	-0.81	**	0.80	**
unclassified *Parcubacteria group*	-0.77	**	0.72	*
unclassified *Rhodobacteraceae*	0.63	*	-0.72	*
unclassified *Rhodobacterales*	-0.69	*	0.69	*
Undibacterium	-0.69	*	0.66	*
Vulcaniibacterium	0.67	*	-0.61	*
Afipia	-0.82	**	0.57	ns
Aquabacterium	0.82	**	-0.42	ns
Arenimonas	-0.81	**	0.55	ns
Azotobacter	-0.80	**	0.59	ns
Bosea	-0.78	**	0.58	ns
Bradyrhizobium	0.78	**	-0.31	ns
Bryobacter	0.73	**	-0.40	ns
Caenimonas	0.72	*	-0.40	ns
Dyella	-0.72	*	0.54	ns
environmental Bacteria	-0.60	*	-0.02	ns
Gemmataceae	-0.71	*	-0.08	ns
Hassallia	0.78	**	-0.31	ns
Herpetosiphon	0.67	*	-0.36	ns
Inhella	-0.69	*	-0.36	ns
Limnobacter	0.67	*	-0.36	ns
Luteimonas	-0.65	*	0.55	ns
Methylotenera	-0.65	*	0.48	ns
Novosphingobium	0.65	*	-0.32	ns
Oscillochloris	0.64	*	-0.53	ns
Paracoccus	0.63	*	-0.54	ns
Pirellula	-0.60	*	0.35	ns
Pseudarthrobacter	-0.60	*	0.32	ns
Rhizobium	-0.60	*	0.29	ns

Rhodoplanes	-0.60	*	0.13	ns
Roseiflexus	-0.60	*	0.40	ns
Sandaracinus	0.95	***	-0.59	ns
Sphingobium	0.87	***	-0.50	ns
unclassified *Actinobacteria*	0.86	***	-0.43	ns
unclassified *Cytophagaceae*	-0.60	*	0.25	ns
unclassified *Flavobacteriales (miscellaneous)*	0.60	*	-0.14	ns
unclassified *Rhodospirillaceae*	-0.60	*	-0.02	ns
unclassified *Xanthomonadaceae*	0.61	*	-0.50	ns
Variovorax	-0.60	*	0.08	ns
Acidovorax	-0.59	ns	0.88	***
Algoriphagus	0.36	ns	-0.85	***
Azohydromonas	0.19	ns	-0.83	**
Bdellovibrio	0.10	ns	-0.82	**
Brevundimonas	-0.36	ns	-0.82	**
Burkholderia	-0.41	ns	0.82	**
Candidatus Nitrosocosmicus	0.02	ns	0.81	**
Candidatus Thermofonsia Clade 2	0.56	ns	0.80	**
Collimonas	-0.48	ns	0.78	**
Cupriavidus	-0.41	ns	0.78	**
Dongia	-0.37	ns	0.78	**
Herbaspirillum	-0.07	ns	-0.77	**
Hyphomicrobium	-0.37	ns	0.76	**
Klebsiella	-0.50	ns	0.76	**
Mesorhizobium	0.30	ns	-0.75	**
Microbacterium	-0.52	ns	0.73	**
Nitrososphaera	-0.23	ns	-0.72	*
Noviherbaspirillum	-0.04	ns	-0.72	*
Paeniglutamicibacter	-0.36	ns	0.71	*
Parabacteroides	-0.17	ns	0.69	*
Pedosphaera	-0.24	ns	0.69	*
Pelomonas	-0.29	ns	0.68	*
Planomicrobium	0.35	ns	-0.67	*
Sediminibacterium	-0.21	ns	0.67	*
Sinorhizobium	0.40	ns	-0.67	*
Stenotrophomonas	-0.58	ns	0.66	*
Streptomyces	-0.45	ns	0.65	*

unclassified *Anaerolineae*	0.32	ns	-0.65	*
unclassified *Caulobacteraceae*	-0.47	ns	0.65	*
unclassified *Comamonadaceae*	-0.52	ns	0.64	*
unclassified *Gemmatimonadetes*	-0.16	ns	0.63	*
unclassified *Nitrosopumilales*	-0.17	ns	-0.62	*
unclassified *Opitutae*	0.17	ns	-0.61	*
unclassified *Oxalobacteraceae*	-0.41	ns	0.61	*
unclassified *Phyllobacteriaceae*	0.45	ns	-0.61	*
unclassified *Planctomycetes*	-0.38	ns	0.61	*
unclassified *Sphingomonadales*	0.26	ns	-0.61	*
unclassified *Thaumarchaeota (miscellaneous)*	-0.26	ns	0.60	*
unclassified *Thermomicrobiales*	0.39	ns	0.60	*
unclassified *Verrucomicrobia (miscellaneous)*	-0.57	ns	0.59	*
unclassified *Verrucomicrobiaceae*	0.00	ns	-0.59	*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns not significant. n=3.

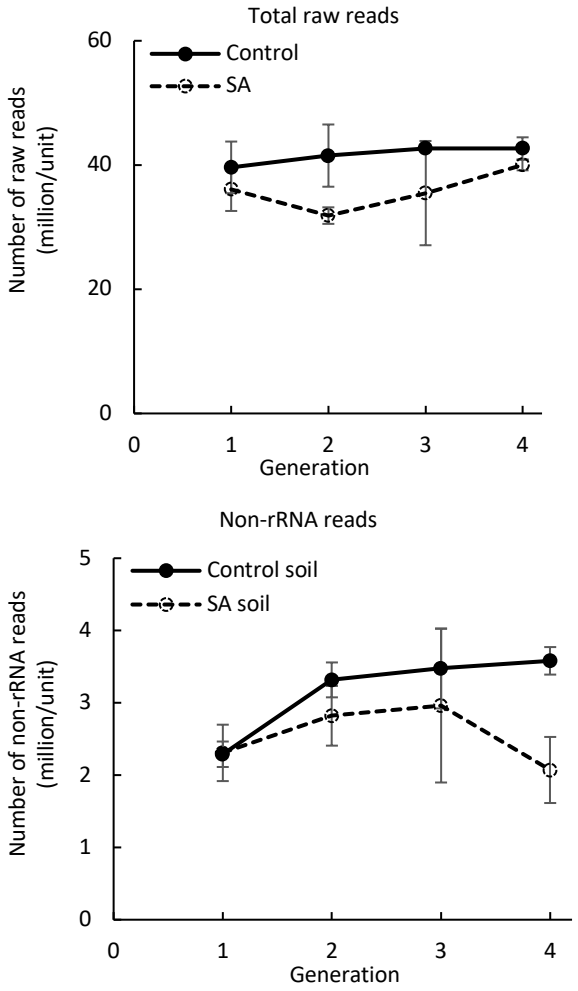


Fig. S1 Number of total raw reads (mean ± SE) (left) and non-rRNA reads (mean ± SE) (right) in the metatranscriptomic datasets over four generations in SA treatments and control. N=3.

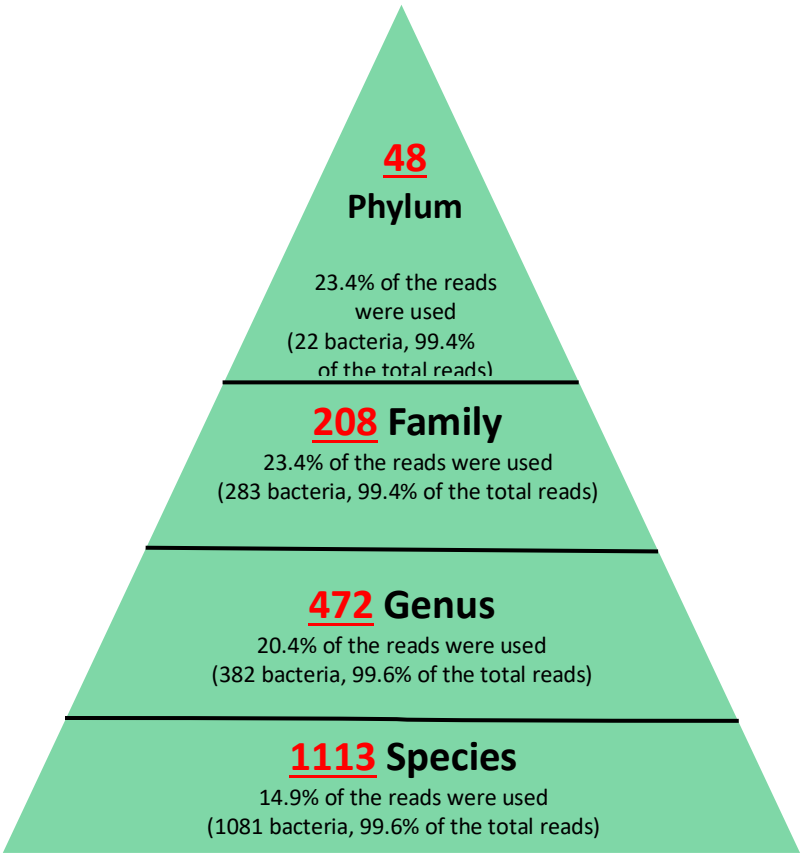


Fig. S2 Heinrich's triangle visualizing information on microbial composition at different classification levels of all 24 rhizosphere soil samples identified with MEGAN against NCBI. The numbers of identified phyla, families, genera and species are shown and the percentage of reads used for each classification level is noted. At phylum level, 2 Archaea, 22 bacteria, 22 eukaryotes and 2 viruses were found; at family level, 3 Archaea, 134 bacteria, 69 eukaryote (only fungi were counted) and 2 viruses were found; at genus level, 4 Archaea, 382 bacteria, 83 eukaryotes (9 fungi, 27 nematodes, 27 algae and 20 others) and 3 viruses were found; at species level, 12 Archaea, 1086 bacteria, 12 eukaryotes and 3 viruses were found. In the Heinrich's triangle, only the information of bacteria is listed, this includes numbers of bacteria and their read percentage against all the reads in that taxonomic level.

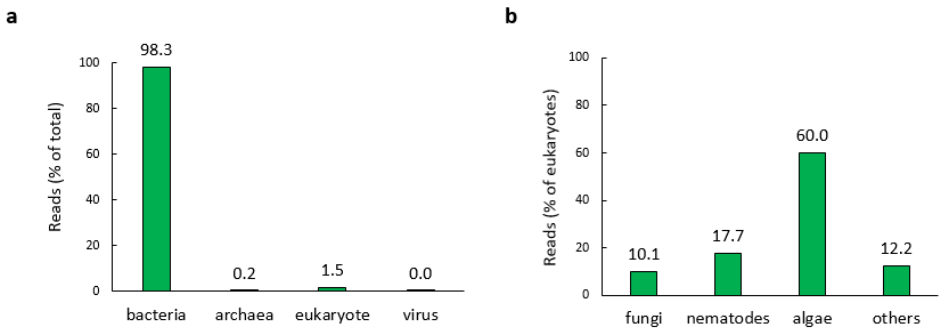


Fig. S3 The percentage of reads at different taxonomic levels. (a) The percentage of reads mapping to bacteria, archaea, eukaryote and virus reads of the total mapped reads. (b) The percentage of reads mapping to fungi, nematodes, algae and others in the total eukaryote reads.

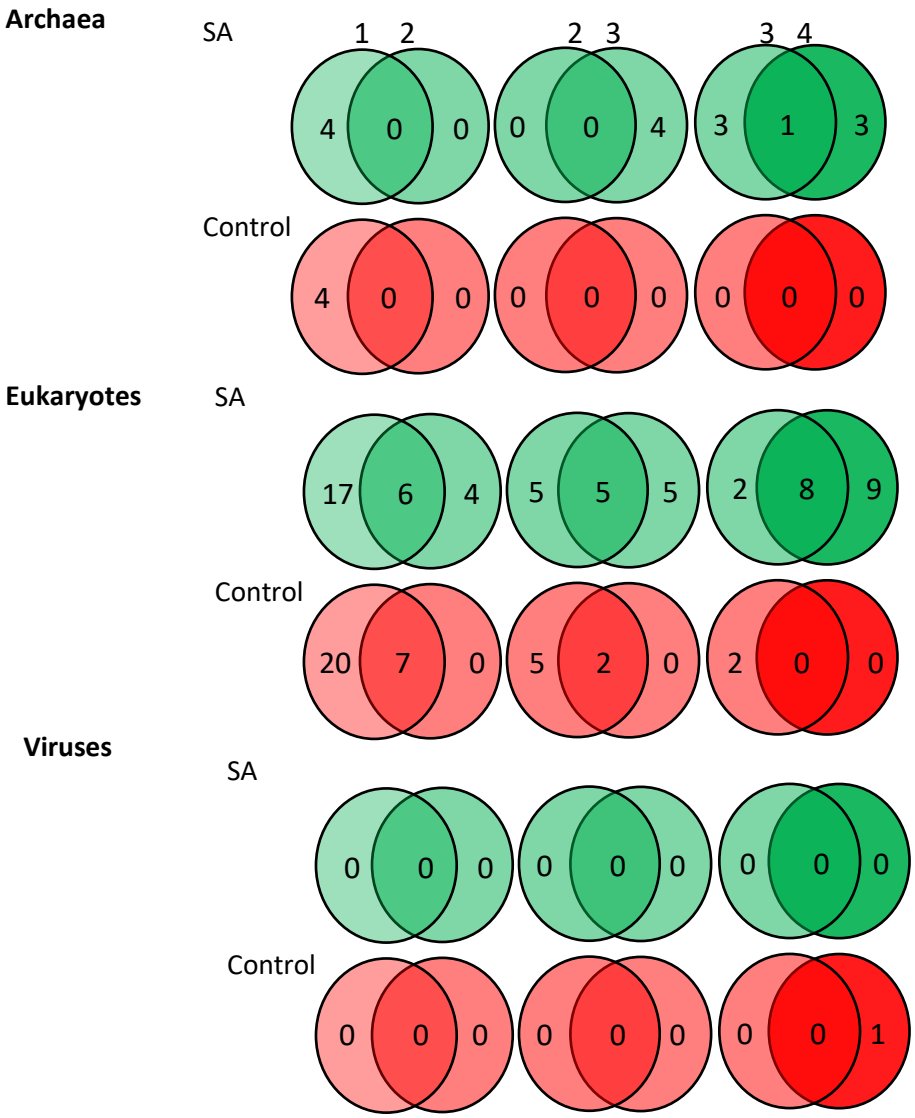


Fig. S4 Venn diagram showing the immigration and extinction numbers at genus level for Archaea, eukaryotes and viruses at each generation in the rhizosphere soil samples of SA-treated or control *J. vulgaris* plants.

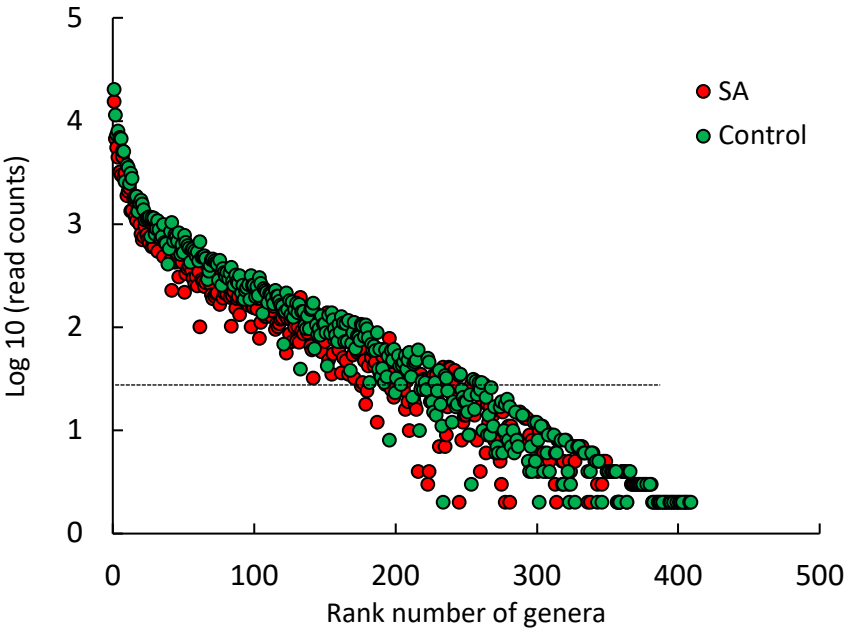


Fig. S5 Scatter plot showing log $_{10}$ transformed read counts of all 408 genera in rhizosphere soil samples of SA-treated and control plants against genus abundance. Rank number was based on the values of total read counts of all genera from the sum of the reads in SA and control treatments. The genus with highest abundance corresponds to the rank number 1. The cut-off line is based on < 0.01% of the total reads.

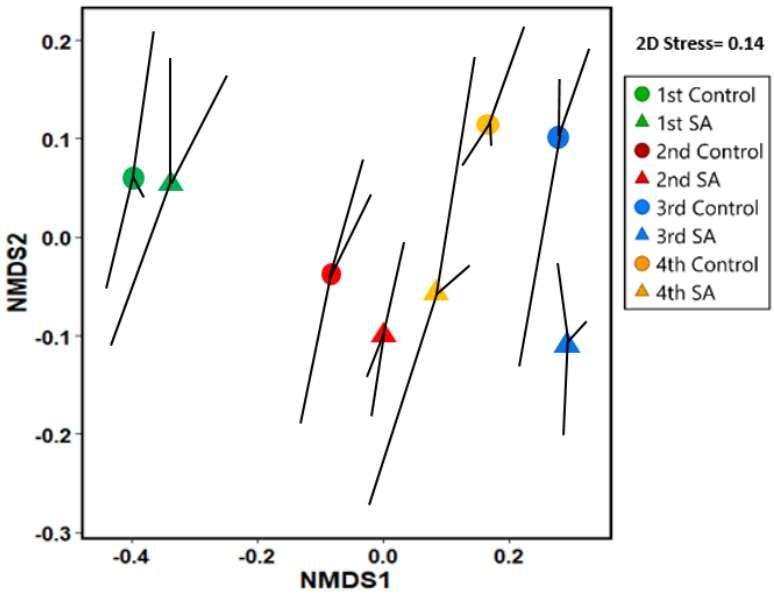


Fig. S6 Nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis distance representing the taxonomic information from the bacterial genera of 24 rhizosphere soil samples. For each treatment combination, the centroid is connected to the three replicates.

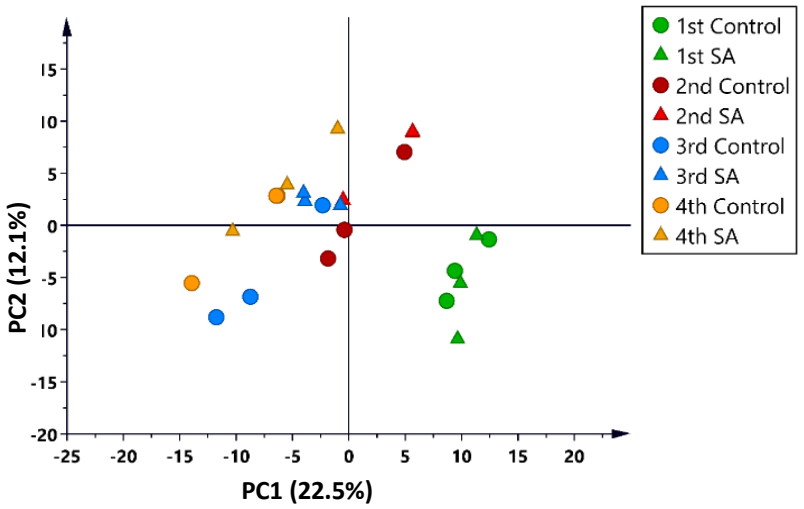
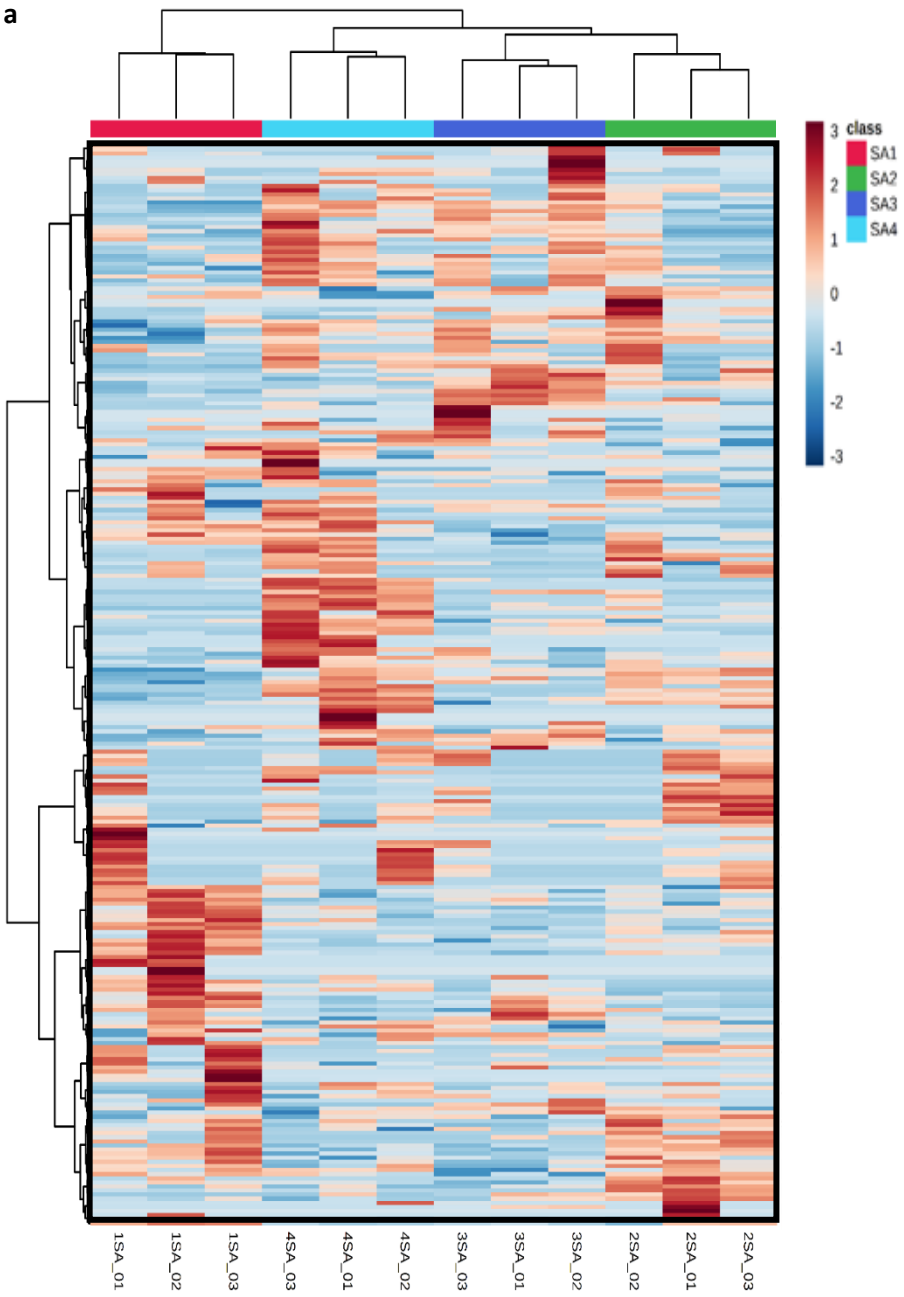


Fig. S7 Principal component analysis (PCA) representing the taxonomic information on the genera of 24 rhizosphere soil samples of SA-treated and control *J. vulgaris* plants. PCA scores are based on relative abundance at genus level.


a
class
SA1
SA2
SA3
SA4
3
2
1
0
-1
-2
-3
1SA_01
1SA_02
1SA_03
4SA_03
4SA_01
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3SA_03
3SA_01
3SA_02
2SA_02
2SA_01
2SA_03

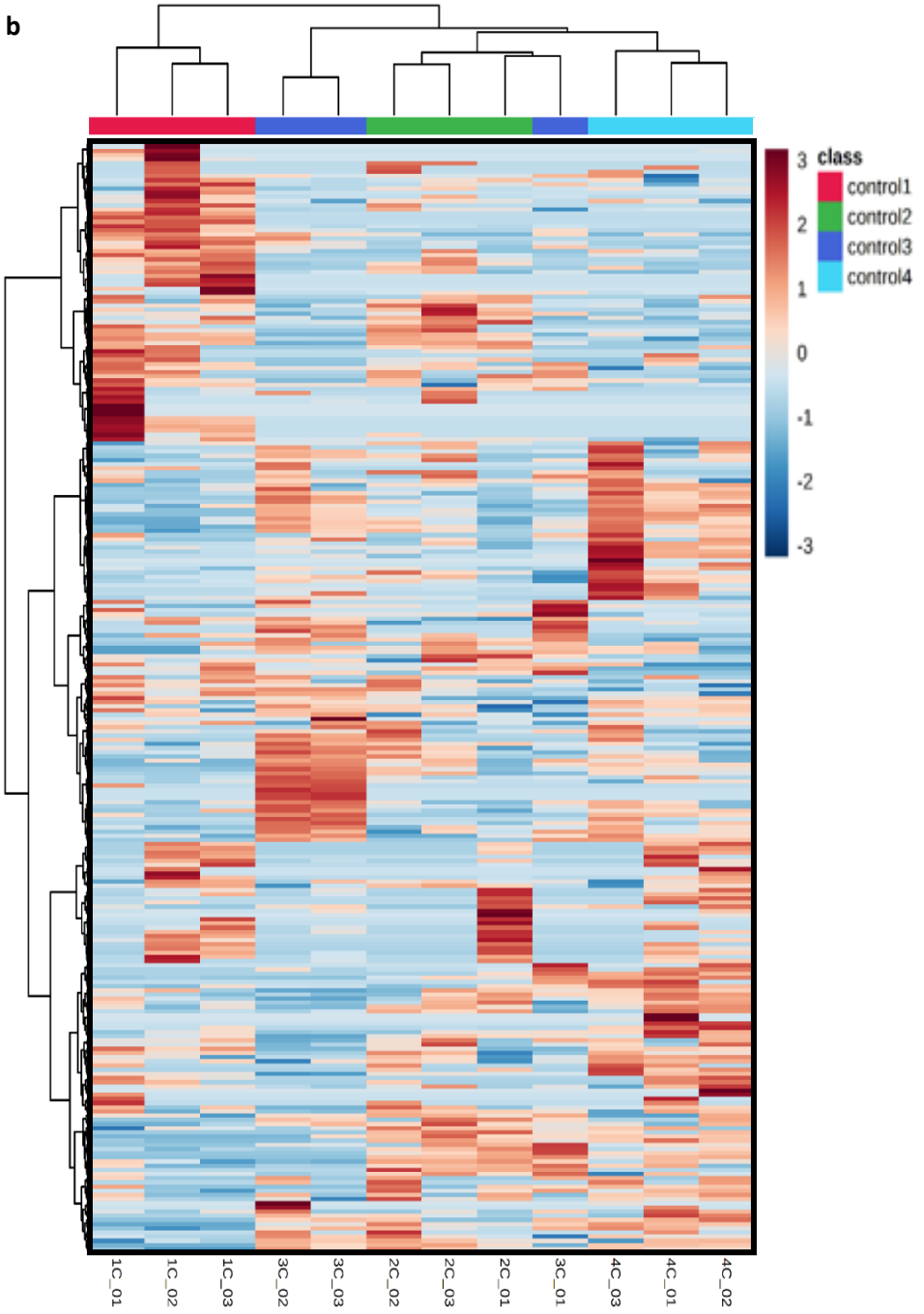
b
class
control1
control2
control3
control4
3
2
1
0
-1
-2
-3
1C_01
1C_02
1C_03
3C_02
3C_03
2C_02
2C_03
2C_01
3C_01
4C_03
4C_01
4C_02

Fig. S8 Heatmap with a hierarchical clustering analysis of 264 genera of rhizosphere soil of SA-treated plants (a) and 270 genera of soil samples of control plants (b). The hierarchical clustering analysis was calculated with Pearson distance and the Ward clustering algorithm based on the relative hit numbers of each genus. The color code represents the values of log2 transformed row-centered relative hit numbers. SA1, SA2, SA3 and SA4 represent SA treatments from the 1st generation, 2nd generation, 3rd generation and 4th generation. Control 1, control2, control3 and control4 represent control treatments from the 1st generation, 2nd generation, 3rd generation and 4th generation. Each treatment in each generation is represented by three replicates indicated as 01, 02 and 03 respectively. E.g. the code 1C_01 represent the first generation control's first replicate.