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PLANT & CELL PHYSIOLOGY

Nutrient Requirements Shape the Preferential Habitat of *Allorhizobium vitis* VAR03-1, a Commensal Bacterium, in the Rhizosphere of *Arabidopsis thaliana*

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(Received 3 May 2024; Accepted 23 August 2024)

A diverse range of commensal bacteria inhabit the rhizosphere, influencing host plant growth and responses to biotic and abiotic stresses. While root-released nutrients can define soil microbial habitats, the bacterial factors involved in plant–microbe interactions are not well characterized. In this study, we investigated the colonization patterns of two plant disease biocontrol agents, *Allorhizobium vitis* VAR03-1 and *Pseudomonas protegens* Cab57, in the rhizosphere of *Arabidopsis thaliana* using Murashige and Skoog (MS) agar medium. VAR03-1 formed colonies even at a distance from the roots, preferentially in the upper part, while Cab57 colonized only the root surface. The addition of sucrose to the agar medium resulted in excessive proliferation of VAR03-1, similar to its pattern without sucrose, whereas Cab57 formed colonies only near the root surface. Overgrowth of both bacterial strains upon nutrient supplementation inhibited host growth, independent of plant immune responses. This inhibition was reduced in the VAR03-1 Δ*recA* mutant, which exhibited increased biofilm formation, suggesting that some activities associated with the free-living lifestyle rather than the sessile lifestyle may be detrimental to host growth. VAR03-1 grew in liquid MS medium with sucrose alone, while Cab57 required both sucrose and organic acids. Supplementation of sugars and organic acids allowed both bacterial strains to grow near and away from *Arabidopsis* roots in MS agar. These results suggest that nutrient requirements for bacterial growth may determine their growth habitats in the rhizosphere, with nutrients released in root exudates potentially acting as a limiting factor in harnessing microbiota.

Keywords: Commensal bacteria • Nutrient requirements • Organic acids • Plant–microbe interactions • Rhizosphere • Sugars

Introduction

Plants harbor a diverse microbial community encompassing symbionts, commensals and pathogens. Although commensals represent the predominant lifestyle of plant-associated microbes, it is crucial to acknowledge that commensalism, regardless of the life form, remains understudied (Mathis and Bronstein 2020). Commensal bacteria benefit from interaction with host plants without eliciting overt harm (Drew et al. 2021). Unlike pathogens, which can evade host defenses and exploit the host for infection (Chen et al. 2021), or root symbionts, which receive nutrients from plants in exchange for services (Bell et al. 2021), commensal bacteria encounter challenges in navigating host immunity and competing for resources to ensure their survival (Yu et al. 2019, Teixeira et al. 2021).

Plants perceive surrounding bacteria through microbial-associated molecular patterns (MAMPs) and induce an immune response known as pattern-triggered immunity (PTI) (Ngou et al. 2022). While most commensal bacterial strains in *Arabidopsis* harbor non-immunogenic flg22, a 22-amino-acid peptide derived from bacterial flagellin (Colaianni et al. 2021), PTI can also be evoked by MAMPs of pathogenic bacteria. To evade this immune response, commensal bacteria have developed various strategies, such as altering root pH through gluconic acid secretion (Yu et al. 2019) and suppressing PTI via type II secretion system (Teixeira et al. 2021). While not all commensals can suppress plant immune responses, the

Plant Cell Physiol. 00(00): 1–18 (2024) doi:https://doi.org/10.1093/pcp/pcae091, Advance Access publication on 24 August 2024, available online at https://academic.oup.com/pcp

intriguing suppression of plant immune responses by certain commensal strains provides opportunities for non-suppressor commensals to colonize plant roots (Teixeira et al. 2021).

Upon evading plant immunity, commensal bacteria must compete with other microbes for plant-derived nutrients. Some commensals exhibit preferences for host plants (Wippel et al. 2021), and others display habitat preferences along the root axis from where preferable nutrients are secreted (Wei et al. 2021, Loo et al. 2024). Root exudates offer a diverse yet limited nutrient pool, favoring bacteria with diverse metabolomic genes that afford flexibility in utilizing available sources (Matilla et al. 2007, Nogales et al. 2008). Hence, exploring the dynamics of plant–commensal microbe interactions has emerged as an intriguing area of research for understanding the complexity of plant–microbe interactions.

Allorhizobium vitis VAR03-1 is a biocontrol agent for crown gall disease in grapevines caused by a pathogenic *A. vitis* harboring tumor-inducing (Ti) plasmid (Kawaguchi et al. 2005, 2007, Kuzmanović et al. 2018, Noutoshi et al. 2020b). Our previous research has identified that the biocontrol activity of VAR03-1 mainly relies on the production of rhizoviticin (Ishii et al. 2024), a phage tail-like bacteriocin that is toxic to other *A. vitis* strains or related species. Interestingly, rhizoviticin-deficient VAR03-1 mutants still showed disease-suppressive activity. This may be due to competition for host surface habitat with pathogens. Therefore, we are interested in the colonization mechanism of this commensal bacterium. The biocontrol activity of VAR03-1 against crown gall has been demonstrated on various host plants, but no adverse effects on these hosts have been reported, indicating a commensal lifestyle of VAR03-1 on them (Kawaguchi et al. 2005, 2007, 2008, 2012).

Here, we investigated the growth pattern of VAR03-1 in the rhizosphere of *Arabidopsis thaliana* using the Murashige and Skoog (MS) agar system and compared it with that of another biocontrol agent, *Pseudomonas protegens* Cab57. Their growth area on and around *Arabidopsis* roots was clearly different and seemed to depend on their nutrient requirements for growth. Supplementation of sucrose in the medium resulted in bacterial overgrowth leading to host growth inhibition, highlighting the importance of strict quality and quantity control of nutrient supply from the roots to regulate the microbial community.

Results

Colonization patterns of *A. vitis* strains and *P. protegens* Cab57 in the *Arabidopsis* rhizosphere

To characterize the root colonization behavior of VAR03-1 in the rhizosphere, we used *Arabidopsis* as a model inoculation system. VAR03-1 cells were mixed into MS agar without any sugars, and *Arabidopsis* seedlings were grown semi-vertically to observe bacterial growth in the rhizosphere. VAR03-1 (a final Optical Density at 600 nm (OD_{600}) value of 5×10^{-4}) enhanced the primary root (PR) growth of *Arabidopsis* (**Fig. 1A**). Please note that we consistently observed a trend of VAR03-1 enhancing *Arabidopsis* root growth throughout the study (refer to different experimental trials in **Fig. 1E**). However, statistical significance was not achieved in some experiments due to the instability of seedling growth on MS agar without sucrose after transplantation. The shoot weight was not significantly affected (**Fig. 1B**). Around the PR, colonies of VAR03-1 were observed, with an uneven distribution along the root axis. A higher population density was found in the upper part of the PR compared to the lower part (**Fig. 1C**). To determine whether this colonization pattern is specific to VAR03-1, we used the other strains of *A. vitis*: VAR06-30 (a non-pathogenic and non-antagonistic strain) and VAT03-9 (Ti) (a pathogenic strain). These two *A. vitis* strains also showed similar growth patterns (**Fig. 1D**) and promoted the lengthening of *Arabidopsis* PR without significantly affecting shoot growth (**Fig. 1E–G**), suggesting that this colonization behavior is a common feature of *A. vitis* in its interaction with *Arabidopsis* as a commensal organism. To further understand bacterial colonization activity, we used *P. protegens* Cab57, another biocontrol agent, as a control. Interestingly, no visible colonies of Cab57 were observed around the PR (**Fig. 1D**). However, Cab57 reduced the length of the *Arabidopsis* PR, despite not significantly affecting the shoots (**Fig. 1E–G**). This suggests that Cab57 may colonize the surface or interior of the plant root tissues at levels not detectable by visible colonies, possibly exerting a negative effect on root growth.

Difference in the nutrient requirements of the commensal bacteria tested

To understand the reason behind the differences in colonization patterns observed in these bacterial genera, we examined their proliferation in liquid MS medium. Neither bacterial species showed an increase in the MS medium supplemented only with vitamins (**Fig. 2A–E**). This indicates that their growth in the *Arabidopsis* rhizosphere, as shown in **Fig. 1**, relies on nutrients, primarily carbon sources, derived from the host roots. To test whether these bacteria have specific requirements or preferences for carbon sources for their growth, we added various types of sugars commonly found in root exudates to the MS medium at different concentrations (Badri et al. 2009, Chaparro et al. 2013). Song et al. (2022) reported that 5-day-old *Arabidopsis* Col-0 seedlings grown hydroponically secrete approximately 40 mg/l (0.12 mM) of sucrose in 24 h. In *Arabidopsis* research, 1% sucrose (29.2 mM) is one of the standard concentrations. Therefore, we tested a range of sugar concentrations from 0.1 to 25 mM. As a result, VAR03-1 was able to grow in MS medium supplemented with all tested sugars (glucose, fructose, sucrose and mannitol) at concentrations $\geq$1 mM (**Fig. 2A–D**). Among the sugars tested, sucrose provided the best growth of VAR03-1 at a minimum concentration of 10 mM (**Fig. 2C**). Next, we compared the growth of VAR03-1 and Cab57 in liquid MS medium supplemented with 1% sucrose. Under these conditions, VAR03-1 reached an OD_{600} value of approximately 0.9 for 20 h, whereas Cab57 only reached an OD_{600} value of 0.2, with no further increase even with extended incubation (**Fig. 2E**).

In addition to sugars, organic acids are reported to be present in root exudates, some of which are recognized

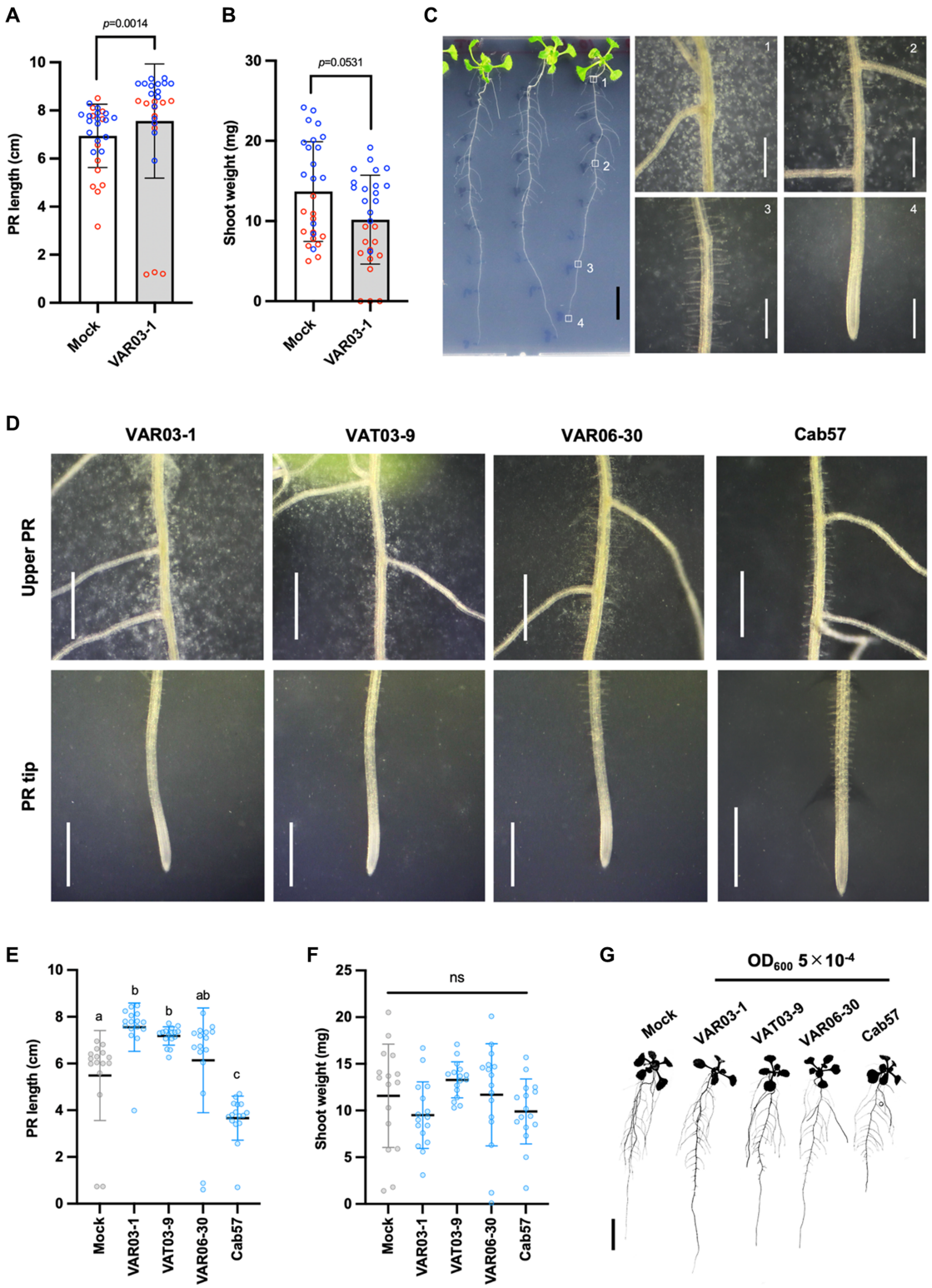

Fig. 1 Colonization of *A. vitis* strains in the *Arabidopsis* rhizosphere in MS agar medium. (A, B) PR length (A) and shoot fresh weight (B) of *Arabidopsis* plants 8 d after transplanting on MS agar medium inoculated with VAR03-1 ($OD_{600} = 5 \times 10^{-4}$). Statistical significance was analyzed using the Mann–Whitney *U* test ($n = 28$). Data from two individual experimental sets were used. (C) Colonies of VAR03-1 along the PR of *Arabidopsis* grown in MS agar medium, with magnification on specific parts of the PR indicated by boxes 1, 2, 3 and 4. Black bar, 1 cm. White bars, 1 mm. (D) Colonies of *A. vitis* strains and *P. protegens* Cab57 around *Arabidopsis* upper PR and root tip regions. Bars, 1 mm. (E, F) PR length (E) and shoot weight (F) of *Arabidopsis* plants 8 d after transplanting on MS agar medium inoculated with *A. vitis* strains or Cab57 ($OD_{600} = 5 \times 10^{-4}$ each). Statistical significance was analyzed using the Brown–Forsythe ANOVA and Dunnett's T3 multiple comparison test ($n = 16$) (E) and ANOVA followed by Tukey's test ($n = 16$) (F). Letters indicate significant differences. (G) Morphology of *Arabidopsis* seedlings 8 d after transplanting on MS medium inoculated with *A. vitis* strains and Cab57. Bar, 1 cm. All experiments were repeated at least twice with similar results.

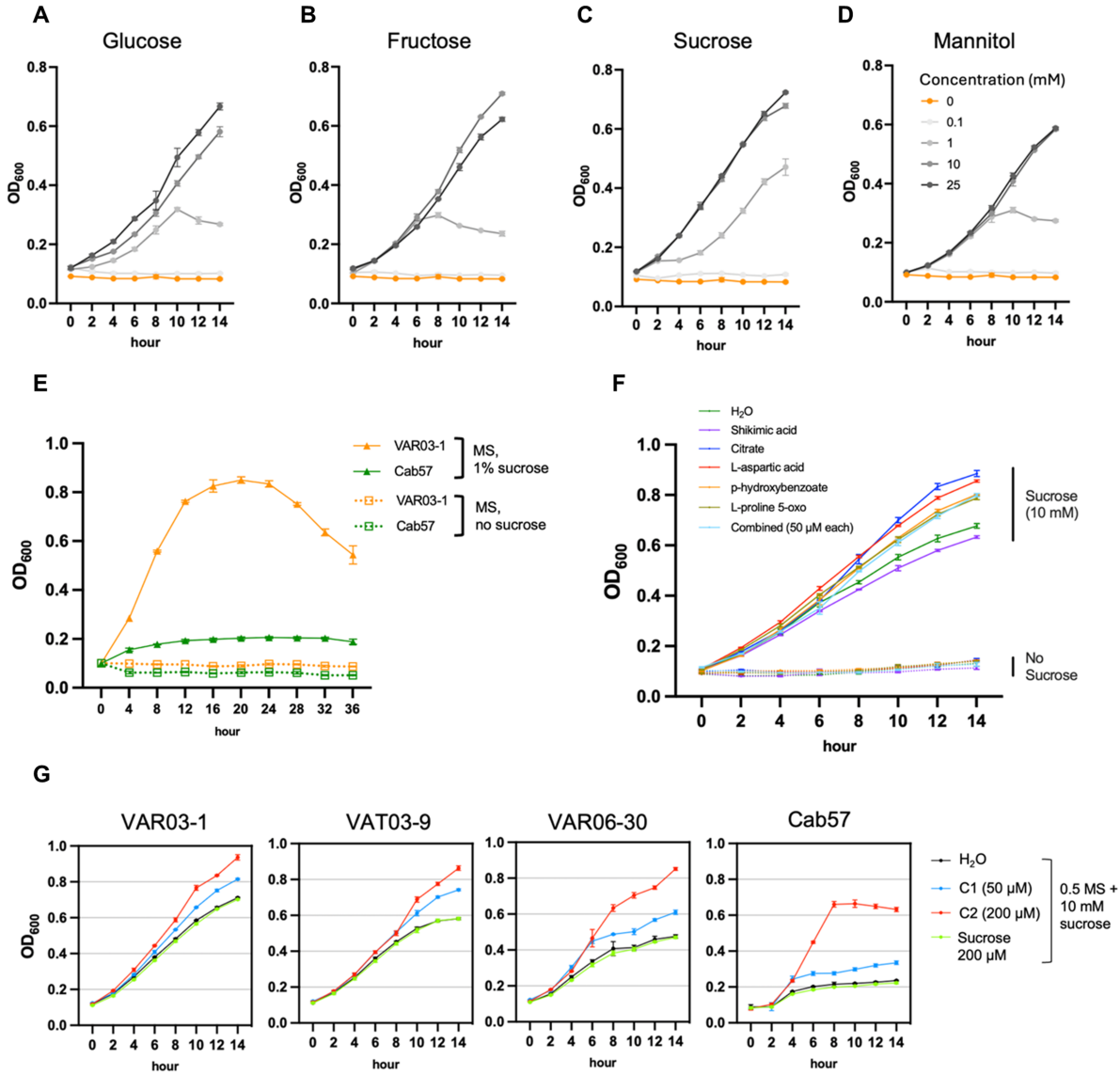

Fig. 2 Growth of *A. vitis* and *P. protegens* Cab57 in liquid MS medium supplemented with sugars and/or organic acids. (A–D) Growth of *A. vitis* VAR03-1 in liquid MS medium supplemented with different sugars (glucose, fructose, sucrose and mannitol). OD_{600} was measured at the indicated times during cultivation. (E) Growth of VAR03-1 and Cab57 in liquid MS medium with or without 1% sucrose. (F) Growth of VAR03-1 in liquid MS medium supplemented with five organic acids (shikimic acid, citrate, L-aspartic acid, *p*-hydroxybenzoate or L-proline 5-oxo) (200 μM each) and a mixture of the five organic acids (50 μM each) with or without 10 mM sucrose. (G) Growth of *A. vitis* strains and Cab57 in liquid MS medium supplemented with a mixture of four organic acids (citrate, L-aspartic acid, *p*-hydroxybenzoate and L-proline 5-oxo) (C1, 50 μM or C2, 200 μM) or 200 μM sucrose with 10 mM sucrose. All experiments were repeated at least twice with similar results.

by pathogenic bacteria for the full induction of virulence (Anderson et al. 2014). We evaluated the growth of VAR03-1 in liquid MS medium supplemented with 200 μM of five selected organic acids (shikimic acids, citrate, aspartic acid, *p*-hydroxybenzoate and proline 5-oxo). These five organic acids have been identified in *Arabidopsis* root exudates and are shown to influence the induction of virulence genes in *Pseudomonas syringae* at an effective concentration of 200 μM (Anderson et al. 2014). However, VAR03-1 growth was not induced by supplementation with 200 μM of each organic acid alone (**Fig. 2F**, dotted line). Additional bacterial growth was only observed when 10 mM sucrose was also supplemented along with the organic acids (**Fig. 2F**). This result indicates that sucrose is a major limiting factor for the growth of VAR03-1. The OD_{600} of VAR03-1 culture reached approximately 0.6 in sucrose-supplemented MS medium and increased to about 0.7–0.9 when citrate, L-aspartic acid, *p*-hydroxybenzoate or L-proline 5-oxo was added (**Fig. 2F**). In contrast, shikimic acid did not produce an additive effect on the growth of VAR03-1 (**Fig. 2F**). Supplementation with all five organic acids together

in the MS medium with sucrose did not enhance growth further than the single organic acid supplements. These results suggest that the presence of at least one of these four organic acids is sufficient to achieve the additive growth effect observed with sucrose.

Next, we investigated whether the nutrient requirement (a combination of sucrose and organic acids) observed in VAR03-1 is a common feature among *A. vitis* strains. VAR03-1, VAT03-9 and VAR06-30 were cultured in MS medium containing 10 mM sucrose and/or 50 or 200 μM of a mixture of four organic acids (citrate, aspartic acid, *p*-hydroxybenzoate and proline 5-oxo), and their growth curves were compared. As shown in **Fig. 2G**, all *A. vitis* strains demonstrated similar dependence on sucrose and organic acids for their growth. Interestingly, their degree of dependence on each type of organic acid varied slightly among these strains. While sucrose is the main limiting factor for the growth of VAR03-1, VAR06-30 requires both sucrose and organic acids for full growth. VAT03-9 exhibited an intermediate growth pattern between VAR03-1 and VAR06-30. This observation is consistent with the number of visible colonies around the PR shown in **Fig. 1D**. In the same experimental setting, we also assessed the nutrient requirements of Cab57. When grown in MS medium supplemented with sucrose and organic acids, Cab57 reached an OD_{600} value of around 0.6, indicating that Cab57 relies on these molecules for growth, with a particularly significant contribution from organic acids (**Fig. 2G**).

Interaction of VAR03-1 and *Arabidopsis* with exogenously applied sucrose

Our results demonstrated that sucrose is the primary carbon source essential for the growth of VAR03-1, suggesting that sucrose may play a significant role in the interaction of VAR03-1 and plants. To explore the role of sucrose in this interaction, we supplemented MS agar with a concentration series of sucrose (ranging from 0.1 to 10 mM). In the presence of sucrose at concentrations of 1 mM or higher, the PR length of *Arabidopsis* was significantly reduced by VAR03-1 (**Fig. 3A**). This sucrose-dependent inhibitory effect was also observed in the upper-ground parts of the plant at 1 mM, becoming more pronounced as the sucrose concentration increased to 10 mM (**Fig. 3B**). In the rhizosphere, the number and density of VAR03-1 colonies surrounding the *Arabidopsis* PR increased in a concentration-dependent manner (**Fig. 3C**). Although VAR03-1 colonies were distributed across the plate, likely due to their ability to utilize sucrose for growth, the densest populations were still observed in the upper-middle regions of the PR under sucrose-supplemented condition, even at 10 mM (**Fig. 3D**). To determine whether this sugar-dependent growth inhibition was specific to sucrose, we tested the effect of other sugars (glucose, fructose, mannitol and arabinose) on *Arabidopsis* growth in the presence of VAR03-1 (**Fig. 3E**). At a concentration of 10 mM, glucose, mannitol and arabinose, but not fructose, significantly inhibited PR length. Additionally, VAR03-1 colonies increased in response to these sugars in a manner similar to that observed with sucrose (**Supplementary Fig. S1**).

We hypothesized that the excessive proliferation of VAR03-1 might be responsible for the observed inhibition of *Arabidopsis* growth. To test this, we examined the *Arabidopsis* growth on MS agar supplemented with higher concentrations of VAR03-1 (final OD_{600} values of 5×10^{-3} and 5×10^{-2}, representing 10- and 100-fold increases, respectively, over the concentration used in **Fig. 1**). These higher concentrations of VAR03-1 reduced both PR length and shoot weight in *Arabidopsis*, even in the absence of exogenous sucrose (**Fig. 3F–H**). Although the inhibition was less pronounced than in the presence of sucrose, similar effects were observed (**Fig. 3H**). Notably, the inoculation of heat-killed (HK) VAR03-1 did not inhibit *Arabidopsis* growth, indicating that the inhibitory effect is associated with the activities of live bacterial cells (**Fig. 3F, G**). In sucrose-supplemented MS agar medium with VAR03-1, the onset of PR length inhibition occurred 4 d after planting young *Arabidopsis* seedlings (**Supplementary Fig. S2**). This result suggests that a certain threshold number of bacterial cells, which increased over 3 d, is necessary to exert a negative effect on *Arabidopsis* PR. Taken together, these results indicate that the proliferation of VAR03-1 cells above a certain level, driven by sugar supplementation, results in the inhibition of *Arabidopsis* seedling growth.

To further clarify this point, we searched for VAR03-1 mutants generated via transposon (Tn)-mediated random mutagenesis that had lost their growth inhibitory activity on *Arabidopsis* in sucrose-supplemented MS agar medium. Two mutants, Δ65 and Δ86, were successfully isolated, both exhibiting reduced inhibitory effects (**Supplementary Fig. S3A, B**). The Δ86 mutant completely lost its inhibitory effect on both root and shoot growth, while the Δ65 mutant showed a partial reduction; the inhibitory effect on shoot growth was largely diminished, but the effect on root remained similar to that of the wild-type (WT) strain. Both mutants demonstrated slightly slower growth in King's B (KB) medium compared to the WT strain (**Supplementary Fig. S3C**). In MS medium containing sucrose, the Δ86 mutant exhibited minimal growth, reaching only 24.2% of the WT strain's growth over a 16-h culture period. Meanwhile, the Δ65 strain reached 63.5% of the WT strains' growth within the same period (**Supplementary Fig. S3D**).

Based on these growth characteristics, the Δ65 mutant displayed a colonization pattern similar to the WT strain, with visible colonies around the PR that increased in density with sucrose supplementation (**Supplementary Fig. S3E**). In contrast, the Δ86 mutant did not form visible colonies around the PR and appeared to adhere to the root surface (**Supplementary Fig. S3E**). The density of the Δ86 mutant colonies on the root surface progressively increased in the presence of 10 mM sucrose (**Supplementary Fig. S3E**). The higher magnification observations revealed that the Δ86 mutant produced a small number of colonies at a short distance from the PR, and this pattern remained similar under 0.1 and 1 mM sucrose conditions.

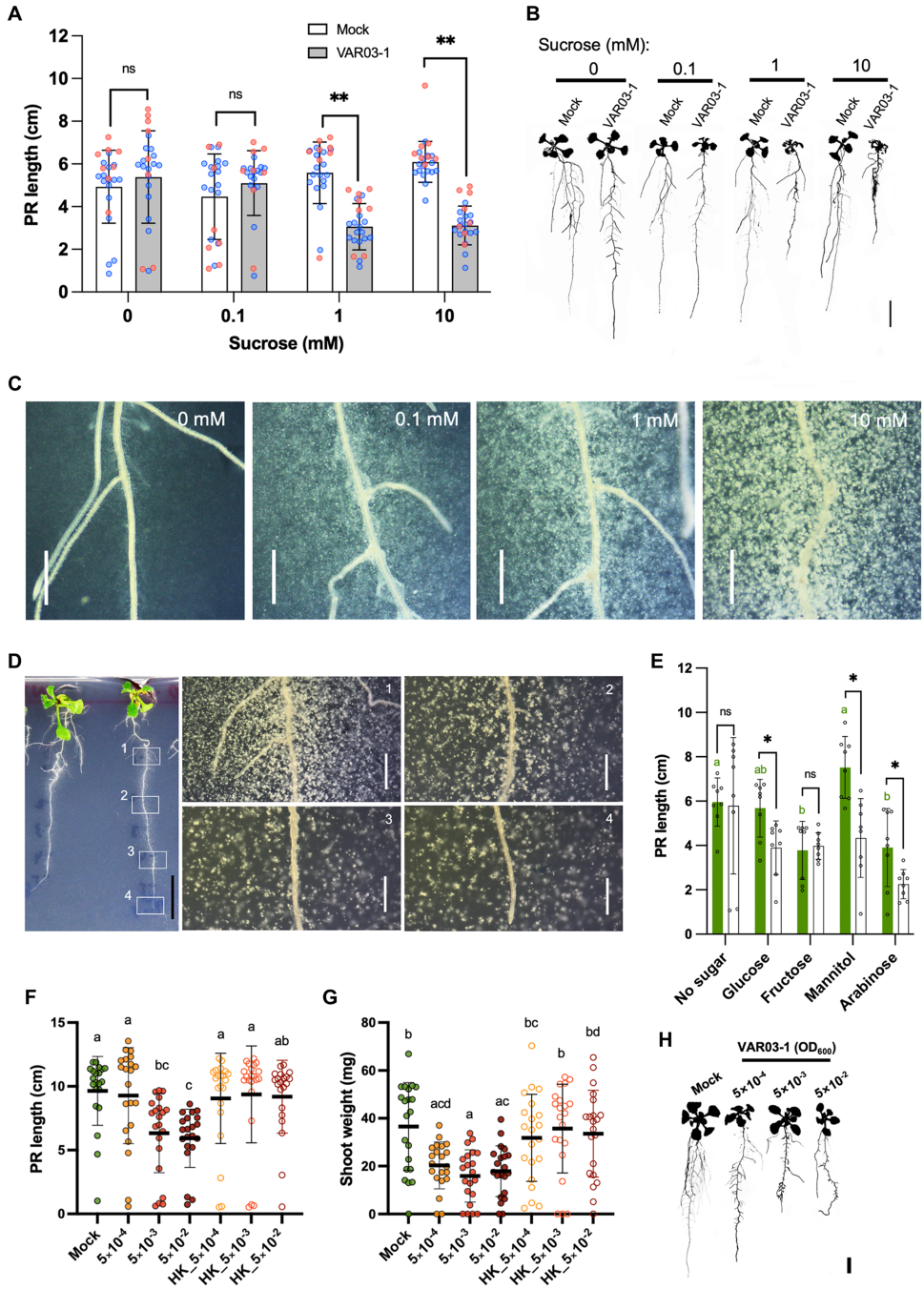

Fig. 3 Effects of sucrose on the *Arabidopsis* growth with *A. vitis* VAR03-1 in MS agar medium. (A, B) PR length (A) and morphology (B) of *Arabidopsis* plants grown on MS agar medium with VAR03-1 ($OD_{600} = 5 \times 10^{-4}$) supplemented with different concentrations of sucrose (0.1, 1 and 10 mM). Data from two distinct experimental sets were used. Statistical significance was analyzed using Mann–Whitney *U* test ($n = 24$). ns, not significant; $^{**}P < 0.001$. Bar, 1 cm. (C) Colonies of VAR03-1 around the upper PR regions of *Arabidopsis* roots in MS agar medium supplemented with different concentrations of sucrose (0.1, 1 and 10 mM). (D) Colonies of VAR03-1 along the PR of *Arabidopsis* grown in MS agar medium supplemented with 10 mM sucrose. Magnification images from some specific area along the roots indicated by boxes 1, 2, 3 and 4 were provided accordingly. Enlarged images of the specific area indicated by boxes 1, 2, 3 and 4 are provided. Black bar, 1 cm. White bars, 1 mm. (E) Length of *Arabidopsis* PR 8 d after transplanting on MS agar medium supplemented with different sugars (glucose, fructose, mannitol and arabinose, 10 mM each). Statistical significance was analyzed using Welch's *t*-test ($n = 8$) for comparison between mock and bacterial treatment in each sucrose assay. $^{*}P < 0.05$. (F, G) PR length (F) and shoot fresh weight (G) of *Arabidopsis* plants 14 d after transplanting on MS agar medium inoculated with VAR03-1 at different concentrations as indicated. Statistical significance was analyzed using the Kruskal–Wallis test followed by Dunn's test ($n = 21$) (F) and the Brown–Forsythe ANOVA followed by Dunnett's T3 test ($n = 21$) (G). $^{**}P < 0.001$; $^{*}P < 0.05$; ns, not significant. (H) Morphology of *Arabidopsis* seedlings 14 d after transplanting on MS medium inoculated with VAR03-1 at the indicated concentrations. Bar, 1 cm. All experiments were repeated at least twice with similar results.

A relatively thicker bacterial layer was observed only at 10 mM sucrose.

We identified the Tn insertion sites using the plasmid-rescue method, revealing that the Δ65 and Δ86 mutants contained Tns within the *cobJ* and *mprF* genes, respectively (**Supplementary Fig. S3G, H**). The *cobJ* gene encodes precorrin-3B methyltransferase, which is involved in the biosynthesis of cobalamin, a cofactor of several enzymatic reactions, including cobalamin-dependent ribonucleotide reductase for deoxynucleotide synthesis (Cowles et al. 1969). The *mprF* gene encodes the multiple peptide resistance factor, a bifunctional protein that synthesizes aminoacyl phospholipids or ions and translocates them to the outer leaflets of bacterial membranes (Song et al. 2021, Lee et al. 2022). This function is essential for bacterial survival against cationic antimicrobial peptides (Ernst and Peschel 2011) and adaptation to acidic environment (Sohlenkamp et al. 2007). Our results are consistent with previous studies that screened for less-colonizing bacterial mutants, identifying causal genes related to metabolism (Cole et al. 2017, Sivakumar et al. 2019). Currently, it remains unclear whether the function of each causal protein directly influences the sucrose-dependent growth inhibition of *Arabidopsis* plants. However, the defective proliferation phenotypes of both mutants in sucrose-containing MS medium were at least consistent with their diminished impact on *Arabidopsis* growth inhibition, where mutants with slower growth colonized poorly and hence induced less inhibition. Therefore, these results support the hypothesis that bacterial overgrowth is a cause of the inhibitory effect on *Arabidopsis* growth.

Bacterial-induced inhibition of *Arabidopsis* growth in the presence of exogenous sucrose exhibits non-strain-specific behavior

To determine whether the growth inhibition observed in the presence of sucrose was specific to VAR03-1, we evaluated two other *A. vitis* strains and *P. protegens* Cab57. Under sucrose-free conditions, all *A. vitis* strains, including the pathogenic VAT03-9, slightly promoted PR growth, indicating a consistent effect regardless of pathogenicity (**Fig. 1E, G**). However, with sucrose supplementation, significant growth inhibition was observed in both root and shoot parts across all *A. vitis*–treated seedlings compared to the control (**Fig. 4A–C**). Notably, the severity of the growth inhibition varied among the strains: VAT03-9 caused more pronounced PR and shoot inhibition than VAR03-1, while VAR06-30 induced a similar level of PR inhibition but significantly less shoot inhibition compared to VAR03-1. Meanwhile, Cab57 significantly reduced PR length even in the absence of sucrose (**Fig. 1E, G**). Upon sucrose supplementation, Cab57 induced growth inhibition on PR length in a concentration-dependent manner (**Supplementary Fig. S4A**). Shoot size remained similar across all sucrose concentrations tested, although it increased with sucrose supplementation. At 10 mM sucrose, Cab57 induced more severe PR inhibition than any of the *A. vitis* strains and also showed an inhibitory effect on shoot growth similar to that of the *A. vitis* strains (**Fig. 4A–C**). Visible colonies of Cab57 were not observed in MS agar medium at any site along the PR under no-sucrose conditions, but a small number of colonies were detected with sucrose, increasing with higher sucrose concentrations (**Supplementary Fig. S4C**). At 1 and 10 mM sucrose, *Arabidopsis* root morphology was severely affected with reduced PR length and increased number of lateral roots. Interestingly, swelling of the PR tip was observed in the presence of Cab57 specifically under 1 mM sucrose conditions (**Supplementary Fig. S4C–E**), which may be due to the accumulation of mucilage. These results suggest that bacterial-induced growth inhibition in the presence of sucrose is not unique to VAR03-1, as evidenced by the inhibitory effects observed in all *A. vitis* strains and *P. protegens* Cab57.

Relationship between nutrient requirements and bacterial growth patterns in the *Arabidopsis* rhizosphere

To further confirm the relationship between nutrient requirements and bacterial colonization patterns in the *Arabidopsis* rhizosphere, we transplanted *Arabidopsis* seedlings onto MS agar containing 10 mM sucrose together with a mixture of four organic acids (citrate, aspartic acid, *p*-hydroxybenzoate and 5-oxo-proline). These acids, at varying concentrations (50, 100 or 200 μM each), had previously enhanced the growth of VAR03-1 in vitro (**Fig. 2F**). In this setup, seedling growth inhibition was exacerbated by the supplementation of organic acids in a concentration-dependent manner (**Fig. 5**; **Supplementary Fig. S5**). In the presence of VAR03-1, seedling growth inhibition was further intensified by organic acid supplementation compared to sucrose alone (**Fig. 5A**, top row). Although colony formation of VAR03-1 around *Arabidopsis* roots was observed with both sucrose and organic acids, no visible increase in bacterial colony density from organic acid supplementation was apparent at any position along the PR (**Fig. 5A**; upper part, second row; lower part, third row; tip, fourth row). Quantifying bacterial cells in the agar medium might indicate increased bacterial titers, but it is technically challenging at this time. For Cab57, organic acids also exacerbated seedling growth inhibition, although statistical differences in PR length and shoot weight compared to sucrose alone were not detected (**Fig. 5B**, top row; **Supplementary Fig. S5**). In contrast to VAR03-1, closer magnification revealed that Cab57 colonies surrounding the PR, and even in the agar medium away from the roots, appeared to become denser and larger with higher concentrations of organic acids (**Fig. 5B**, middle and bottom rows). The colony formation patterns of VAR03-1 and Cab57 on agar medium supplemented with sucrose and organic acids were consistent with their nutrient requirements for growth in liquid MS culture (**Fig. 2G**). For both bacterial species, colony density appeared to be higher around plant roots compared to distant areas, suggesting the necessity of additional plant-derived nutrients beyond sucrose and the tested organic acids for full growth in the rhizosphere.

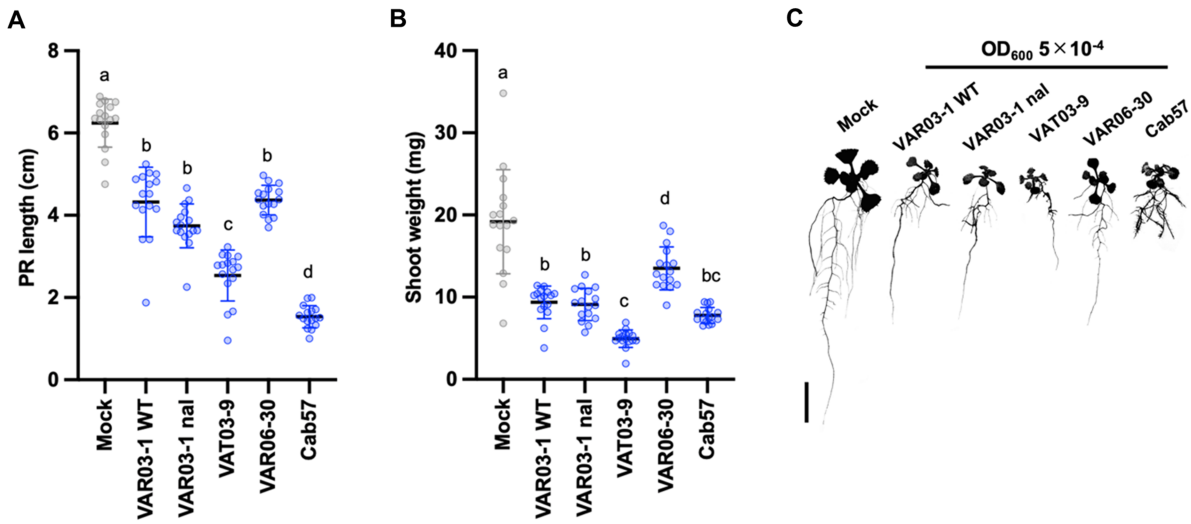

Fig. 4 Effect of sucrose on *Arabidopsis* growth in MS agar medium inoculated with *A. vitis* strains and *P. protegens* Cab57. (A–C) PR length (A), shoot fresh weight (B) and morphology (C) of *Arabidopsis* plants 8 d after transplanting onto 10 mM sucrose-containing MS agar medium inoculated with *A. vitis* strains or Cab57 ($OD_{600} = 5 \times 10^{-4}$). Statistical significance was analyzed using the Brown–Forsythe ANOVA followed by Dunnett's T3 multiple comparison test ($n = 16$) (A) and ANOVA followed by Tukey's test ($n = 16$) (B). Letters indicate significant differences. Bar, 1 cm. All experiments were repeated at least twice with similar results.

VAR03-1-induced *Arabidopsis* growth inhibition in the mutants for PTI

Plant growth inhibition triggered by immune responses to surrounding microbes often results from a trade-off between growth and defense (Boller and Felix 2009). Such inhibition could be abolished in plant mutants with compromised immune responses corresponding to the underlying causal factors (Okada et al. 2021). To investigate the cause of the VAR03-1-induced growth suppression in the presence of sucrose, we examined whether the inhibition induced by overgrowth of VAR03-1 was associated with PTI by employing *Arabidopsis* mutants *efr1*, *bak1-5/bkk1-1* and *bik1*, which are defective in the perception of elf18, a potential MAMP for *A. vitis* (Kunze et al. 2004) and MAMP-triggered signaling (Lu et al. 2010, Roux et al. 2011), respectively. As shown in **Fig. 6A, B**, both mutants exhibited growth inhibition in response to VAR03-1 in the presence of sucrose, similar to the WT.

Next, to confirm these results, we evaluated the growth inhibition of these *Arabidopsis* mutants in response to supplementation with a high concentration of VAR03-1 under sucrose-free conditions. Similar to the WT, all the tested *Arabidopsis* mutants showed reductions in PR length and shoot weight in response to VAR03-1 supplementation, at least at $OD_{600} = 5 \times 10^{-2}$, and in some cases at 5×10^{-3} (**Fig. 6C, D**). This suggests that VAR03-1-induced growth inhibition, regardless of the addition of exogenous sucrose, was not dependent on PTI, at least mediated by gene products tested.

To further investigate the involvement of PTI in the interaction between VAR03-1 and *Arabidopsis*, we compared the root and shoot weight of *Arabidopsis* seedlings (WT, *efr1* and *bak1-5/bkk1-1*) grown with VAR03-1 at $OD_{600} = 5 \times 10^{-4}$ and 5×10^{-3}. This comparison revealed that the growth inhibition triggered by VAR03-1 at an OD_{600} value of 5×10^{-3} was more pronounced in these *Arabidopsis* mutants, particularly in *bak1-5/bkk1-1*, compared to the WT (**Fig. 6E, F**). To estimate the reason for this difference, we cultivated these *Arabidopsis* seedlings in a hydroponic culture supplemented with VAR03-1 at $OD_{600} = 5 \times 10^{-4}$ and 5×10^{-3} and then counted the number of cells both on the root surface and in the liquid medium. Higher numbers of colonies were detected on the roots of *bak1-5/bkk1-1* compared to the WT at both inoculation concentrations (**Fig. 6G**). Additionally, increased colony numbers were observed in all plant genotypes at $OD_{600} = 5 \times 10^{-3}$ compared to those at $OD_{600} = 5 \times 10^{-4}$. It is noteworthy that this increased population of VAR03-1 in the mutant was observed only on the root surface, not in the medium (**Fig. 6H**). This result implies that plant immunity, particularly mediated by BAK1-BKK1, plays a specific role in regulating the VAR03-1 population in *Arabidopsis* roots. In terms of morphological alterations, the *bak1-5/bkk1-1* mutant facilitated higher levels of VAR03-1 colonization on the roots compared to the WT, and this increased population of VAR03-1 resulted in more severe growth inhibition in the mutant.

Arabidopsis growth inhibition induced by VAR03-1 in the defense hormone mutants

To further analyze the involvement of plant immunity in the plant–microbe interaction, we used *Arabidopsis* mutants impaired in salicylic acid biosynthesis and signaling (*npr1-5*, *sid2-2* and *eds1-2*) and ethylene signaling (*etr1-1*). None of the tested mutants showed a reduction in the inhibitory effects on both PR length and shoot weight compared to the WT in

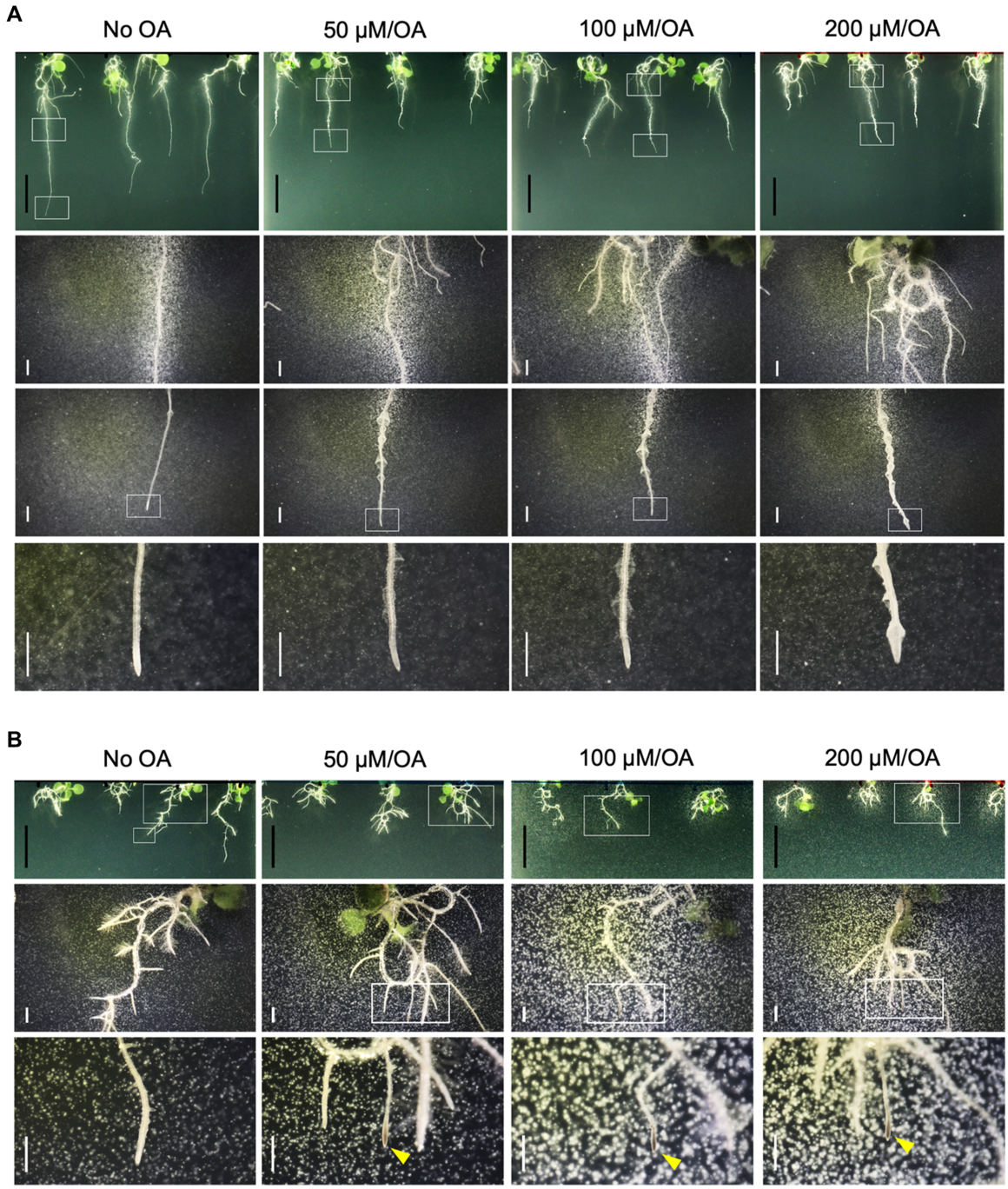

Fig. 5 Growth and bacterial colonization of VAR03-1 (A) and Cab57 (B) colonies around *Arabidopsis* seedlings 5 d after transplantation into MS agar supplemented with 10 mM sucrose with or without a combination of four organic acids (citrate, aspartic acid, *p*-hydroxybenzoate and proline 5-oxo) at different concentrations (50, 100 or 200 μM each). Seedling morphology and bacterial colonies on MS agar plate (first row) and the enlarged image at upper (second row) and tip (third row) parts of PR. The fourth row in (A) provides a further magnified view of the PR tip of the boxes in the third row. Black bars, 1 cm. White bars, 1 mm. All experiments were repeated at least twice with similar results.

the presence of VAR03-1 under sucrose supplement conditions (**Fig. 7A, B**).

For further confirmation, we assessed the expression level of *FRK1* and *PDF1.2*, marker genes for PTI, in both root and shoot tissues of *Arabidopsis* seedlings 8 d after transferring to MS agar containing VAR03-1 supplemented with different concentrations of sucrose. The expressions of these genes were generally higher in the presence of VAR03-1 compared to the uninoculated condition, except for *FRK1* expression in roots (**Fig. 7C, D**), indicating the immune response of *Arabidopsis* seedlings

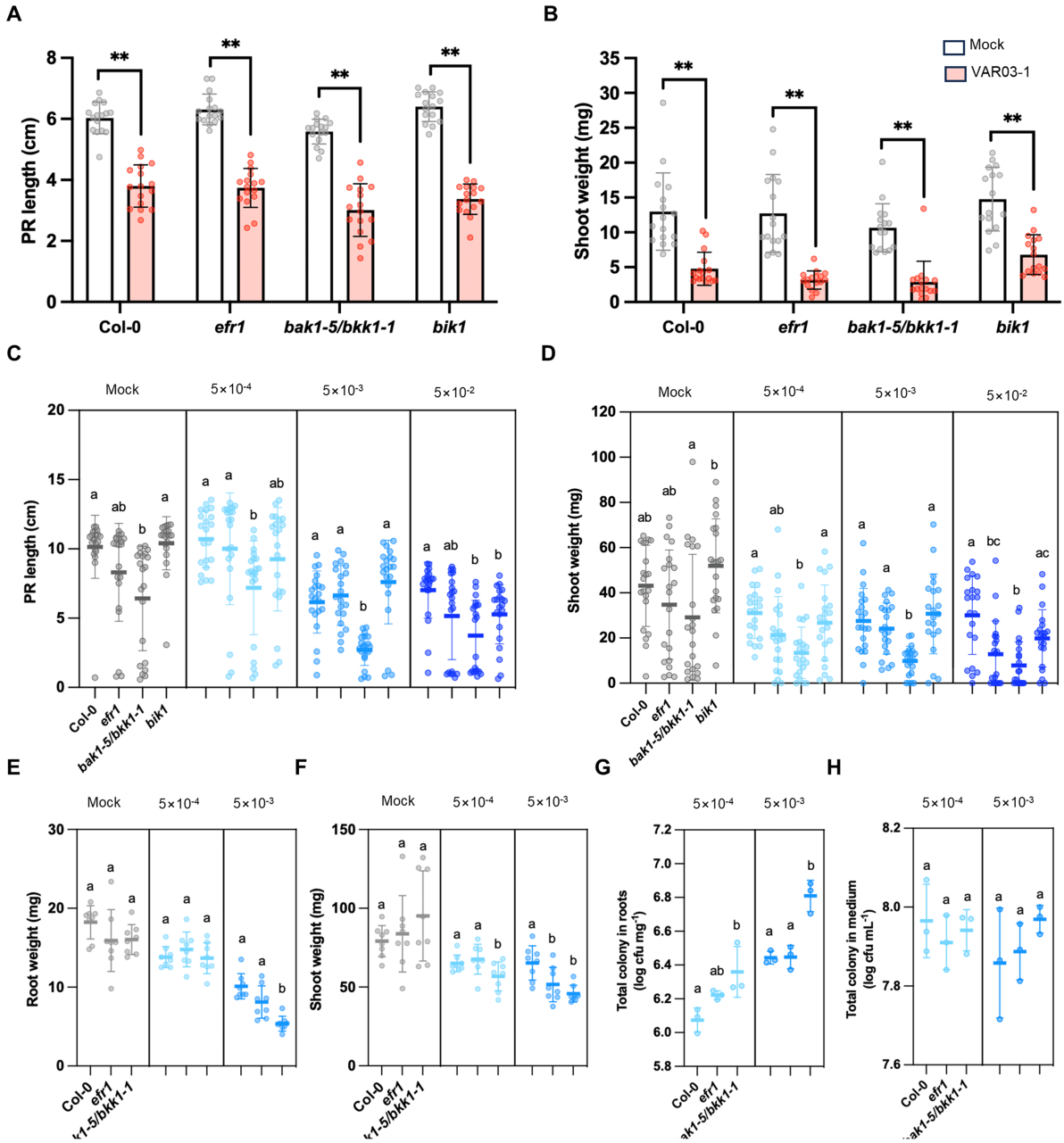

Fig. 6 Effect of sucrose in the presence of VAR03-1 or high concentrations of VAR03-1 cells on the growth of *Arabidopsis* PTI mutants. (A, B) PR length (A) and shoot fresh weight (B) of *Arabidopsis* mutants (Col-0 (WT), *efr1*, *bak1-5/bkk1-1* and *bik1*) 8 d after transplanting on MS agar medium with VAR03-1 ($OD_{600} = 5 \times 10^{-4}$) supplemented with 10 mM sucrose. Statistical significance was analyzed using Student's *t*-test ($n = 32$) (A) and the Mann–Whitney *U* test ($n = 16$) (B). $^{**}P < 0.001$. (C, D) PR length (C) and shoot fresh weight (D) of *Arabidopsis* mutants (Col-0 (WT), *efr1*, *bak1-5/bkk1-1* and *bik1*) 14 d after transplanting on MS agar medium with different concentrations of VAR03-1 ($OD_{600} = 5 \times 10^{-4}$, 5×10^{-3} and 5×10^{-2}). Statistical significance was analyzed using the Kruskal–Wallis test followed by Dunn's comparison test ($n = 21$) (C) and the Brown–Forsythe ANOVA followed by Dunnett's T3 ($n = 21$) (D). ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.001$. (E, F) Fresh weights of roots (E) and shoots (F) of *Arabidopsis* seedlings (Col-0 (WT), *efr1* and *bak1-5/bkk1-1*) 10 d after transplanting into a sucrose-free hydroponic culture inoculated with high concentrations of VAR03-1 ($OD_{600} = 5 \times 10^{-4}$ and 5×10^{-3}). Statistical significance was analyzed using one-way ANOVA and Tukey's test ($n = 8$). ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.001$. (G, H) Colony forming units (CFU) on roots (G) and medium (H) of *Arabidopsis* seedlings (Col-0 (WT), *efr1* and *bak1-5/bkk1-1*) 10 d after transplanting in hydroponic culture inoculated with high concentrations of VAR03-1 ($OD_{600} = 5 \times 10^{-4}$ and 5×10^{-3}). Statistical significance in different genotypes for the same VAR03-1 concentrations and in different concentration samples for the same genotypes using two-way ANOVA and Tukey's test ($n = 3$). Letters indicate significant differences among genotypes in each bacterial concentration. All experiments were repeated at least twice with similar results.

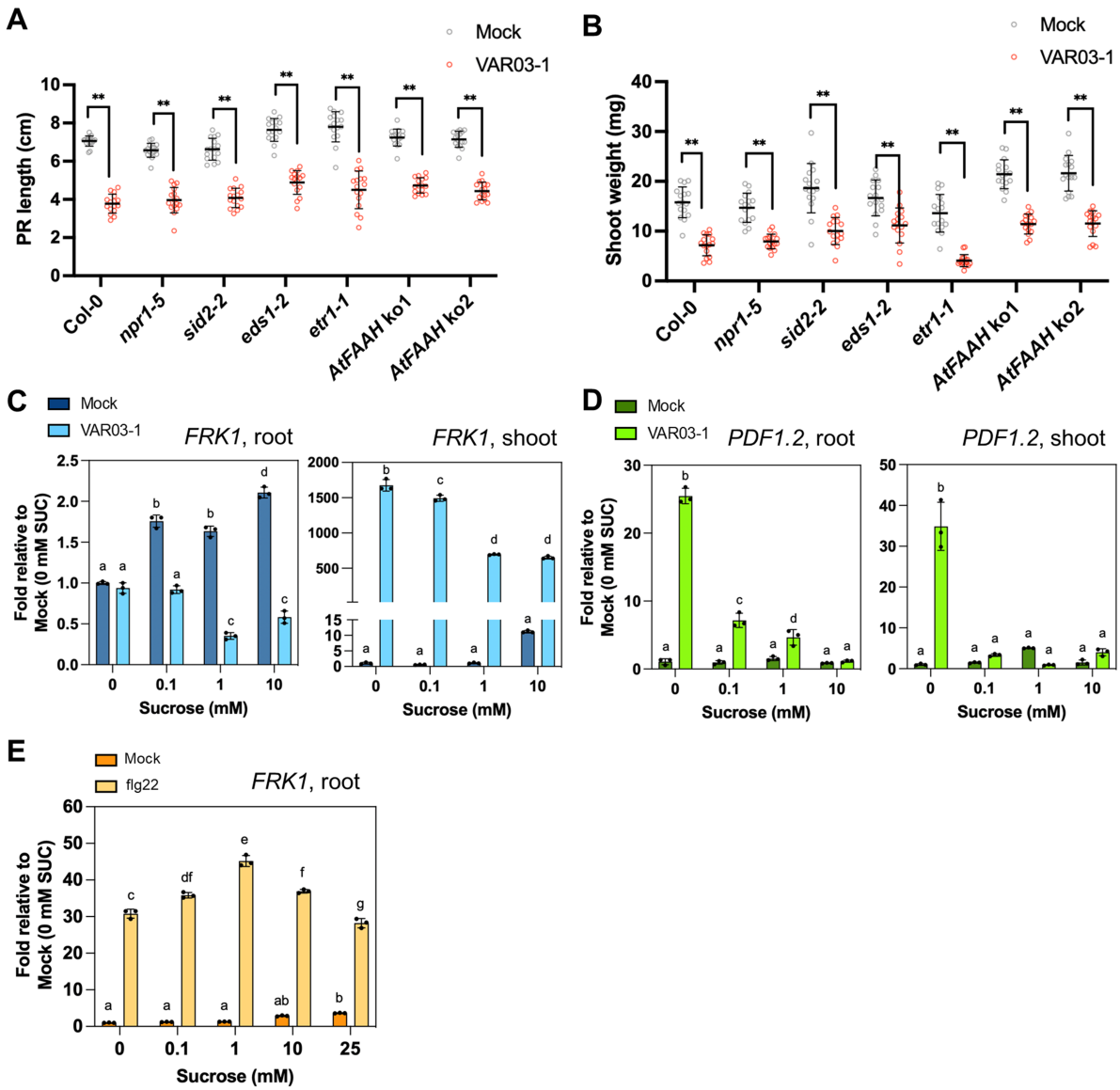

Fig. 7 Effect of VAR03-1 on the growth of *Arabidopsis* mutants related to defense hormones and fatty acid metabolism in the presence of sucrose. (A, B) PR length (A) and shoot fresh weight (B) of *Arabidopsis* plants (Col-0 (WT), *npr1-5, sid2-2, eds1-2, etr1-1* and *faah*) 8 d after transplanting on VAR03-1-inoculated MS agar medium containing 10 mM sucrose. Statistical significance of each mock treatment was analyzed using Student's *t*-test ($n = 16$). $^{**}P < 0.001$. (C, D) Expression of *FRK1* (C) and *PDF1.2* (D) in shoot and root parts of *Arabidopsis* seedlings 8 d after transplanting on VAR03-1-inoculated MS agar supplemented with different concentrations of sucrose. (E) Expression of *FRK1* in roots of *Arabidopsis* seedlings 3 d after application of 0.5 μM flg22 in MS agar supplemented with different concentrations of sucrose. Statistical significance was analyzed using ANOVA followed by Tukey's test ($n = 3$). All experiments were repeated at least twice with similar results.

to VAR03-1. In root tissues, *FRK1* gene expression was significantly downregulated in the presence of 1 and 10 mM sucrose (**Fig. 6C**). In shoot parts, a substantial induction of the *FRK1* gene was detected in response to VAR03-1 without sucrose, which decreased in the presence of 1 and 10 mM sucrose. As for the *PDF1.2* gene, its expression was induced by VAR03-1 in both roots and shoots, and it was decreased by sucrose in a concentration-dependent manner. Unlike the *FRK1* gene, a reduction of *PDF1.2* gene expression was observed even at 0.1 mM sucrose (**Fig. 7D**). To evaluate the effect of sucrose itself on *Arabidopsis* responsiveness to PTI, we measured the expression of *FRK1* induced by flg22 in the root tissues of *Arabidopsis* seedlings grown on sucrose-containing media. As a result, no significant reduction in the induction levels of *FRK1* in response to flg22 was observed at sucrose concentrations ranging from 0.1 to 10 mM compared to the sucrose-free medium (**Fig. 7E**). Plant growth suppression was observed even in the situation where defense gene expressions were attenuated in response to the overproliferation of VAR03-1 under conditions with sucrose (**Figs. 3A, B, 7C, D**), suggesting that the plant immune

response may not be the primary cause of this inhibitory effect.

N-acyl-L-homoserine lactone seems not to be involved in the VAR03-1-induced *Arabidopsis* growth inhibition

Bacteria produce *N*-acyl-L-homoserine lactones (AHLs) as signaling molecules to sense population density, which is crucial for controlling growth and facilitating the transition from a free-living state to biofilm formation (Verstraeten et al. 2008). The exogenous application of long-chain AHLs has been reported to inhibit *Arabidopsis* growth (Ortíz-Castro et al. 2008, Palmer et al. 2014). This inhibition is abolished in the knockout mutant and enhanced in the overexpressor of the *AtFAAH* (fatty acid amide hydrolase) gene, suggesting that ethylene production stimulated by homoserine from the metabolic conversion of AHLs by FAAH in *Arabidopsis* might be responsible for this effect (Palmer et al. 2014). To test whether AHLs contribute to VAR03-1-induced *Arabidopsis* growth inhibition in the presence of sucrose, we compared the response of *AtFAAH* mutants to the WT. Under our experimental conditions, two independent *AtFAAH* mutants showed reduced root length on MS medium containing both VAR03-1 and sucrose at a similar level to the WT (**Fig. 7A, B**). *A. vitis* strains, either virulent or non-virulent strains, have been reported to produce some long-chain AHLs, mediated by the *avsI* gene on their chromosome, which are basically impermeable through bacterial membranes (Li et al. 2006, Wang et al. 2008, Savka et al. 2011). We confirmed the presence of the *avsI* gene sequence in the VAR03-1 genome (Noutoshi et al. 2020b). Hence, we assume that VAR03-1 also produces long-chain AHLs. Similar research on *P. aeruginosa* has shown that long-chain AHLs are impermeable through bacterial membranes (Pearson et al. 1999). If this is also the case for VAR03-1, then the total external AHLs may not reach concentrations sufficient to induce plant-growth suppression, even though the ability of exogenously applied long-chain AHLs to inhibit plant growth. Taken together, we conclude that the AHLs produced by VAR03-1 may not significantly contribute to this growth inhibition.

Involvement of RecA in VAR03-1-induced inhibition of *Arabidopsis* growth in the presence of sucrose

Commensal bacteria form biofilms on the root surface during colonization, and extracellular DNAs or polysaccharides play a critical role as the structural basis for biofilm formation (Verstraeten et al. 2008, Ravaioli et al. 2020). Biofilm formation is regulated by RecA, a positive regulator of the Save Our Soul pathway, which responds to environmental stress (Turnbull et al. 2016, Kaushik et al. 2022, Wu et al. 2022). The loss of *recA* has been reported to reduce virulence in pathogenic bacteria (Martínez et al. 1997, Fuchs et al. 1999, Aranda et al. 2011, Corral et al. 2020). Therefore, we investigated whether biofilm formation is related to VAR03-1-induced growth inhibition of *Arabidopsis* in the presence of sucrose. To test this, we evaluated the effect of the *ΔrecA* mutant of VAR03-1 on the growth of *Arabidopsis* seedlings in MS agar containing sucrose. The growth inhibition induced by the *ΔrecA* mutant in both shoot and root parts was significantly reduced compared to the WT strain (**Fig. 8A–C**). The growth rate of *ΔrecA* was slightly lower than that of the WT strain in the liquid MS medium supplemented with sucrose, but its reduction level was smaller than those of Δ65 and Δ86 (**Supplementary Fig. S3D**). Although not quantitatively evaluated, the colony density of *ΔrecA* in the surrounding area of the PR appeared to be decreased compared to the WT strain, and instead, the cell layer on the root surface appeared to be increased (**Fig. 8D**). To further explore this result, we examined the biofilm formation of the *ΔrecA* mutant and found it to be higher than that of the WT strain (**Fig. 8E**). Correspondingly, the number of free-living (planktonic) cells was reduced in the *ΔrecA* mutant compared to the WT strain in this in vitro experimental system (**Fig. 8F**). These results suggest that RecA plays a negative role in the biofilm formation of VAR03-1. Given that the excessive sucrose-dependent growth inhibition of *Arabidopsis* seedlings was attenuated by the mutation in RecA, the deleterious activity of the increased VAR03-1 population may be associated more with bacterial behaviors typical of a free-living lifestyle rather than a sessile lifestyle.

Discussion

In this study, we explore the dynamics of the interaction between VAR03-1 and *Arabidopsis* under gnotobiotic conditions. VAR03-1 is a biocontrol agent for crown gall disease in grapevines. Disease suppression by antagonistic bacteria typically relies on its antibiotic activity against pathogens, as well as the induction of disease resistance in host plants. Additionally, competition for habitat on plant tissues plays an important role in this mechanism. Therefore, understanding the principles of rhizosphere colonization by biocontrol agents is necessary for expanding the use of biocontrol methods in crop protection, promoting environmentally friendly and sustainable agriculture.

We used *Arabidopsis* as a model to investigate the interaction of VAR03-1 with plants, despite initial uncertainties about whether VAR03-1 could establish interaction with Brassicaceae, including *Arabidopsis*. Since *A. vitis* strains are frequently isolated from grapevines as the causal agents of crown gall disease, they may have a colonization advantage over other bacteria, including those within the same genus of *Rhizobium*, in grapevines. However, VAR03-1 has also been shown to protect other plants such as apples, pears, roses and sunflowers from crown gall (Kawaguchi et al. 2007, 2008, 2012), suggesting its potential to colonize a range of plant species beyond Vitaceae. In our experiments, VAR03-1 was able to significantly increase its population in the vicinity of the *Arabidopsis* root system. It also promoted an increase in *Arabidopsis* PR length. These

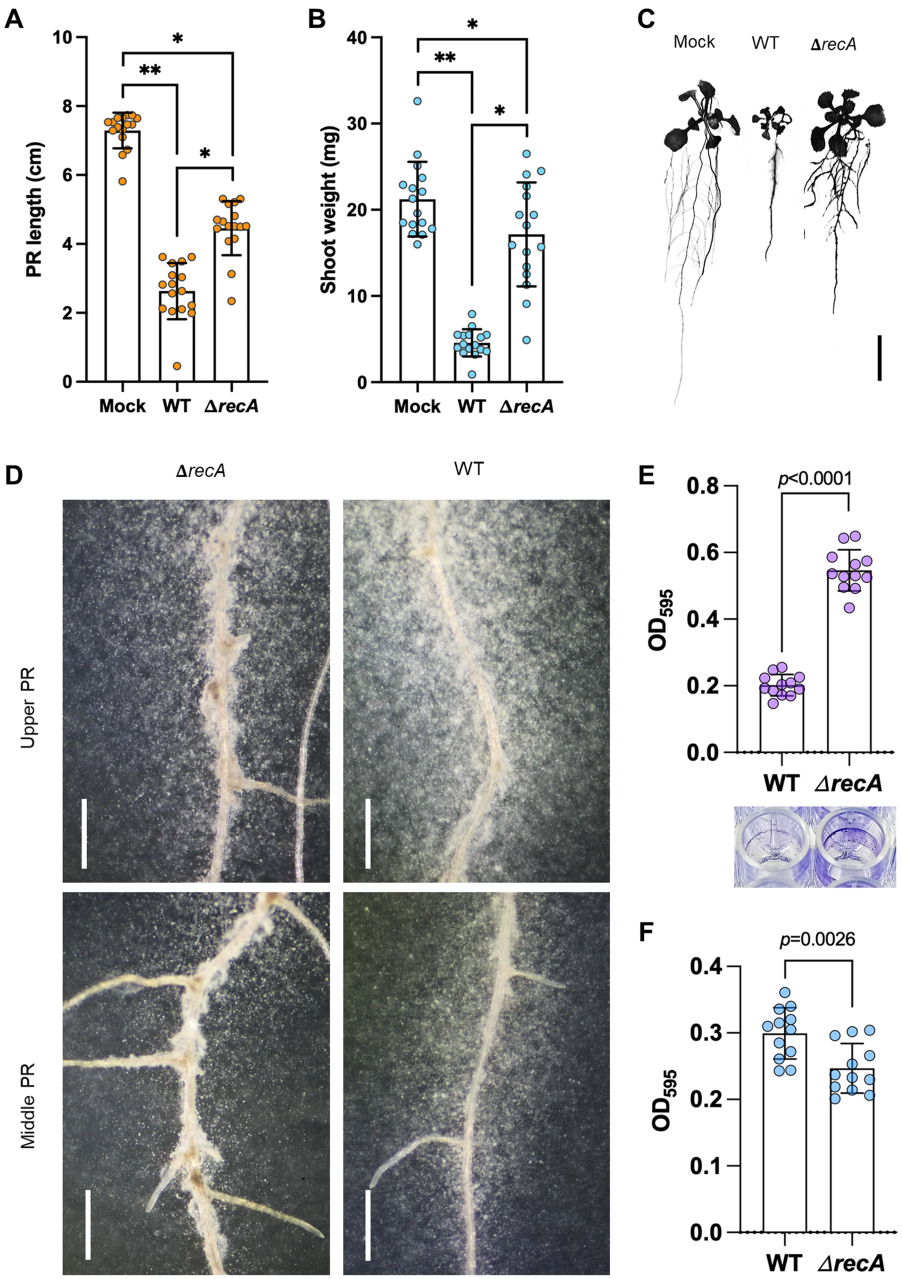

Fig. 8 Effect of the *ΔrecA* mutant of VAR03-1 on the growth of *Arabidopsis* in the presence of sucrose. (A, B) PR length (A), shoot fresh weight (B) and morphology (C) of *Arabidopsis* plants 8 d after transplanting on 10 mM sucrose-containing MS agar medium inoculated with VAR03-1 (WT and *ΔrecA*, $OD_{600} = 5 \times 10^{-4}$). Statistical significance was analyzed using the Kruskal–Wallis test followed by Dunn's test (A) and ANOVA followed by Tukey's test (B) ($n = 16$). *$P < 0.05$; **$P < 0.001$; ns, not significant. Bar, 1 cm. (D) Colonies of VAR03-1 (WT and *ΔrecA*, $OD_{600} = 5 \times 10^{-4}$) around the upper PR regions of *Arabidopsis* roots 8 d after transplanting to MS agar containing 10 mM sucrose. (E, F) Biofilm formation (E) and planktonic cells (F) of VAR03-1 WT and *ΔrecA* mutant after 6 d of incubation in ATM medium. All experiments were repeated at least twice with similar results.

findings suggest that VAR03-1 can effectively interact with *Arabidopsis*, at least to some extent, resembling a commensal bacterium (Garrido-Oter et al. 2018). Using a small model plant like *Arabidopsis* allows us to study the root colonization patterns of VAR03-1 and its effects on plant–microbe interactions. However, the applicability of these findings to other crops needs to be carefully validated.

We compared the colonization behavior of two different biocontrol bacterial agents, VAR03-1 and Cab57, from different genera, in the *Arabidopsis* rhizosphere. To our surprise,

their growth patterns in the root area appear to be distinctly different. This variation seems to be attributable to their differing nutrient requirements. Recently, Loo et al. (2024) reported that the composition of metabolic molecules of *Arabidopsis* roots varies depending on the position along the roots. Particularly, the distribution of Sugar Will Eventually be Exported Transporters, which control sugar sequestration, and sugars, including sucrose, are more abundant in the roots near the shoots compared to the tips. The sugars released from the roots may diffuse into the agar in our experimental setup, creating a concentration gradient. Given that sucrose or other sugars are major limiting factors for the growth of VAR03-1, VAR03-1 was able to form visible colonies in the *Arabidopsis* rhizosphere where sugar concentrations meet the growth demands of VAR03-1. Our results are consistent with previous findings that, in the rhizosphere, the presence of sugars in root exudates can profoundly influence microbial community dynamics, with particularly sucrose playing a significant role in shaping the microbial community during the early vegetative stage of maize (Lopes et al. 2022). The supplementation of sugars into the medium enables the further proliferation of VAR03-1 in this culture system. The background level of the number and density of VAR03-1 colonies elevated even at positions distant from *Arabidopsis* roots; however, the population gradient (predominant colonization near the upper part of the roots) was still observed. This suggests the presence of other limiting factors, including root exudates, for the full growth of VAR03-1 beyond sugars. This hypothesis is also supported by the varying effects of the *A. vitis* strains on PR length and shoot weight under 10 mM sucrose, which cannot solely be explained by their growth rates in the sucrose-containing liquid MS medium. The dependence of growth on organic acids may partly explain this observed difference. Since the composition of metabolites in root exudates varies among plant species (McLaughlin et al. 2023), differences in the requirement for these molecules as nutrients may contribute to host specificity and competition within the microbial community in the rhizosphere. Additionally, the catabolism or detoxification of host-specific specialized metabolites may also play a role in selectivity (Shimasaki et al. 2021). This concept is exemplified by the colonization pattern of Cab57. It colonized only the root surface and did not proliferate in the surrounding area of the PR. This behavior could be because Cab57 requires organic acids in addition to sugars for growth. With the supplementation of sugars, it began to form visible colonies near the roots and exhibited significant growth at the root tips when 1 mM sucrose was added. This habitat preference aligns with the observation that organic acids are predominantly released from root tips rather than other parts of the root (Loo et al. 2024). Consistently, the supplementation of organic acids with sucrose allowed Cab57 to grow in the agar. In the MS agar medium, Cab57 could not form visible colonies in the vicinity of the PR, suggesting that *Arabidopsis* may limit the total amount of nutrients released from roots to a level insufficient for the growth of Cab57, even at the root tip area. This restriction could be attributed to the deleterious effect of Cab57 on plant growth, as observed in *Arabidopsis*. This notion is supported by studies indicating that transcriptional changes in several SWEET in root tissues are closely associated with pathogen infections (Zhou et al. 2023, Loo et al. 2024). Furthermore, plants have been shown to limit or alter root exudate compositions in response to microbial infections (Gu et al. 2016).

Our study suggests a relationship between the habitat preference of commensal bacteria and their nutrient requirements in the rhizosphere. However, further analysis is needed to confirm whether such a relationship exists in soil. A previous study demonstrated a skewed distribution pattern of bacterial communities associated with particular genera in the apical, middle and rear parts of root sections in the soil rhizosphere where *Brachypodium distachyon* was grown (Wei et al. 2021).

For the biocontrol of plant diseases, it has been empirically established that potential antagonistic microorganisms belonging to the same genus as the pathogens can be effective. For example, VAR03-1 has been shown to effectively suppress crown gall caused by the pathogenic *A. vitis* (Kawaguchi et al. 2005, 2007). Our study provides evidence supporting this experiential knowledge. Because the habitat preference of soil bacteria varies significantly based on their metabolic characteristics, as demonstrated in this study, it is prudent to select candidate biocontrol agents for specific diseases from microbes that exhibit antagonistic activity and belong to the same genus as the target pathogen. Some strains of *Pseudomonas fluorescens* have been identified as effective biocontrol agents for several crop diseases (Couillerot et al. 2009). *Pseudomonas protegens* strains, including CHA0, Pf-5, Cab57 and others, protect various crops against fungal pathogens and oomycetes (Défago et al. 1990, Pfender et al. 1993, Takeuchi et al. 2014). The ability of *P. fluorescens* to colonize is crucial for providing protection to *Arabidopsis*, even against pathogens from the same genus *Pseudomonas* (Wang et al. 2022). Particularly for soil-borne pathogens, these bacteria may offer crop protection, predominantly in an epiphytic or endophytic manner near the root tip.

A study by Burr et al. (1995) found that *A. vitis* survived in decaying grapevine roots and canes buried in soil for 23 months. The decomposed plant materials, primarily consisting of cellulose and hemicellulose, were broken down into their monomer sugars within a few months (Gunina and Kuzyakov 2015). The metabolic features of *A. vitis* strains may contribute to their long-lasting survival and their role in the grapevine-associated microbiome by producing various metabolites after consuming sugars.

Sucrose supplementation resulted in the suppression of host growth by both VAR03-1 and Cab57. A study by Liu et al. (2012) in an aquatic ecosystem demonstrated that changes in nutrient availability favoring bacterial growth can shift interactions between aquatic algae and bacteria from commensalism to competition. This shift leads to a decrease in algal biomass, driven by increased bacterial proliferation and competition for nutrients. A similar depletion of nutrients by overgrown

bacteria may occur in our experimental setting. Alternatively, other bacterial-derived molecules besides AHLs, such as bacterial toxins, could contribute to this inhibition (Stroo et al. 1988, Bolton et al. 1989).

In our results, the *ΔrecA* mutation of VAR03-1 reduced its harmful activity to *Arabidopsis* in the presence of sucrose. Unlike Δ65 and Δ86, the *ΔrecA* mutant exhibited almost similar growth in liquid MS medium with only a slight reduction compared to the WT strain, but its inhibitory activity in the presence of sucrose was lower than that of the WT strain. This may be due to the decreased colonization (growth) in the surrounding areas of the roots. As colonies like biofilm on the root surface appeared to be increased, this may indicate a shift in bacterial habitat from a free-living to a sessile mode due to the loss of *recA*, as suggested by in vitro experiments. The effect of RecA on biofilm formation varies between bacterial species. For instance, the loss of *recA* leads to decreased biofilm formation in *Escherichia coli* (Beloin et al. 2004, Recacha et al. 2019), *Streptococcus mutans* (Inagaki et al. 2009) and *Bacillus cereus* (Gao et al. 2020), while it increases biofilm formation in *Acinetobacter baumannii* (Ching et al. 2024) and *P. aeruginosa* (Yahya et al. 2023). In *Staphylococcus lugdunensis*, the biofilm formation of *A. vitis* may depend more on exopolysaccharide (EPS) rather than environmental DNA from spontaneously bursting cells via RecA activity (Ravaioli et al. 2020). The increased biofilm formation observed in the *ΔrecA* mutant of VAR03-1 could be due to an increase in EPS or a behavioral change. Our study suggests that the growth inhibitory activity caused by the increased population of VAR03-1 in the rhizosphere is likely linked more to the biological activity associated with its free-living style. Disturbances affecting persistence and stability in commensalism with host plants, such as sucrose supplementation, should provide clues to understanding the molecular mechanisms and biological significance underlying commensalism. For this purpose, simplified and controllable experimental settings, alongside studies involving synthetic communities or soil systems, are crucial.

Materials and Methods

Plant material and growth conditions

Seeds were surface-sterilized using a 0.1% (v/v) sodium hypochlorite solution in a 1.5-ml tube, followed by shaking for 10 min at room temperature. The seeds were then rinsed seven times with sterile distilled water. During the last rinse, a portion of the water was retained to maintain seed submergence in the tube. Seeds were then stratified under dark conditions at 4°C for 3–4 d before being sown on plates containing half-strength MS agar [containing 0.1% (v/v) Gamborg vitamins and 0.8% (w/v) agar] supplemented with 1% sucrose. Plates were incubated in a growth chamber under long-day conditions (16 h photoperiod) at 22°C for further experiments. *AtFAAH* knockout mutants were a generous gift from Professor Kent D. Chapman from the University of North Texas.

Bacterial strains and growth conditions

All WT strains of *A. vitis*, including an antagonistic strain VAR03-1 (Noutoshi et al. 2020b), a pathogenic strain VAT03-9 (Ti) (Noutoshi et al. 2020c) and a non-pathogenic and non-antagonistic strain VAR06-30 (Noutoshi et al. 2020a), and *P. protegens* Cab 57 (Takeuchi et al. 2014) were cultured in KB medium without antibiotics. For VAR03-1 *ΔrecA* (Ishii et al. 2024), KB medium containing 30 μg/ml nalidixic acid was used. Prior to experiments, glycerol stock cultures were streak-inoculated onto KB agar with or without antibiotics and incubated at 27°C for 24 h for *A. vitis* strains and 12 h for *P. protegens* Cab57. The colonies were then transferred to KB liquid medium with or without antibiotics and incubated at 28°C with shaking at 165 rpm for 18 h for *A. vitis* strains and 12 h for *P. protegens* Cab57.

Bacterial growth assay

The bacterial culture was harvested by centrifugation (3,500 × *g*, 10 min) and resuspended in 10 mM $MgCl_2$. The bacterial suspension was then adjusted to an OD of 10 at 600 nm (OD_{600}). Subsequently, 150 μl of bacterial suspension was added to 15 ml of liquid MS [supplemented with 0.1% (v/v) Gamborg vitamins with or without sugars] (a final OD_{600} value of 0.1). Cultures were then incubated at 27°C (165 rpm), and OD_{600} was monitored every 2 h for 14–16 h. For supplementation of organic acids, 150 μl of 20 mM stock solution was added to 15 ml of MS medium prior to transferring the bacterial suspension, resulting in a final organic acid concentration of 200 μM (Anderson et al. 2014).

Bacterial inoculation on *Arabidopsis* seedlings

Bacterial inoculation was performed on square plates containing MS agar supplemented with 0.1% Gamborg vitamins and 0.8% agar, with or without sucrose or alternative sugars or organic acids. The bacterial suspension was then adjusted to an OD_{600} value of 0.5. Fifty microliters of bacterial suspension was added to 50 ml of warm MS agar and poured onto a square plate (resulting in a final OD_{600} value of 5×10^{-4}, equivalent to approximately 10^5 c.f.u./ml). For mock treatment, 50 μl of 10 mM $MgCl_2$ was used. After solidification, 5-day-old *Arabidopsis* seedlings were transferred to the square plate (8 seedlings/plate) and sealed with micropore tape. Two replicate plates were prepared for each treatment. The plates were arranged in a near-vertical orientation to facilitate root penetration into the agar. The plates were then kept in a growth chamber for 8–14 d until the roots almost touched the bottom of the plate. The photographs of the plates were taken using a mirrorless camera, and the PR length was measured using ImageJ software. Bacterial colonies around the roots were observed using a stereomicroscope. Finally, the shoots were separated from the roots and weighed.

In experiments with different initial concentrations of VAR03-1, the collected bacterial suspension was adjusted to OD_{600} values of 5, 0.5 and 0.05, resulting in final OD_{600} concentrations of 5×10^{-2}, 5×10^{-3} and 5×10^{-3}, respectively. Then, 500 μl of the bacterial suspension was mixed with 50 ml of warm MS agar without sucrose and poured onto a square plate. For the control (mock), 500 μl of 10 mM $MgCl_2$ was used.

Flg22 treatment for gene expression analysis

After a 3-day stratification period, sterile *Arabidopsis* seeds were germinated on MS agar plates. To minimize the potential effect of high sucrose on subsequent responses to flg22, seeds were not germinated on MS agar plates supplemented with 1% sucrose. Instead, they were germinated on MS agar plates with different sucrose concentrations to be tested (0, 0.1, 1, 10 and 25 mM). Five-day-old seedlings grown on MS agar with 1, 10 and 25 mM sucrose and 6-day-old seedlings grown on MS agar with 0 and 0.1 mM sucrose were selected for the experiment. This selection was made to standardize seedling size, as seedlings grown at lower sucrose concentrations tend to be smaller. Seedlings were then transferred to a square plate containing MS agar supplemented with 0.5 μM flg22 or sterile water with the same sucrose concentration as their germination medium. The plates were then incubated in a growth chamber for 3 d before RNA extraction.

Root colonization assay

For root colonization assessment, a hydroponic culture system using a 12-well plate was used instead of a square plate agar system. This system allowed the evaluation of bacterial colonies both attached to the roots and free in the medium. The liquid MS medium was supplemented with a bacterial suspension to achieve a final OD_{600} value of 5×10^{-4}. Five milliliters of this mixture was dispensed into each well. Sterile silicone rubber with slits, aligned with the position of each well in a row, was placed on top of each row. A 7-day-old *Arabidopsis* seedling was carefully positioned in the slit of each well, ensuring that the shoot protruded above the rubber while the roots were in contact with the medium below. This setup effectively sealed the surface of the well, leaving only the slit housing the seedling open, thus minimizing evaporation of medium. The plate was placed in a sterile, clear plastic box, sealed with micropore tape and incubated in a growth chamber. After 10–12 d of incubation, three or four representative seedlings from each treatment were selected for evaluation of root colonization. Roots were detached and transferred to a 2-ml tube. Roots were then washed twice with sterile water by vigorous pipetting and placed on filter paper to remove excess moisture before weighing. Roots were then transferred to a microtube containing 400 μl of sterile water and homogenized using a tissue homogenizer. An additional 600 μl of sterile water was added to create a 1,000 μl root suspension. Simultaneously, 1 ml of the medium was collected from the wells in which the selected seedlings were growing. Serial dilutions were then performed on both the root suspension samples and the medium from each seedling. Three 6-μl drops of each dilution were dispensed to three square plates containing KB agar. After allowing the drops to dry for approximately 3 min, the plates were sealed with micropore tape and incubated at 10°C for 3–7 d. The colonies that appeared on each drop were counted using a stereomicroscope.

Gene expression analysis

Inoculated *Arabidopsis* seedlings were harvested from the square plates at the designated time points. Roots and shoots were collected in separate tubes. Due to the small size of the seedlings, composite samples of roots or shoots from five to 15 seedlings were prepared in one tube. The tubes were immediately frozen in liquid nitrogen. RNA was extracted using the ISOSPIN Plant RNA kit (Nippon Gene, Tokyo, Japan), and cDNA was synthesized using the PrimeScript™ RT reagent kit with a gDNA eraser (Takara Bio, Kusatsu, Japan). Relative expression levels of *FRK1* and *PDF1.2* were quantified using Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) and the LightCycler 96 (Roche, Basel, Switzerland). *UBQ10* was selected as a housekeeping gene. Primers are listed in **Supplementary Table S1**.

Tn-mediated random mutagenesis

The production of the VAR03-1 mutant population by Tns has been described previously (Ishii et al. 2024). A total of 112 out of about 200 mutants were mixed in MS agar containing 1% sucrose at a final OD_{600} value of 5×10^{-4}, and the mutants with relatively less effect on growth suppression of *Arabidopsis* seedlings were selected. The identification of the transposition site based on the plasmid rescue method was also described previously (Ishii et al. 2024).

Biofilm formation assay and free-living cell measurement

The biofilm formation assay was performed on a 96-well plate according to Abarca-Grau et al. (2011) with some modifications. Briefly, bacterial culture grown overnight in liquid adherence test medium (ATM) (containing 2 g/l mannitol) supplemented with biotin (2 mg/l) was adjusted to an OD_{600} value of 0.04 in fresh liquid ATM medium. Subsequently, 200 μl of this bacterial suspension was dispensed into each well of a 96-well plate and incubated statically at 27°C. After 6 d, the liquid bacterial culture was transferred to a new 96-well plate for OD_{595} measurement of free-living (planktonic) cells. The original plate was then thoroughly rinsed three times with distilled water and air-dried for 45 min. Two hundred microliters of 0.1% crystal violet was added to each well and incubated for 45 min to stain sessile bacteria. The plates were then rinsed three times with distilled water and air-dried for 45 min. Two hundred microliters of 95% ethanol was added to each well, and 150 μl was transferred to a new 96-well plate. Biofilm formation was measured at OD_{595}.

Supplementary Data

Supplementary data are available at *PCP* online.

Data Availability

The data supporting the findings of this study are available within the article and its **supplementary data**.

Funding

Japan Society for the Promotion of Science (JSPS) KAKENHI (Grant-in-Aid for Challenging Research (Pioneering) JP20K20572 to Y.N.); Basic fund from Okayama University to Y.N.

Acknowledgments

Bacterial strains of *A. vitis* were kindly provided by Okayama Prefecture, Japan. *Pseudomonas protegens* Cab57 was obtained from the Genebank Project, NARO, Japan. *Escherichia coli* S17-1 λpir was obtained from the National BioResource Project *E. coli* at the National Institute of Genetics.

Author Contributions

Y.N. conceived of the study and designed the experiments. N.M.H., J.B. and M.W. performed all experiments. H.M., K.T., Y.I. and Y.N. provided equipment and facilities. N.M.H. and Y.N. drafted the manuscript, and N.M.H. and Y.N. revised and edited the draft manuscript. N.M.H., J.B., M.W., H.M., K.H., Y.I. and Y.N. contributed to the analyses, data interpretation and critical revision of the manuscript.

Disclosures

The authors have no conflicts of interest to declare.

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Plant Cell Physiol. 00(00): 1–18 (2024) doi:https://doi.org/10.1093/pcp/pcae091, Advance Access publication on 24 August 2024, available online at https://academic.oup.com/pcp