

Dimethyl Disulfide Produced by the Naturally Associated Bacterium *Bacillus* sp B55 Promotes *Nicotiana attenuata* Growth by Enhancing Sulfur Nutrition^W

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***Bacillus* sp B55, a bacterium naturally associated with *Nicotiana attenuata* roots, promotes growth and survival of wild-type and, particularly, ethylene (ET)-insensitive 35S-ethylene response1 (*etr1*) *N. attenuata* plants, which heterologously express the mutant *Arabidopsis thaliana* receptor ETR1-1. We found that the volatile organic compound (VOC) blend emitted by B55 promotes seedling growth, which is dominated by the S-containing compound dimethyl disulfide (DMDS). DMDS was depleted from the headspace during cocultivation with seedlings in bipartite Petri dishes, and ³⁵S was assimilated from the bacterial VOC bouquet and incorporated into plant proteins. In wild-type and 35S-*etr1* seedlings grown under different sulfate (SO₄²⁻) supply conditions, exposure to synthetic DMDS led to genotype-dependent plant growth promotion effects. For the wild type, only S-starved seedlings benefited from DMDS exposure. By contrast, growth of 35S-*etr1* seedlings, which we demonstrate to have an unregulated S metabolism, increased at all SO₄²⁻ supply rates. Exposure to B55 VOCs and DMDS rescued many of the growth phenotypes exhibited by ET-insensitive plants, including the lack of root hairs, poor lateral root growth, and low chlorophyll content. DMDS supplementation significantly reduced the expression of S assimilation genes, as well as Met biosynthesis and recycling. We conclude that DMDS by B55 production is a plant growth promotion mechanism that likely enhances the availability of reduced S, which is particularly beneficial for wild-type plants growing in S-deficient soils and for 35S-*etr1* plants due to their impaired S uptake/assimilation/metabolism.**

INTRODUCTION

Bacteria can affect plant performance through a variety of mechanisms. Among these, improving access to nutrients, particularly to the limiting macronutrient N, is a direct means of plant growth promotion (PGP). The symbiosis between legumes and diazotrophic rhizobia is the best-studied example of this. Improved access to other nutrients such as phosphorus, iron, or sulfate (SO₄²⁻) has also been reported in mutualistic plant-microbe associations (Kloepper et al., 1980; Rodríguez and Fraga, 1999; Kertész and Mirleau, 2004; Banerjee and Yesmin, 2009). In addition, bacteria can alter plant hormone homeostasis by producing phytohormones (e.g., auxins [indole-3-acetic acid], cytokinins, or gibberellins) or by lowering ethylene (ET) levels by metabolizing the plant ET precursor, 1-aminocyclopropane-1-carboxylic acid, as an N source (Glick et al., 2007).

In addition, bacterial volatile organic compounds (VOCs) have been shown to have PGP effects. The VOCs of *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937 increase *Arabidopsis thaliana* growth (Ryu et al., 2003). The PGP effects were largely attributed to two volatiles, namely, 3-hydroxybutan-2-one (acetoin) and 2,3-butanediol. Several other studies have examined bacterial bioactive volatile compounds that promote or suppress plant growth (Farang et al., 2006; Splivallo et al., 2007; Vespermann et al., 2007; Kai et al., 2009, 2010; Kai and Piechulla, 2009; Gutiérrez-Luna et al., 2010; Zou et al., 2010; Blom et al., 2011a, 2011b; Velazquez-Becerra et al., 2011; Weise et al., 2012; Yu and Lee, 2013), but the underlying mechanisms of these effects remain largely unknown (Wenke et al., 2012b). Profiling studies in *Arabidopsis* seedlings provided the first insights into changes in the transcriptome and proteome elicited by exposure to bacterial VOCs (Zhang et al., 2007; Kwon et al., 2010) and revealed the importance of hormone signaling, particularly that of indole-3-acetic acid and abscisic acid (Zhang et al., 2007, 2008).

The phytohormone ET plays multiple roles in plant development and defense (Pierik et al., 2006; van Loon et al., 2006), regulating processes such as seedling emergence, flower development, and fruit abscission, as well as biotic and abiotic stress responses (Davidson, 1949; Wilson, 1966; Rasmussen and Cooper, 1968; Pegg, 1976; Pegg and Cronshaw, 1976; Egley, 1980; Kahl et al., 2000). Recently, several studies have highlighted ET's prominent role in mediating mutualistic plant-microbe interactions (Penmetta and Cook, 1997; Iniguez et al., 2005; Long et al., 2010)

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and due to its gaseous nature, studies on microbial VOC-mediated signaling processes in plants have focused on ET (Bailey and Weisskopf, 2012). Indeed, *ethylene insensitive2* mutants are non-responsive to VOC-mediated PGP by *B. amyloliquefaciens* IN937 and *Piriformospora indica* (Ryu et al., 2003; Camehl et al., 2010). However, other ET-insensitive mutants respond to the VOCs similarly to the wild type. Furthermore, ET emission by VOC-treated plants was found to be unaltered (Ryu et al., 2003). On the other hand, recent transcriptome and proteome analyses of VOC-exposed *Arabidopsis* plants revealed regulation of genes and proteins involved in ET signaling and biosynthesis (Kwon et al., 2010). Although these studies suggest a role for ET in microbial VOC-mediated interactions, unambiguous proof is lacking.

Although studies with well-established plant and bacterial model organisms have been essential in discovering the roles of specific metabolites and mechanisms of PGP, their importance in native plant–bacteria interactions remains largely unstudied. We have previously shown that ET signaling determines the culturable bacterial endophyte community in roots of Coyote tobacco (*Nicotiana attenuata*) in the natural environment and identified natural root-associated bacteria that improve *N. attenuata* growth and survival (Long et al., 2010). *Bacillus* sp B55, isolated from ET-insensitive transgenic *N. attenuata* plants (*35S-etr1*, which heterologously express the mutant *Arabidopsis* receptor ETR1-1), causes dramatically increased survival and growth of *35S-etr1* plants grown in nature (Meldau et al., 2012). However, the mechanisms of B55's PGP remain elusive.

Here, we report one mechanism responsible for B55's remarkable PGP effects on *N. attenuata* plants. We show that VOCs emitted by B55 promote wild-type and *35S-etr1* seedling growth and identify an S-containing VOC, dimethyl disulfide (DMDS), that is released by B55 and confers the observed PGP effects in *N. attenuata* plants. We find that *35S-etr1* plants are impaired in SO_4^{2-} uptake and S metabolism and that DMDS and VOCs emitted from B55 ameliorate these defects. Our data demonstrate that plants can benefit from their interaction with PGP bacteria by obtaining reduced S (i.e., in the form of bacterial DMDS), which helps to fulfill their S requirements.

RESULTS

B55 VOCs Promote Wild-Type and *35S-etr1* Seedling Growth

To evaluate whether volatiles contribute to the previously described PGP effect of B55 on wild-type and *35S-etr1* plants (Meldau et al., 2012), we cocultivated wild-type and *35S-etr1* seedlings (on GB5 agar, containing all essential macro- and micronutrients, including 2 mM SO_4^{2-}) with B55 (on 0.5× yeast peptone dextrose agar [YPDA]) on bipartite Petri dishes, where seedlings and bacteria had contact only through a shared headspace. After 12 d, the surface area of seedlings exposed to the B55 VOCs was 5 and 8 times higher than that of wild-type and *35S-etr1* control plants, respectively (Figures 1A and 1B). B55 VOC-exposed seedlings of both genotypes also produced more true leaves (Figure 1C), more lateral roots per cm of primary root length (Figure 1D), and longer roots (Figure 1E) than did controls, with larger increases observed for *35S-etr1* seedlings

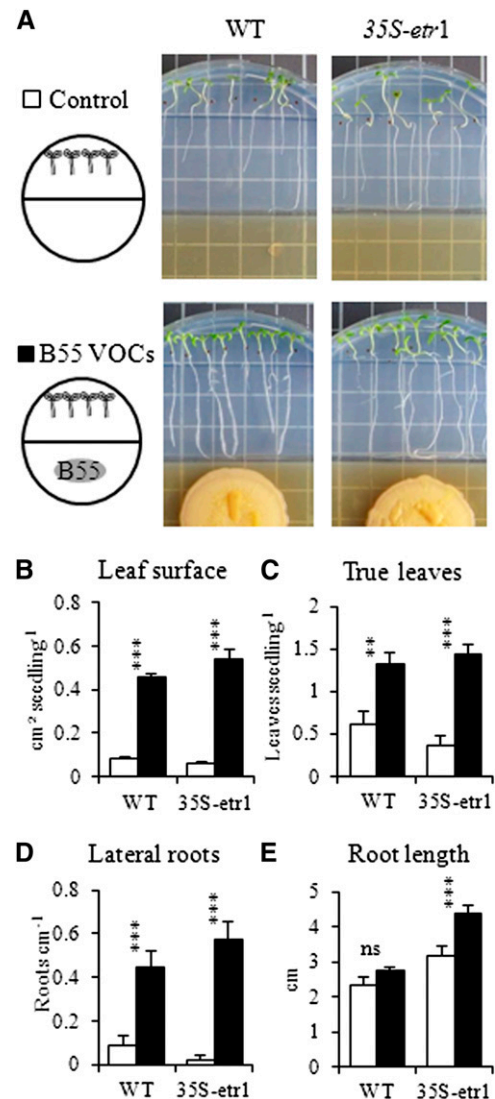


Figure 1. Effect of B55 VOCs on Seedling Growth.

(A) Experimental design and effect of B55 VOCs. Wild-Type (WT) and *35S-etr1* seedlings were cocultivated with or without B55 for 12 d in bipartite Petri dishes containing GB5 medium (seedlings) and 0.5× YPDA (B55).

(B) Mean (\pm SE) seedling surface area.

(C) Mean (\pm SE) number of true leaves.

(D) Mean (\pm SE) number of lateral roots.

(E) Mean (\pm SE) primary root length.

For (B) to (E), a Student's *t* test between mock- and B55 VOC-treated seedlings was performed with **P < 0.01 and ***P < 0.001. ns, not significant. *n* = 4 Petri dishes with 20 [(B) and (C)] or seven seedlings [(D) and (E)].

compared with the wild type. Chlorophyll *a* and *b* contents were lower in *35S-etr1* seedlings compared with the respective wild-type treatment group, and the exposure to B55 VOCs did not change chlorophyll *a* and *b* contents of wild-type and *35S-etr1* seedlings (see Supplemental Figure 1 online). These data demonstrate that VOCs emitted from B55 are sufficient to promote plant growth in *N. attenuata* wild-type and *35S-etr1* seedlings grown on GB5.

DMDS Is Emitted by B55 and Taken up by Seedlings

We next analyzed by solid phase microextraction (SPME) gas chromatography–mass spectrometry (GC–MS) the composition of the headspace volatile bouquet of 12-d-old B55 cocultivated with and without wild-type seedlings on a bipartite Petri dish. The S-containing metabolite DMDS (CAS 624-92-0) proved to be an abundant VOC produced by B55 (and not by media or seedlings) that was depleted from the headspace upon cocultivation with seedlings (Figure 2; see Supplemental Figure 2 online). Cocultivation of a standardized inoculation of B55 with increasing numbers of seedlings correlated with decreases in DMDS headspace concentration (see Supplemental Figure 3A online). Transcript abundances of B55 genes coding for enzymes involved in bacterial DMDS production (*L-METHIONINE- γ -LYASE*, *CYSTATHIONINE- γ -LYASE*, and *CYSTATHIONINE- β -LYASE*) were not affected by the presence of seedlings (see Supplemental Figure 3B online). Taken together, these results are consistent with the idea that DMDS is absorbed and potentially assimilated by the seedlings rather than that its production suppressed by the presence of seedlings.

We further found that a singly cultivated, 14-d-old B55 colony accumulated ~ 1 ng DMDS per cm^2 of colony in its headspace in one day. Due to the rapid removal of DMDS by the seedlings, in situ

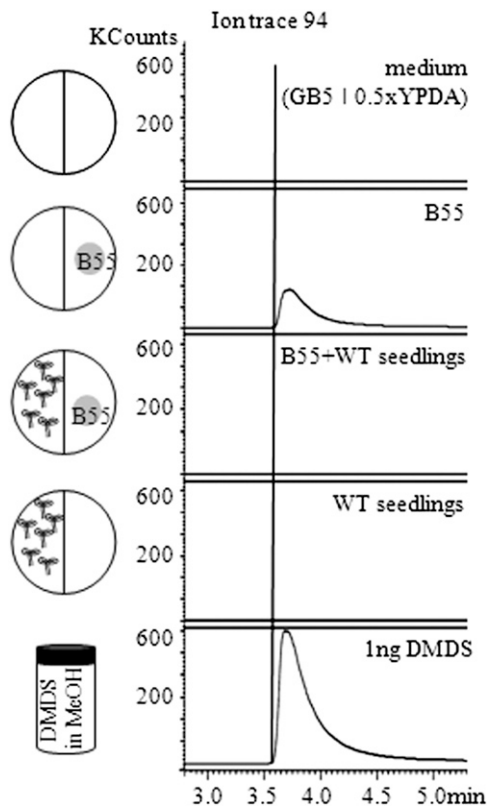


Figure 2. Depletion of Headspace DMDS after Cocultivation of B55 with Seedlings.

GC–MS spectra of the selected molecule (ion 94) from cultivation medium, B55, cocultivation (B55+WT seedlings), wild-type (WT) seedlings, and a DMDS standard.

quantification of DMDS released by B55 in the presence of seedlings is difficult. In general, DMDS production was promoted when B55 colonies were grown on a full medium, such as YPDA, at high incubation temperatures, and under light, and DMDS production grew with colony age (see Supplemental Figures 4A to 4C online).

35S-etr1 Plants Absorb More ^{35}S Than Do Wild-Type Plants

To test if DMDS was absorbed and assimilated by seedlings, the incorporation of volatile S into seedling protein was evaluated. We performed ^{35}S -labeling experiments using bipartite Petri dishes, in which B55 and seedlings had contact only through the shared headspace and in which B55 was grown on minimal medium (M9) containing ^{35}S -labeled Na_2SO_4 as the sole S source (Figure 3A). ^{35}S from a ^{35}S -containing VOC (most probably DMDS) was incorporated into wild-type seedling proteins when the seedling growth medium was S deficient (Figure 3B; see Supplemental Figure 5 online). Although gel exposure was extended for 3 months, no radioactively labeled protein was detected for wild-type seedlings grown on S-replete media, likely due to insufficient sensitivity of the imaging screen.

Since *35S-etr1* seedlings realize a greater benefit compared with wild-type seedlings from inoculation with B55 (Meldau et al., 2012) and exposure to B55 VOCs (Figure 1), we evaluated whether *35S-etr1* seedlings absorbed more DMDS than wild-type seedlings. Using tripartite Petri dishes (Figure 4A), we found that S-starved seedlings (grown on MM2, which lacks S) absorbed larger amounts of ^{35}S than did seedlings grown on SO_4^{2-} -rich media (MM2, 2 mM SO_4^{2-}) (Figure 4B). Most interestingly, *35S-etr1* seedlings assimilated more ^{35}S than did wild-type seedlings, particularly under high SO_4^{2-} supply (Figure 4B). These findings led to the hypotheses that (1) bacterial volatile S contributes to *N. attenuata*'s S nutrition when grown in an SO_4^{2-} -depleted environment and that (2) *35S-etr1* seedlings have higher S requirements.

35S-etr1 Seedlings Are Impaired in S Metabolism

To evaluate the importance of S nutrition for *N. attenuata* wild-type and *35S-etr1* seedlings, we grew seedlings on MM2 containing different SO_4^{2-} concentrations (0, 50, or 1000 μM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$). As expected, for 20-d-old wild-type seedlings, leaf surface area and chlorophyll a concentration correlated positively with increasing SO_4^{2-} concentration (Figures 5A, first column, to 5C, open bars; see Supplemental Figure 6 online). The total free amino acid concentrations decreased with increasing SO_4^{2-} concentration (Figure 5D, open bars). Lateral root number also decreased slightly with increasing SO_4^{2-} (Figure 6A, open bars). However, *35S-etr1* seedlings did not show these correlations. Even under high SO_4^{2-} supply (1000 μM) *35S-etr1* seedlings appeared abnormal, with enlarged, swollen, and yellowish leaves (Figure 5A, third column) and increased seedling surface area compared with the wild type (Figure 5B, open bars). The chlorophyll a content was only half that of the wild type and the lateral root number was consistently lower in *35S-etr1* seedlings (Figures 5C and 6A, open bars). Compared with the wild type, total free amino acid concentrations were higher in *35S-etr1* seedlings (Figure 5D, open bars).

Sulfate concentration in the culture medium also affected the levels of S-containing metabolites in the plants. In wild-type seedlings, the concentrations of free Met and reduced and

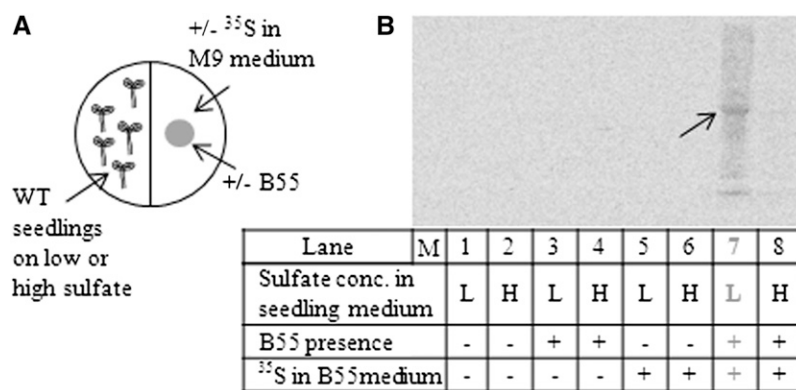


Figure 3. Incorporation of ^{35}S -Labeled Volatile Sulfur into *N. attenuata* Seedling Protein.

(A) Setup of the experiment. WT, the wild type.

(B) Radioactivity screen and table summarizing treatment combinations. The arrow depicts the incorporation of ^{35}S into wild-type seedling protein. L, low SO_4^{-2} concentration; H, high SO_4^{-2} concentration; M, marker.

oxidized glutathiones (GSH and GSSG) increased with medium SO_4^{-2} concentration (Figures 5E to G, open bars). Compared with the wild type, the levels of free Met, GSH, and GSSG in *35S-etr1* seedlings grown in the presence of SO_4^{-2} were reduced (Figures 5E to 5G, open bars). To test whether *35S-etr1* plants are impaired in SO_4^{-2} uptake, we performed an SO_4^{-2} uptake experiment using radioactively labeled $\text{Na}^{35}\text{SO}_4$. As expected, S starvation dramatically induced ^{35}S assimilation by the roots in both genotypes. However, whereas S-replete wild-type and *35S-etr1* plants assimilated similar amounts of ^{35}S , the S starvation induced SO_4^{-2} uptake by *35S-etr1* plants was one-third lower than that in the wild type ($P < 0.001$) (Figure 7).

Due to their ET insensitivity, *35S-etr1* plants overproduce ET constitutively as well as in response to biotic elicitation (von Dahl et al., 2007). ET production is tightly coupled to the Yang cycle in which the S-containing amino acid Met is recycled (Miyazaki and Yang, 1987). Our results suggest that *35S-etr1* plants have an unregulated S metabolism. Hence, we analyzed how the availability of SO_4^{-2} influenced ET production. In line with the previous report (von Dahl et al., 2007), the ET-insensitive *35S-etr1* plants had higher basal ET emissions compared with the wild type, irrespective of the SO_4^{-2} supply (see Supplemental Figure 7 online). We hypothesized that deregulation in the S assimilatory pathway and/or the Yang cycle (Met cycle), which are related to ET overproduction, could account for the growth phenotype of *35S-etr1* and hence analyzed transcript abundances of key enzymes in S assimilation and Cys biosynthesis (i.e., high affinity SULFATE TRANSPORTER [SULTR], ADENYLYLSULFATE REDUCTASE [APR], SULFITE REDUCTASE [SIR], and O-ACETYLSELINE [THIOL] LYASE [OAS-TL]), Met biosynthesis (i.e., CYSTATHIONINE- Γ -SYNTHETASE [CGS] and METHIONINE SYNTHASE [MS]), and Yang cycle (S-ADENOSYLMETHIONINE SYNTHETASE [SAMS], METHYLTHIOADENOSINE NUCLEASE [MTN], and METHYLTHIORIBOSE KINASE [MTK]). Wild-type and *35S-etr1* seedlings were grown for 18 d on MM2 medium, supplemented with different concentrations of SO_4^{-2} (0, 50, or 1000 μM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$). Transcript levels of all genes except Na-MTN were significantly affected by the SO_4^{-2} concentration ($P < 0.05$) (Figure 8,

open bars; see analysis of variance [ANOVA] table in Supplemental Table 1 online). The high affinity Na-SULTR, Na-APRs, Na-SIR, Na-SAM 140, and Na-MTK were strongly induced when seedlings were SO_4^{-2} starved. Additionally, transcript levels of the Na-APRs, Na-OAS-TLs, Na-CGS, Na-MS, Na-SAM 113, and Na-MTK were also significantly affected by plant genotype ($P < 0.05$): Control *35S-etr1* seedlings accumulated significantly higher transcript levels than the wild type.

Taken together, these findings are consistent with the hypothesis that *35S-etr1* seedlings are impaired in S metabolism.

DMDS Promotes Wild-Type and *35S-etr1* Seedling Growth

To evaluate the contribution of DMDS to wild-type and *35S-etr1* seedling S nutrition, we applied the pure compound to the head-space of the seedlings. To find an effective dose, we first spotted

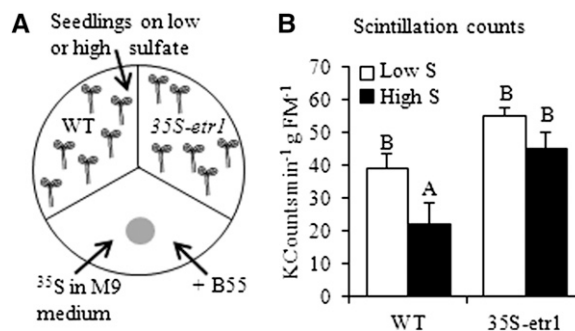


Figure 4. Uptake of ^{35}S -Labeled Volatile Sulfur by *N. attenuata* Wild-Type and *35S-etr1* Seedlings.

(A) Setup of the experiment. WT, the wild type.

(B) Scintillation counts (\pm SE) of wild-type and *35S-etr1* total seedling tissue after 20 d of cocultivation with B55. A protected least significant difference (PLSD) test of an ANOVA between B55 VOC-treated wild-type and *35S-etr1* seedlings grown on low or high sulfate concentration was performed. Different letters denote statistical differences at $P < 0.05$. S, sulfate; FM, fresh mass. $n \geq 6$ Petri dishes with 15 seeds.

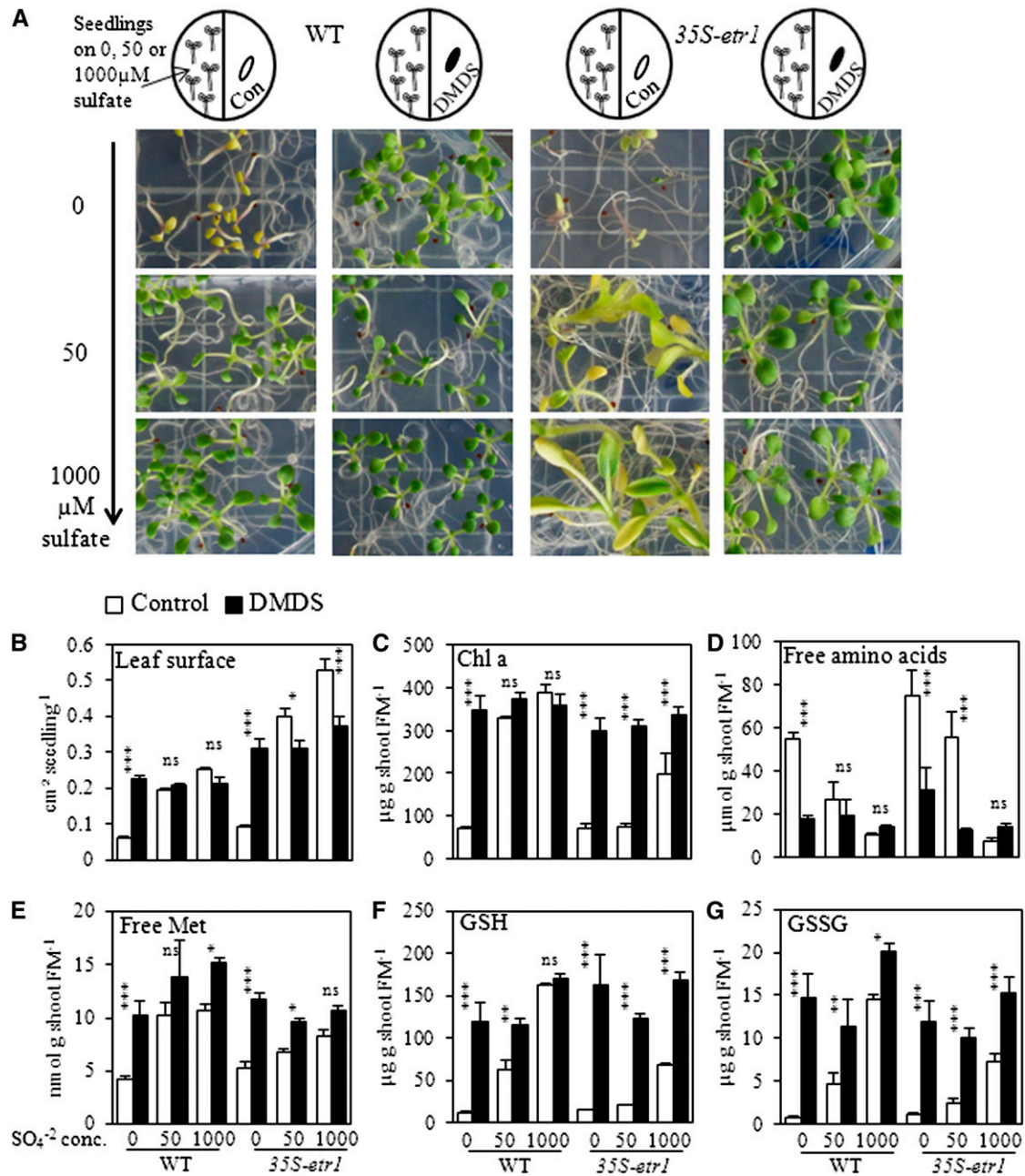


Figure 5. Effect of Genotype and DMDS on Seedling Shoot Growth and S-Containing Compounds.

(A) Experimental design and effect of DMDS on wild-type (WT) and *35S-etr1* seedlings. Seedlings were grown for 20 d in bipartite Petri dishes containing MM2 medium supplemented with different sulfate concentrations (0, 50, and 1000 μM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$).

(B) to (G) Mean (\pm SE) shoot surface area **(B)**, mean (\pm SE) chlorophyll *a* (Chl *a*) concentration **(C)**, mean (\pm SE) total amino acid concentration **(D)**, mean (\pm SE) free Met concentration **(E)**, mean (\pm SE) GSH concentration **(F)**, and mean (\pm SE) GSSG concentration **(G)** of mock- or DMDS-treated wild-type and *35S-etr1* seedlings. A PLSD test of an ANOVA between mock- and DMDS-treated seedlings was performed with * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. FM, fresh mass; ns, not significant. $n =$ four Petri dishes with 20 seeds.

different amounts of DMDS (0, 0.5, 5, 50, 500, or 2000 μg) to a cotton bud located in one compartment of a bipartite Petri dish with wild-type seedlings sown on MM2, supplemented with different $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ concentrations (0, 50, or 1000 μM), in the other compartment. After 17 d, we evaluated DMDS dose- and

SO₄²⁻ concentration-dependent PGP effects (see Supplemental Figure 8A online). The application of 2000 μg DMDS completely inhibited seed germination and seedling growth. For SO₄²⁻-starved seedlings (0 μM SO₄²⁻), PGP was observed with 50 μg DMDS and was highest when 500 μg DMDS was applied. Seedlings grown on

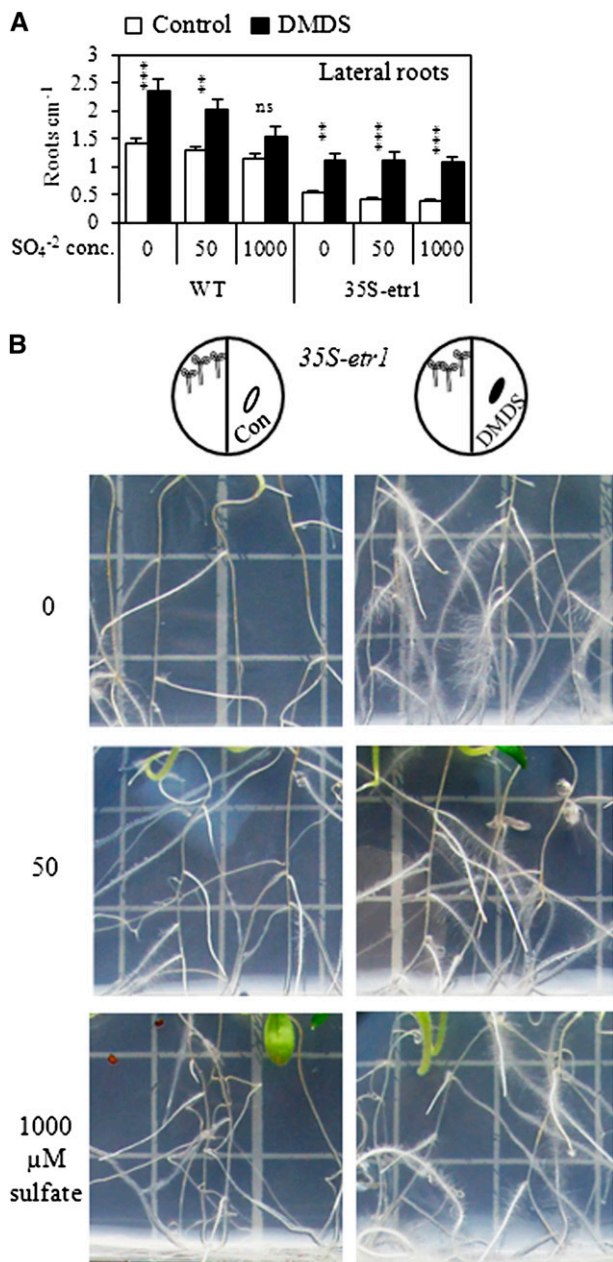


Figure 6. Effect of Genotype and DMDS on Seedling Root Architecture.

Wild-type (WT) and *35S-etr1* seedlings were grown for 17 d in bipartite Petri dishes containing MM2 medium (vertical placement) supplemented with different sulfate concentrations (0, 50, and 1000 μM MgSO₄ × 7H₂O) and treated with or without DMDS.

(A) Mean (±SE) lateral root number. A PLSD test of an ANOVA between mock- and DMDS-treated seedlings was performed with **P < 0.01 and ***P < 0.001. ns, not significant. *n* = four Petri dishes with greater than or equal to four seeds.

(B) Effect of DMDS on *35S-etr1* root hair formation.

50 or 1000 μM SO₄²⁻ experienced no PGP in terms of seedling surface area, irrespective of how much DMDS was added. At a DMDS concentration of 500 μg, the growth of these seedlings was slightly inhibited (see Supplemental Figure 8B online). Since the SO₄²⁻ concentration of *N. attenuata*'s native soils is low (~22.5 μM) and PGP effects on in vitro-grown seedlings in SO₄²⁻-depleted media were highest after the application of 500 μg DMDS (see Supplemental Figure 7 online), all further DMDS application experiments were performed with 500 μg (0.5 μL) DMDS per Petri dish.

When seedlings were grown on MM2 supplemented with different concentrations of SO₄²⁻ and exposed to DMDS, PGP effects were strongest for SO₄²⁻-starved seedlings (0 μM SO₄²⁻; Figures 5 and 6, closed bars), irrespective of the plant genotype. For 20-d-old wild-type seedlings grown on 0 μM SO₄²⁻ medium, addition of DMDS increased seedling surface area, chlorophyll *a* content (Figures 5A to 5C), and the number of lateral roots dramatically (Figure 6A). DMDS application to wild-type seedlings grown under medium (50 μM) and high (1000 μM) SO₄²⁻ supply resulted in less pronounced PGP effects (Figures 5A to 5C). However, when exposed to DMDS, the number of lateral roots increased substantially for wild-type seedlings grown on 50 μM and 1000 μM SO₄²⁻, respectively (Figure 6A).

In contrast with the wild type, *35S-etr1* seedlings realized dramatic PGP effects from the exposure to DMDS, irrespective of SO₄²⁻ concentration (Figure 5A). Seedling surface area in the presence of DMDS was increased markedly in seedlings grown on 0 μM SO₄²⁻, but decreased in seedlings grown on 50 and 1000 μM SO₄²⁻ (Figure 5B). Chlorophyll *a* content in *35S-etr1* seedlings increased in the presence of DMDS at each SO₄²⁻ concentration (Figure 5C), as did the number of lateral roots (Figure 6A). Strikingly, *35S-etr1* seedlings, which are normally deficient in root hair production, displayed increased root hair production upon exposure to DMDS (Figure 6B).

Total amino acid concentrations of DMDS-treated seedlings grown on 0 and 50 μM SO₄²⁻ decreased in the wild type and

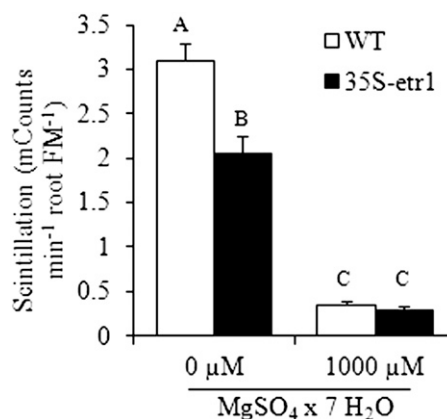


Figure 7. Effect of Genotype on Sulfate Uptake.

S-starved and S-replete plants (24 d old) were incubated for 30 min in ³⁵SO₄²⁻-spiked Hoagland solution. The mean (±SE) ³⁵S incorporated into the roots was quantified by scintillation counting. A PLSD test of an ANOVA between wild-type (WT) and *35S-etr1* plants grown on low or high sulfate concentration was performed. Different letters depict statistical differences at P < 0.001. FM, fresh mass. *n* = 6.

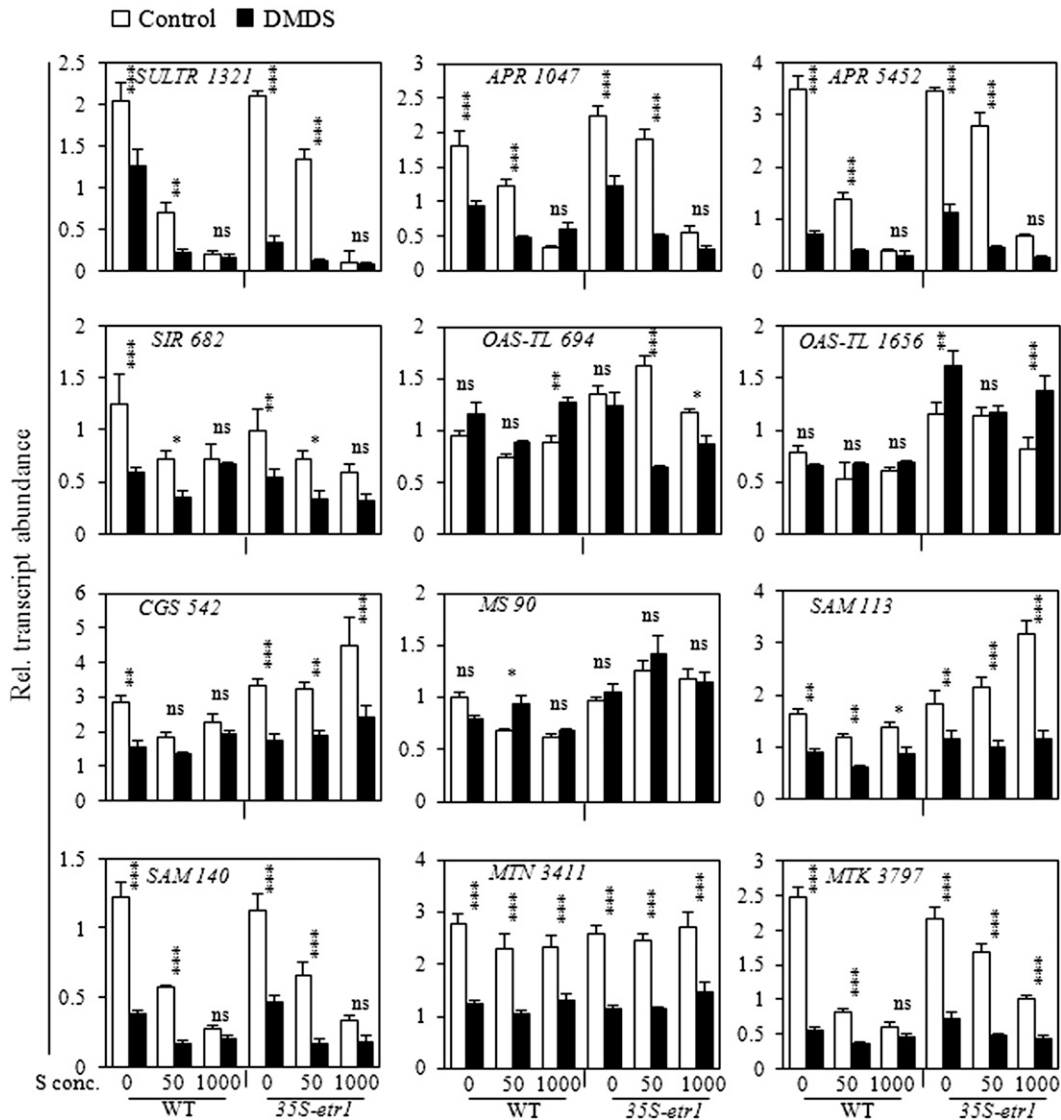


Figure 8. Effect of Genotype and DMS on *N. attenuata* Transcript Abundance of Genes Involved in S Assimilation, Met Biosynthesis, and Recycling in the Yang Cycle.

Wild-type (WT) and *35S-etr1* seedlings were grown for 18 d in bipartite Petri dishes containing MM2 medium supplemented with different sulfate concentrations (0, 50, and 1000 μM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$) and treated with or without DMS. Transcript abundances of a high affinity (*SULTR*, two *APRs*, *SIR*, two *OAS-TLs*, *CGS*, *MS*, *SAMS*, *MTN*, and *MTK*) were quantified by quantitative PCR. For gene similarity to *Arabidopsis* and primers used, see Supplemental Tables 1 and 3 online. A PLSD test of an ANOVA between mock- and DMS-treated seedlings was performed with * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (see Supplemental Table 1 online). ns, not significant. $n \geq 4$, except for Na-*SULTR 1321*, $n \geq 3$.

35S-etr1, but increased in seedlings grown under high SO_4^{2-} supply (Figure 5D, closed bars). The addition of DMS increased free Met concentration in SO_4^{2-} -deprived (0 μM SO_4^{2-}) wild-type and *35S-etr1* seedlings but had less effect on seedlings grown under medium and high SO_4^{2-} supply (Figure 5E). While the DMS exposure led to increases in GSH and GSSG levels in wild-type seedlings only under low (0 μM SO_4^{2-}) and medium (50 μM SO_4^{2-}) SO_4^{2-} supply, GSH and GSSG concentrations of *35S-etr1* seedlings were dramatically enhanced under all SO_4^{2-} concentrations (Figures 5F and 5G).

Collectively, these data demonstrate that exposure to volatile DMS positively effects plant growth and concentrations of S-containing metabolites, especially when the supply of inorganic S is limited.

DMS Application Affects Transcript Abundance of Genes Involved in SO_4^{2-} Metabolism

Assimilatory sulfate reduction is an energy-demanding process for plants (Takahashi et al., 2011) and the uptake of reduced S

(e.g., in the form of DMDS) theoretically allows a seedling to forgo these costs and invest more energy in other physiological processes, such as growth or reproduction. Accordingly, we were interested in whether DMDS fumigation downregulated the expression of genes involved in the S reduction pathway, Met biosynthesis, and Yang cycle (Figure 8). Transcript levels of Na-SULTR, Na-APRs, Na-SIR, Na-CGS, Na-SAMs, Na-MTN, and Na-MTK significantly decreased after the addition of DMDS (see Supplemental Table 1 online). Generally, DMDS effects were more dramatic for seedlings grown under low (0 μM) and medium (50 μM) SO_4^{-2} supply than under high (1000 μM). The expressions of Na-OAS-TLs in the wild type and 35S-*etr1* were less strongly affected by DMDS. For these genes, no general trend was inferred. Na-MS transcript abundance was not affected by DMDS, with the exception of wild-type seedlings grown on 50 μM SO_4^{-2} , in which Na-MS transcripts increased slightly (Figure 8).

PGP Effects Elicited by the B55 VOC Bouquet Are Smaller Than DMDS-Induced Effects

We tested whether exposure of wild-type and 35S-*etr1* seedlings to the complete B55 VOC bouquet would mimic the growth responses observed for pure DMDS exposure when seedlings were grown under different SO_4^{-2} supply. Therefore, we grew wild-type and 35S-*etr1* seedlings in one compartment of a bipartite Petri dish on MM2, supplemented with different $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ concentrations (0, 50, or 1000 μM) and spotted 5 μL B55 suspension onto the other compartment containing 0.5 \times YPDA (Figure 9A). In terms of surface area, only S-deprived wild-type and 35S-*etr1* seedlings showed a positive reaction to B55 VOCs: Wild-type and 35S-*etr1* seedling surface area was increased by 36 and 66%, respectively (Figure 9B). For all other treatment combinations, no effects were observed. Seedling chlorophyll a content was not affected by B55 VOCs. For wild-type seedlings cultured on 1000 μM SO_4^{-2} , even negative effects were observed (Figure 9C). Interestingly, free Met levels of S-deprived wild-type and 35S-*etr1* seedlings (0 μM SO_4^{-2}) were not altered by exposure to B55 VOCs (Figure 9D, closed bars). However, free Met content increased in wild-type and 35S-*etr1* seedlings grown under medium and high SO_4^{-2} supply (Figure 9D, closed bars). By contrast, the opposite pattern was found for reduced GSH: Whereas wild-type and 35S-*etr1* seedlings grown under medium (50 μM) and high (1000 μM) SO_4^{-2} supply did not show any changes, S-deprived wild type and 35S-*etr1* contained more GSH in the presence of B55 VOCs (Figure 9E).

Root architecture was influenced by B55 VOCs as well. Wild-type seedlings grown under 0, 50, or 1000 μM SO_4^{-2} produced more lateral roots in the presence of B55 VOCs compared with the respective controls, with greater effects at higher SO_4^{-2} levels (Figure 9F). Similar results were found for 35S-*etr1* seedlings (Figure 9F). These data demonstrate that B55 VOCs elicit changes in plant growth but that those changes are attenuated compared with those induced by application of pure DMDS.

DISCUSSION

In this study, we describe a VOC-mediated PGP effect that is based on a nutritional mechanism. Our results support a model

in which the abundant S-containing VOC produced by *Bacillus* sp B55, DMDS, contributes to *N. attenuata*'s S nutrition (Figure 10). We further demonstrate that the ET-insensitive 35S-*etr1* *N. attenuata* plants have impaired SO_4^{-2} uptake and S metabolism (Figures 5 to 9; see Supplemental Figure 7 online) and hence benefit from the interaction with B55 by obtaining reduced S in the form of bacterial DMDS.

Sulfur is the fourth most important macronutrient (after nitrogen, phosphorus, and potassium) and is essential for plant and animal life. Being incorporated into compounds such as the essential amino acids Cys and Met, the universal methyl donor S-adenosyl Met (SAM), several coenzymes, vitamins, thio-redoxins, and glutathiones, S availability determines crop quality and quantity (Zhao et al., 1993, 1999; Hawkesford and De Kok, 2006). Sulfur deficiency directly affects a plant's primary metabolism, in large part through suppression of the photosynthetic machinery via decreased chlorophyll content and reduced synthesis of ribulose-1,5-bisphosphate carboxylase/oxygenase, restricting CO_2 assimilation (Burke et al., 1986; Gilbert et al., 1997). Concentrations of nonprotein thiol compounds and amino acid composition are also affected by S deprivation (Buchner et al., 2004; Koralewska et al., 2008; Khan et al., 2010).

Generally, S is taken up by the roots from surrounding soil in the form of SO_4^{-2} . However, plants are also capable of using atmospheric S, sulfur dioxide and H_2S , to meet their S needs. These otherwise toxic volatiles are valuable S sources, particularly when S supply to the root is limited (DeKok et al., 1997; Stuiver and De Kok, 2001; Buchner et al., 2004; Durenkamp and De Kok, 2005; Koralewska et al., 2008; Chen et al., 2011). We show that exposure of wild-type and 35S-*etr1* seedlings to the B55 VOC bouquet or DMDS dramatically increased seedling vigor (in terms of size, chlorophyll content, and root architecture) as well as the concentration of S-containing compounds (i.e., free Met and glutathiones [GSH and GSSG]) (Figures 1, 5, 6, and 9). The PGP effects of DMDS or B55 VOCs were greatest when *N. attenuata* seedlings were grown under SO_4^{-2} -limiting conditions (Figures 5 and 6; see Supplemental Figure 9 online). Furthermore, the addition of DMDS to *N. attenuata* seedlings substantially lowered transcript abundance of S metabolism-related *N. attenuata* genes (Figure 8). This could reflect an energy-saving strategy, allowing energy that would be used for S reduction and assimilation to be invested in growth. Typically, S deprivation results in low shoot:root ratios (Buchner et al., 2004). Interestingly, the shoot:root ratio of *N. attenuata* seedlings was not affected by SO_4^{-2} concentration (data not shown). However, we found that DMDS and B55 VOCs exposure shortened the length of the primary root and increased lateral root formation and root hair production (Figures 6A and 9F).

Our observations that DMDS promotes plant growth differ in part from those of Kai et al. (2010) in that fumigation of *Arabidopsis* seedlings with DMDS suppressed growth irrespective of the concentration applied. In our experimental setup (MM2 medium supplemented with different amounts of SO_4^{-2}), we found positive effects of 500 μg DMDS per Petri dish on the growth of *Arabidopsis*, as we did with *N. attenuata* seedlings (see Supplemental Figure 10 online). Although the minimum effective dose of synthetic DMDS required to elicit PGP effects (50 μg /Petri dish) was substantially higher than the minute

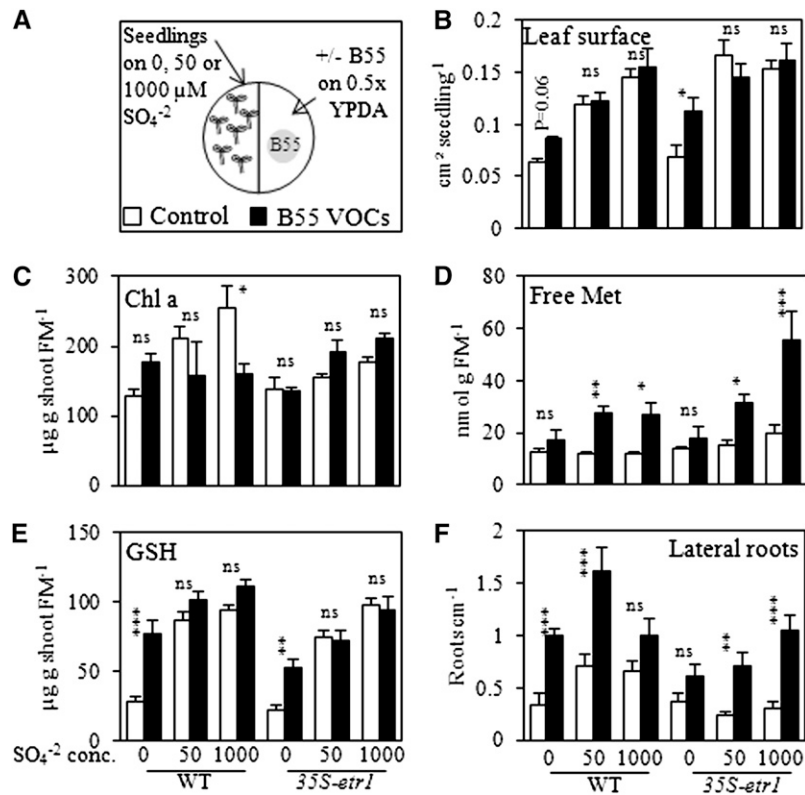


Figure 9. Effect of B55 VOCs on Seedling Grown under Different Sulfate Supply.

(A) Experimental design.

(B) to (E) Mean (\pm SE) shoot surface area (B), mean (\pm SE) chlorophyll *a* (Chl *a*) concentration (C), mean (\pm SE) free Met concentration (D), and mean (\pm SE) GSH concentration (E) of mock- or VOC-treated wild-type and *35S-etr1* seedlings. $n \geq 3$ Petri dishes with 20 seeds.

(F) Mean (\pm SE) lateral root number of vertically grown, mock- or VOC-treated wild-type and *35S-etr1* seedlings. $n \geq 3$ Petri dishes with four seeds. For (B) to (F), a PLSD test of an ANOVA between mock- and VOC-treated seedlings was performed with * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. FM, fresh mass; ns, not significant. Wild-type (WT) and *35S-etr1* seedlings were cocultivated for 15 d (B) to (E) or 11 d (F) with or without B55 in bipartite Petri dishes. Seedlings were grown on MM2 medium supplemented with different sulfate concentrations (0, 50, and 1000 μM $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$); B55 was grown on 0.5 \times YPDA.

amounts we measured as emitted by B55 (1 ng per cm^2 colony and day), these values likely underestimate the natural flux of DMDS between bacteria and host plants. A large portion of the synthetic DMDS was rapidly lost from the Petri dish system immediately upon its application and by diffusion out of the system during incubation. When these sources of experimental error are accounted for, it is possible that the DMDS concentrations of the two systems are similar.

Since direct headspace measures of in situ B55 DMDS production in the presence of seedlings was thwarted by its rapid removal by the seedlings (see Supplemental Figure 2 online), we analyzed the transcriptional abundance of B55 genes involved in DMDS production in the presence or absence of seedlings. Unfortunately, knowledge of sulfurous VOC biosynthesis pathways in bacteria other than lactic acid bacteria is sparse. In lactic acid bacteria, sulfurous VOCs are derived from L-Met catabolism, mainly by the activity of three enzymes: L-METHIONINE- γ -LYASE (MGL), CYSTATHIONINE- γ -LYASE (CGL), and CYSTATHIONINE- β -LYASE (CBL) (Alting et al., 1995; Bruinenberg et al., 1997; Dias and Weimer, 1998). We analyzed the transcript abundance of these

three enzymes in B55 but observed no increase in the presence of seedlings (see Supplemental Figure 3B online).

Although major PGP effects could be attributed to bacterial VOCs in vitro, their role in nature remains untested. To evaluate the contribution of B55 DMDS emission to plant S nutrition in nature, a bacterial mutant unable to produce DMDS would be ideal. However, considering the challenge of transforming Gram-positive bacteria, as well as the fact that the expression of three genes (*MGL*, *CGL*, and *CBL*) involved in DMDS production might need to be silenced, a more tenable approach to elucidating the physiological relevance of DMDS might be through the use of plant lines impaired in SO_4^{2-} reduction. A molecular tool box for transforming *N. attenuata* has been developed over the last decade, but unfortunately no transgenic line impaired in S reduction was available to address our hypothesis that B55 VOCs/DMDS function as PGP agents by providing reduced S to the plant. Our attempts to interrupt *N. attenuata* SO_4^{2-} reduction by transient silencing of *Na-SIR* using virus-induced gene silencing repeatedly failed. However, the use of the ET-insensitive *35S-etr1* line in the analysis of VOC-mediated PGP proved fortuitous: In the process

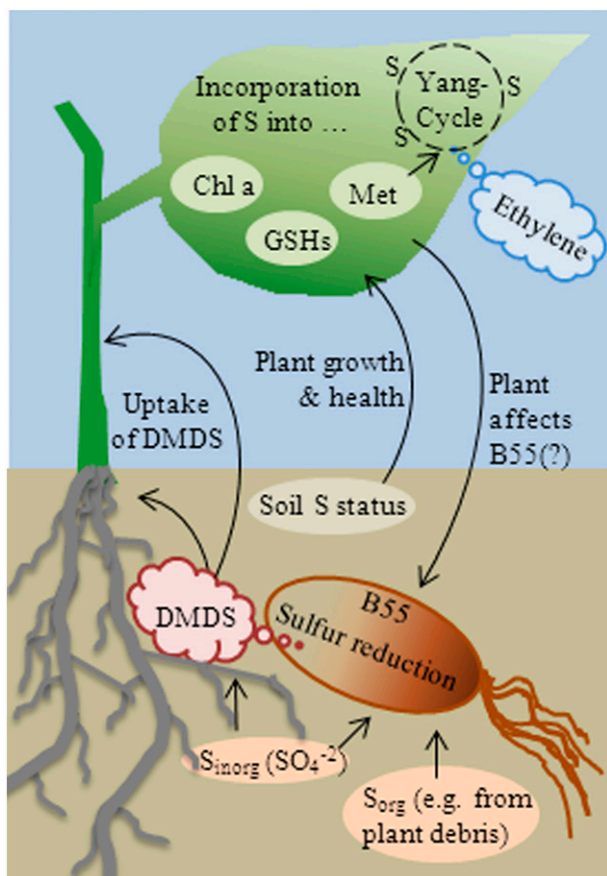


Figure 10. Proposed Model Summarizing the VOC-Mediated Mutualistic Interaction between *Bacillus* sp B55 and its Native Host *N. attenuata*.

S availability can limit plant growth in many habitats. The *N. attenuata* root-colonizing bacterium B55 reduces (organic [org]) S and emits DMDS, which is efficiently assimilated by the plant. This reduced S is incorporated into S-containing compounds (e.g., GSH and GSSG or the amino acid Met) and S-related metabolic pathways, leading to biosynthesis of chlorophyll (Chl a) or ET. We hypothesize that the energy saved by not reducing inorganic (inorg) S contributes to the promotion of plant growth in B55-inoculated plants. Given the observation that B55 populations are larger in ET-insensitive *35S-etr1* seedlings, plants are likely capable of culturing B55.

of unraveling the dramatic PGP effect conferred by B55 onto *N. attenuata* wild-type and *35S-etr1* plants (Meldau et al., 2012), we found that *35S-etr1* seedlings are impaired in S uptake, reduction, and metabolism.

Several lines of evidence demonstrate that *35S-etr1* seedlings are defective in S-related processes. First, irrespective of the SO_4^{2-} concentration in the medium, nontreated (control) *35S-etr1* plants exhibited abnormal growth combined with low concentrations of S-containing compounds and chlorophyll a (Figure 5). Second, ^{35}S -labeling experiments revealed that the induction of SO_4^{2-} uptake of S-starved plants is impaired (Figure 7). The transcript abundance of the high affinity transporter Na-SULTR, however, was not affected by the genotype (Figure 8). This apparent discrepancy might be explained by posttranscriptional

regulatory mechanisms, as proposed by Yoshimoto et al. (2007). Third, *35S-etr1* seedlings accumulated higher levels of free amino acids (Figure 5D), a feature also observed for *Arabidopsis* mutant plants (*sir1-1*) impaired in SO_4^{2-} reduction (Khan et al., 2010) as well as SO_4^{2-} -starved *Brassica oleracea* plants (Buchner et al., 2004). Fourth, transcript rates of genes involved in S metabolism and Met recycling are upregulated in *35S-etr1* (Figure 8), consistent with the idea that this line is impaired in S metabolism.

We further propose that ET-insensitive plants, which constitutively emit large amounts of ET (see Supplemental Figure 6 online) (von Dahl et al., 2007) invest their already limited S into the biosynthesis and recycling of Met (in the Yang cycle) to supply the demands of their ET emissions, which are maintained at the expense of plant growth. To meet a plant's Met demand for prolonged ET production (as in *35S-etr1* plants) and/or under SO_4^{2-} -limiting conditions, efficient recycling of Met (in the Yang cycle) becomes vital in addition to a de novo synthesis (Kushad et al., 1985, 1988; Sauter et al., 2005; Katz et al., 2006; Bürstenbinder et al., 2007). Compared with *N. attenuata* wild-type seedlings, control *35S-etr1* seedlings accumulated higher basal transcript levels of genes involved in Met biosynthesis (Na-CGS542 and Na-MS90), SAM synthesis (Na-SAMS113), and Met recycling in the Yang cycle (Na-MTK3797). These data suggest that *35S-etr1* plants meet their high Met requirements by de novo synthesis of Met as well as by recycling of Met (Figure 8).

The transcriptional analyses of *N. attenuata* genes suggest that Met biosynthesis in *35S-etr1* plants is enhanced compared with the wild type. Although the basal Met levels in *35S-etr1* plants are, compared with other S-containing metabolites (i.e., GSH or GSSG), relatively high, they were still lower than in the wild-type control (Figure 5; see Supplemental Figure 11 online). Since the transcriptional abundance of a SAMS (Na-SAMS 113) was significantly higher in *35S-etr1* than in the wild type, SAM biosynthesis in *35S-etr1* plants might be increased, thereby consuming free Met. This finding is in contrast with that of Woodson et al. (1992), who found no increase in the transcriptional abundance of SAMS in flowering, ET-producing *Dianthus caryophyllus*. Hence, assessment of SAM concentration in the wild type and *35S-etr1* might be revealing. In addition, the labeling experiment in Figure 4 provides data consistent with the hypothesis that *35S-etr1* seedlings have a higher requirement for (reduced) S (i.e., to complement for defects in S uptake/reduction or for enhanced ET production) and assimilate more labeled S than do wild-type seedlings. However, as the phenotype of *35S-etr1* plants is complemented by reduced S (in the form of DMDS) but not SO_4^{2-} , defects in SO_4^{2-} uptake or reduction are more probable than an increased flux of S through the Yang cycle, especially under low SO_4^{2-} supply. On the other hand, the S-replete *35S-etr1* plants (which displayed no impairments in SO_4^{2-} uptake [Figure 7]) showed growth defects (Figures 5 and 6). Hence, we propose that *35S-etr1*'s impairments cannot simply be attributed to insufficient SO_4^{2-} uptake. Multiple branches in S metabolism might be affected and further molecular dissection is needed to elucidate this.

Together, our results provide evidence supporting the hypothesis that *35S-etr1* plants are impaired in SO_4^{2-} uptake, S metabolism, and the Yang cycle. At the same time, *35S-etr1*

seedlings, which are colonized by B55 at 10 times higher population sizes than the wild type, realize greater fitness benefits compared with the wild type from inoculation with B55 (Meldau et al., 2012), but particularly after the exposure to B55 VOCs or DMDS. Remarkably, B55 VOCs and DMDS partially rescue many of the severe growth phenotypes usually associated with ET insensitivity (Figures 1, 5, and 6; see Supplemental Figure 6 online). We propose that the exposure of *35S-etr1* seedlings to DMDS (pure or in the complete B55 VOC bouquet) compensates for the deficiencies in SO_4^{-2} uptake/reduction, thereby restoring seedling growth and performance in the natural habitat (Meldau et al., 2012).

Our in vitro experiments provide evidence that DMDS is assimilated by seedlings through the headspace (Figure 2; see Supplemental Figures 2 and 3 online). In addition, ^{35}S was incorporated into seedling protein after exposure to the ^{35}S -labeled bacterial VOC bouquet (Figures 3 and 4). We cannot exclude the possibility that other S-containing VOCs, not detected by methods we used (SPME coupled to GC-MS), might also have been transferred through the headspace and assimilated. For example, other sulfurous VOCs, such as methanethiol, dimethyl sulfide, and dimethyl trisulfide, are commonly produced by a range of microbes (Farag et al., 2006; Kai et al., 2010; Minerdi et al., 2011). Furthermore, S-methyl pentanethioate (see Supplemental Table 2 online), which is emitted in trace amounts, might have contributed to the radioactive signal. Hydrogen sulfide was not detected among the B55 VOCs (see Supplemental Figure 12 online), making it unlikely that it contributed to the observed phenotype.

The PGP effects of the B55 VOC bouquet on seedlings grown on low SO_4^{-2} MM2 medium were smaller than those observed after DMDS exposure (Figures 5, 6, and 9). This was particularly true for *35S-etr1* seedlings and might be due to the deliberately chosen difference in cultivation time (15 d of VOC treatment versus 20 d of DMDS treatment). The degree of sulfur starvation in 15-d-old seedlings was likely less than that in 20-d-old ones (compared with controls of Figures 5 and 9) and PGP effects were not yet apparent. However, B55's DMDS production might not have been sufficiently high to compensate for the S deficiency of *35S-etr1* seedlings grown on the low SO_4^{-2} MM2 medium. Furthermore, other (nonsulfurous) VOCs (listed in Supplemental Table 2 online) emitted by B55 might interact with each other, leading to an altered growth response, a hypothesis that requires the development of a transformation system for B55 in order to be tested. Additionally, we found that the bacterial culture medium, 0.5× YPDA, slowed seedling growth when seedlings were cultured on MM2 (cf. Figures 5 and 9). VOC effects in a Petri dish system seem to be not only strongly influenced by the bacterial culture medium, as previously reported by Blom et al. (2011b), but also by the seedling medium or the interaction of the two (cf. Figure 1 [seedlings on GB5] and Figure 9 [seedlings on MM2]).

Various groups of microorganisms and algae emit volatile S-containing compounds (such as methanethiol, DMDS, dimethyl sulfide, or dimethyl trisulfide), as do vascular plants, including *Brassicaceae* sp and *Alliaceae* sp (White, 1982). The sulfurous, bioactive volatiles play diverse roles, affecting organisms across all kingdoms. DMDS was first described as produced by bacteria

isolated from decomposing chicken (Freeman et al., 1976). Surprisingly, DMDS, along with other VOCs emitted by rhizobacteria *Pseudomonas fluorescens* B-4117 and *Serratia plymuthica* IC1270, was found to exert antimicrobial and nematicidal activity (Kai et al., 2009; Wang et al., 2009; Huang et al., 2010; Dandurishvili et al., 2011) and to inhibit the cell-cell communication quorum-sensing network of various bacteria (Chernin et al., 2011). Furthermore, DMDS functions as a plant defense compound against nonspecialist herbivores feeding on *Allium porrum* (Dugravot et al., 2003, 2004) and as both an oviposition repellent and an attractant to different natural enemies of the cabbage root fly *Delia radicum* (Ferry et al., 2007, 2009). Recently, Huang et al. (2012) described DMDS as an elicitor of induced systemic resistance in *N. benthamiana* against *Botrytis cinerea*. Additionally, S-containing VOCs seem to enhance grape vine bud break (Kubota et al., 2003; Vargas-Arispuro et al., 2008). Whether DMDS mediates signaling processes involved in plant defense in *N. attenuata* is currently under investigation.

Most research on VOC-mediated PGP has been performed on a few model plants and the mechanisms remain elusive for the most part, as reviewed by Bailly and Weisskopf (2012) and Wenke et al. (2012b). By examining the interaction between *N. attenuata* and its naturally root-associated *Bacillus* sp B55, we uncovered a new mechanism of PGP: nutrient-driven PGP by the S-containing VOC DMDS, likely functioning by enhancing S availability and reducing the need for energy-demanding S assimilation. Furthermore, we conclude that *35S-etr1* plants realize large benefits from the association with B55 (and the concomitant DMDS emissions) due to impaired S metabolism (Figure 10). This work demonstrates that the effects of bacterial compounds are highly context specific; they depend on plant genotype and bacterial strain, as would be expected of any coevolved facultative interaction. In addition, our results highlight the value of using transgenic lines of a native plant to uncover plant-microbe interactions and their function. This work joins a groundswell of research that highlights the ability of eukaryotes to compensate for deficiencies by recruiting microbial partners.

METHODS

Plant Materials and Bacterial Strain

The 31-time self-pollinated wild-type line of *Nicotiana attenuata* and the ET-insensitive transgenic line *N. attenuata 35S-etr1* (A-03-328-8, fully described in von Dahl et al., 2007) and *Arabidopsis thaliana* Columbia-0 seeds were used in these experiments. Germination procedures have been described elsewhere (Ryu et al., 2003; Long et al., 2010). The plant growth-promoting *Bacillus* sp B55 strain (GenBank accession number JX101913) was isolated from a *35S-etr1* plant grown in native Utah soil in 2008 (Long et al., 2010). Unless noted otherwise, B55 was routinely grown on half-strength yeast peptone dextrose agar (YPDA; Sigma-Aldrich) at 30°C. *N. attenuata* seedlings were grown, depending on the experimental setup, on GB5 (naturally containing 2 mM SO_4^{-2} , supplemented with or without 1.2% Suc; Gamborg's B5 media including vitamins; Duchefa) or on modified minimal medium (MM2) supplemented with different amounts of SO_4^{-2} (0, 50, or 1000 μM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$). One liter MM2 contains 80 mg KNO_3 , 65 mg KCl , 4.8 mg KH_2PO_4 , 288 mg $\text{Ca}(\text{NO}_2)_2 \times 4\text{H}_2\text{O}$, 8 mg NaFeEDTA , 0.75 mg KI , 6 mg $\text{MnCl}_2 \times 4\text{H}_2\text{O}$, 2.65 mg $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 1.5 mg H_3BO_3 , 0.13 mg $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 0.0024 mg $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$,

and 1.2% Suc, pH was adjusted to 6.8; modified after Becard and Fortin, 1988). To avoid magnesium deficiency in the low SO_4^{-2} media and to exclude possible Cl^{2-} effects in high SO_4^{-2} media, the magnesium concentration was balanced by the addition of $50 \mu\text{M MgCl}_2 \times 6\text{H}_2\text{O}$ to all MM2 media. Petri dishes were kept in a Percival growth chamber at 16/8-h day/night cycle, $155 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $30/28^\circ\text{C}$. Depending on the experimental design, *Arabidopsis* seedlings were cultivated on half-strength Murashige and Skoog salts agar medium (Duchefa) or MM2 medium supplemented with different amounts of SO_4^{-2} (0, 50, or $1000 \mu\text{M MgSO}_4 \times 7\text{H}_2\text{O}$) in a York growth chamber (16/8-h day/night cycle, 190 to $220 \mu\text{mol m}^{-2} \text{s}^{-1}$, and 21°C).

Seedling Growth Promotion by B55 VOCs

The effect of B55 VOCs was evaluated by spotting $5 \mu\text{L}$ of bacterial suspension (in sterile water, $\text{OD}_{600} = 1.0$) onto one side of a bipartite Petri dish containing $0.5 \times \text{YPDA}$, while wild-type and *35S-etr1* seeds were transferred onto the other side containing GB5 medium, so that seedlings and bacteria shared only the common headspace. Seedling surface area was quantified after 12 d according to the video tutorial by Zach Jarou (<http://www.chlorofilms.org/index.php/crpVideo/display/videoId/46>) using Adobe Photoshop CS5. Chlorophyll *a* and *b* content was analyzed spectrophotometrically from an 80% acetone extract using a TECAN plate reader. Number of true leaves and lateral root branches was determined by counting, and primary root length was measured. Four replicates with at least 20 seedlings (horizontal placement) or seven seedlings (vertical placement) per Petri dish were performed for each treatment. The experiment was repeated two times.

To test the effect of B55 VOCs on seedlings grown under different SO_4^{-2} supply, wild-type and *35S-etr1* seedlings were germinated in one side of a bipartite Petri dish containing MM2 medium supplemented with different $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ concentrations (0, 50, or $1000 \mu\text{M}$). After 4 d, plates were opened under a safety hood and $5 \mu\text{L}$ of bacterial suspension (in sterile water, $\text{OD}_{600} = 1.0$) or water was applied on the other side of the bipartite dish, containing $0.5 \times \text{YPDA}$. Growth effects in terms of lateral root branches were assessed 11 d after sowing (vertical placement, $n \geq 4$ Petri dishes with four seeds per dish). Seedling surface area, chlorophyll *a* content, free Met, and GSH levels were determined in 15-d-old seedlings (horizontal placement, $n \geq 4$ Petri dishes with ~ 20 seeds per dish). Preliminary experiments had shown that, due to the fast growth of B55, prolonged cocultivation (longer than 15 d) of seedlings with B55 resulted in a sudden seedling growth depression; hence, the experiment was stopped after 15 d. The experiment was repeated twice for the wild type and once for *35S-etr1*.

Analysis of Glutathione (γ -Glutamyl-Cysteinyl-Gly)

The concentrations of GSH and GSSG were determined according to the modified protocol by Rellán-Alvarez et al. (2006). Briefly, 50 to 70 mg fresh nitrogen-ground shoot tissue was extracted with $200 \mu\text{L}$ ice-cooled extraction buffer (5% [w/v] metaphosphoric acid, 0.1% [v/v] formic acid, 1% [w/v] polyvinyl-pyrrolidone, and $10 \text{ ng}/\mu\text{L}$ GSH internal standard [$\text{Gly-}^{13}\text{C}_2,^{15}\text{N}$; Sigma-Aldrich]) for 5 min using a vortex. After centrifugation at 4°C for 20 min at $15,000g$, the pellet was reextracted with $150 \mu\text{L}$ extraction buffer. Two hundred microliters of pooled extract was used for liquid chromatography–mass spectrometry analysis on a Varian 1200 triple quad spectrometer. Separation was performed on a Varian ProStar HPLC system (Econosil CN $5 \mu\text{m}$ $250 \times 4.6 \text{ mm}$; Alltech) with a mobile phase A: water, 0.05% formic acid, and 0.1% acetonitrile; and B: methanol. The elution profile was 0 to 5 min, 5% B; 5 to 12 min, 5 to 60% B; and 12 to 20 min, 60% B. The flow rate of 1 mL min^{-1} was reduced to $250 \mu\text{L min}^{-1}$ by a LC Packings 1:4 fixed splitter. The triple quadrupole mass spectrometer was operated in multiple reaction monitoring mode, using the following ion transitions: (mass-to-charge ratio [m/z] Q1 \rightarrow Q3/

collision energy): reduced GSH (m/z 306 \rightarrow 143/19V); ($\text{Gly-}^{13}\text{C}_2,^{15}\text{N}$)-GSH (m/z 309 \rightarrow 146/19V); and GSSG (m/z 611 \rightarrow 305/25V).

Analysis of Free Met

Free Met content was assessed by extracting approximately 50 mg finely ground shoot sample in 80% methanol for 5 min. The diluted extracts were directly analyzed by liquid chromatography–tandem mass spectrometry after a modified protocol by Jander et al. (2004). Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies), and separation was achieved on a Zorbax Eclipse XDB-C18 column ($50 \times 4.6 \text{ mm}$, $1.8 \mu\text{m}$; Agilent Technologies). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was 0 to 1 min, 3% B; 1 to 2.7 min, 3 to 100% B in A; 2.7 to 3 min 100% B; and 3.1 to 6 min 3% B. The mobile phase flow rate was 1.1 mL min^{-1} . The column temperature was maintained at 25°C . The liquid chromatography was coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source operated in positive ionization mode. The instrument parameters were optimized by infusion experiments with pure standards (amino acid standard mix; Fluka). The ion spray voltage was maintained at 5500 eV. The turbo gas temperature was set at 700°C . Nebulizing gas was set at 70 p.s.i., curtain gas at 35 p.s.i., heating gas at 70 p.s.i., and collision gas at 2 p.s.i. Multiple reaction monitoring was used to monitor analyte parent ion \rightarrow product ion: Multiple reaction monitoring was chosen as follows: Met (m/z 150 \rightarrow 104), ^{13}C , ^{15}N -Met (m/z 156 \rightarrow 109). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. All samples were spiked with ^{13}C , ^{15}N -labeled amino acids (algal amino acids ^{13}C , ^{15}N ; Isotec) at a concentration of $10 \mu\text{g}$ of the mix per milliliter. The concentration of the individual labeled amino acids in the mix had been determined by classical HPLC–fluorescence detection analysis after precolumn derivatization with ortho-phthalaldehyde-mercaptoethanol. Met was quantified using ^{13}C , ^{15}N -labeled Met as an internal standard.

Volatile Collection and Analysis

VOCs of bipartite Petri dishes containing medium ($0.5 \times \text{YPDA}$)(GB5), B55, B55+wild-type seedlings (cocultivation), or wild-type seedlings only were collected 14 d after inoculation by headspace sampling for 20 min with a SPME divinyl-carboxen-PDMS fiber (Sigma-Aldrich) and analyzed on a Varian CP-3800 GC coupled with a Varian Saturn 4000 ion trap mass spectrometer in electron ionization mode (70 eV) (Varian). The sample (SPME fiber) was injected into the gas chromatograph and volatiles separated on a DB-5 column ($30 \text{ m} \times 0.25 \text{ mm i.d.}$, $0.25\text{-}\mu\text{m}$ film thickness; Agilent) with helium at a constant flow of 1 mL min^{-1} as the carrier gas. The injector temperature was at 230°C ; the oven temperature program was 40°C for 5 min, 185°C at $5.0^\circ\text{C min}^{-1}$, and a $30^\circ\text{C min}^{-1}$ ramp to 300°C . Electron ionization spectra were recorded on scan mode from m/z 40 to 300. DMDS was identified by library search and by comparison to synthetic DMDS (98%; Sigma-Aldrich). Quantification of DMDS was performed in the linear range of detection based on calibration curves generated with increasing concentrations of commercial DMDS mixes in methanol. Other volatiles detected and identified by library search are summarized in Supplemental Table 2 online.

^{35}S -Labeling Experiments

Ten microliters of B55 suspension (in sterile water; $\text{OD}_{600} = 1.0$) was inoculated on 25 mL solid M9 medium containing $0.27 \text{ MBq Na}_2^{35}\text{SO}_4$ (Perkin-Elmer) ($+^{35}\text{S}$) as the sole SO_4^{-2} source in one compartment of a bipartite Petri dish. In the other compartment, wild-type seedlings were germinated on modified MM2 medium supplemented with $2 \text{ mM MgSO}_4 \times 7\text{H}_2\text{O}$ ($+\text{SO}_4$) or without $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ ($-\text{SO}_4$) (Figure 3A). The

plates were kept in a hood of the radioactive lab at room temperature at a 16/8-h day/night cycle and were illuminated with a self-built transportable lamp (stand: Trop Aquarienleuchte Typ E4/0-S 1 × 15 W; lamp: Osram L15W light color 954, Lumilux de Luxe [Osram]). Seventeen days after inoculation, seedlings were harvested in liquid N₂ for scintillation measurements and total protein extraction. Proteins were extracted with a buffer containing 100 mM HEPES, pH 7.5, 5 mM EGTA, 5 mM EDTA, 10 mM DTT, 10 mM Na₂VO₄, 10 mM NaF, 50 mM glycerol-2-phosphate, 1 mM PMSF, and 10% glycerol; separated with 10% SDS-PAGE, dried for 30 min on a gel dryer (583; Bio-Rad Laboratories), exposed to a positron imaging plate (FLA 3000 system; Fujifilm), and scanned after 7 d of exposure. For scintillation counting 50 to 100 mg fresh mass of fine-ground frozen seedling tissue was incubated in 1 mL sodium hypochlorite solution for 1 h at 60°C. After cooling to room temperature, 15 mL Hionic-Fluor (Perkin-Elmer) was added, and scintillation counts were measured (Win Spectral 1414; Perkin-Elmer).

To determine whether *35S-etr1* seedlings absorb higher concentrations of volatile, labeled S, the same experiment was performed once more with tripartite Petri dishes: one-third containing wild-type seeds, the other *35S-etr1* seeds, and the last third containing B55. After 20 d of cocultivation, scintillation counts were measured. At least six Petri dishes were used for each treatment. This experiment was performed twice for the wild type and once for *35S-etr1*.

We further examined the SO₄⁻² uptake capacity of wild-type and *35S-etr1* roots, performing a ³⁵SO₄⁻² uptake experiment, modified after Lappartient and Touraine (1996), Shibagaki et al. (2002), and Maruyama-Nakashita et al. (2004). Plants (24 d old) were grown for 4 d in a Hoagland solution without any SO₄⁻² source or with 1000 μM MgSO₄ × 7 H₂O, before SO₄⁻² uptake was induced by the addition of MgSO₄ × 7 H₂O (final concentration: 0.5 mM). Thirty minutes later, plants were transferred to a Hoagland solution containing 0.5 mM MgSO₄ × 7 H₂O and 10 μCi Na³⁵SO₄ mL⁻¹. After 30 min of incubation, plants were washed three times in 0.2 mM CaSO₄ to remove access Na³⁵SO₄, followed by 2 h incubation in 0.2 mM CaSO₄ to allow plasma efflux of extracellular ³⁵S. Afterwards, roots were dissected from shoots, blotted, frozen, and finely ground before scintillation counts were measured. The experiment was performed twice for SO₄⁻²-starved and once for SO₄⁻²-replete plants (*n* = 6).

Application of Synthetic DMDS

Serial concentrations of DMDS in methanol (0.5, 5, 50, 500, and 2000 μg) were applied onto a cotton bud placed in an empty side of a bipartite Petri dish while wild-type seeds were sown in the other side containing MM2 medium (supplemented with different MgSO₄ × 7 H₂O concentrations [0, 50, or 1000 μM]). The volume of 0.5 μL (500 μg) DMDS/Petri dish was found to promote growth best for SO₄⁻²-starved seedlings (see Supplemental Figure 8 online) and further DMDS PGP experiments were performed using this concentration.

Petri dishes containing wild-type or *35S-etr1* seeds sown on MM2 media on the one side and a cotton bud containing DMDS (or not) on the other side were sealed three times with Parafilm and kept in a Percival growth chamber (16/8-h day/night cycle, 30/28°C) for at least 17 d in vertical or horizontal position before PGP effects were evaluated in terms of seedling surface area, chlorophyll a content, lateral root number, amino acid content, free Met, and GSH and GSSG concentration. Five plates with at least 15 or seven seeds were done for each treatment for horizontal and vertical placement, respectively. The experiment was repeated two times.

RNA Extraction and Quantitative Real-Time PCR Conditions

To analyze whether different SO₄⁻² concentrations in the MM2 medium and DMDS application modulate the expression of genes involved in plant S metabolism, quantitative PCR was performed. Wild-type and *35S-etr1* seeds were sown on one side of a two-partite Petri dish containing MM2

medium (supplemented with different MgSO₄ × 7 H₂O concentrations [0, 50, or 1000 μM]), and 0.5 μL pure DMDS was applied or not onto a cotton bud positioned in the other side of a two-partite Petri dish. After 18 d of growth, seedlings were harvested and root and shoot separated and immediately frozen in liquid N₂. RNA was extracted from 200 mg fine-ground frozen material after the protocol of Kistner and Matamoros (2005). The experiment was performed once with *n* ≥ 4.

To test whether the presence of wild-type seedlings affects B55's DMDS production on the transcriptional level, we analyzed the gene expression of three enzymes known to be involved in the biosynthesis of the DMDS precursor methanethiol: B55 (*MGL*, *CGL*, *CBL*).

Bacterial RNA was extracted from 10-d-old colonies cocultured on bipartite Petri dishes with or without wild-type seedlings according to the protocol of Majumdar et al. (1991). The experiment was performed once with *n* ≥ 4. One loop of cells was harvested, suspended in 200 μL sterile water, and mixed with half volumes of cold killing buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 20 mM sodiumazid) and centrifuged at 6500g at 4°C for 10 min. The supernatant was discarded and the pellet washed again with 1 mL killing buffer and centrifuged before the pellet was resuspended in 500 μL lysis buffer (200 mM NaCl and 3 mM EDTA). The resulting suspension was transferred to a 2-mL Eppendorf tube containing 500-μL glass beads (0.25 to 0.5 mm; Karl Roth) and 500 μL phenol-chloroform-isoamylalcohol. The mixture was vortexed for 2 min at highest speed, cooled down on ice for 2 min, and centrifuged at 4°C for 5 min at 15,000g. The water phase was transferred to a new tube and reextracted with 600 μL phenol-chloroform-isoamylalcohol. The supernatant was added to a new Eppendorf tube containing 600 μL chloroform-isoamylalcohol, vortexed for 5 min, and centrifuged for 5 min at 13,000g, before the supernatant was reextracted with 600 μL chloroform-isoamylalcohol. The resulting supernatant was mixed with one-tenth volume of 3 M sodium-acetate solution, pH 5.2, and 2.5 × volume ice-cold ethanol and inverted 10 times. The samples were kept for 2 h at -80°C and then centrifuged at 4°C and 15,000g for 25 min. The pellet was washed with 500 μL 70% ice-cold ethanol (RNA grade) and centrifuged at 4°C and at 15,000g for 20 min. The supernatant was removed and the pellet dried before it was suspended in diethylpyrocarbonate-treated water. The RNA was subjected to a DNase treatment using the RQ1 RNase-Free DNase kit (Promega). cDNA synthesis using either oligo (dT) (plant) or random hexamers (bacteria) was performed according to Wu et al. (2004). Quantitative PCR was run on a Mx3005P qPCR system (Stratagene). The gene-specific PCR products were detected with a qPCR Core Kit for SYBR Green I (Eurogentec). Quantitative PCR conditions were set according to the manufacturer's manual.

Primer Design

Comparison of *Bacillus* sp B55 *MGL*, *CGL*, and *CBL* DNA partial sequences (sequences > 780 N) using the National Center for Biotechnology Information's BLAST (Altschul et al., 1997) revealed high sequence similarity (>99%; data not shown) to the respective gene of the DSMZ strain *Bacillus megaterium* QM B1551, and primers were designed using the *B. megaterium* QM B1551 sequences.

For *N. attenuata* S metabolism transcript analysis by quantitative PCR, protein sequences obtained from The Arabidopsis Information Research (www.arabidopsis.org) of *Arabidopsis* genes involved in S metabolism were BLAST searched (xBLASTn) against in-house databases to find *N. attenuata* homologs. The *N. attenuata* transcriptome sequence with the best similarity to the respective *Arabidopsis* gene was used for primer design. At least one putative homolog coding for each enzymatic step selected for analysis of S assimilation, Met biosynthesis, and recycling in the Yang cycle was included in the analysis. When possible, multiple putative homologs of a gene family were included. The *N. attenuata* *ELONGATION FACTOR1A* and *B. megaterium* QM B1551 *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* genes served as internal standards for normalization. Primer design was done manually and primer properties

were inferred from OligoCalc (Kibbe, 2007). For primer sequences, see Supplemental Tables 3 and 4 online.

Characterization of DMDS Production by B55

The effect of age, colony size, culture medium, light, temperature, and the presence of wild-type seedlings on the Petri dish headspace concentration of DMDS was evaluated. Therefore, DMDS headspace concentration of B55 cultures of different age (ranging from 5 to 15 d), B55 cultures grown on different media (0.125 \times , 0.25 \times , and 0.5 \times YPDA), and under different temperatures (26 and 30°C) and light conditions (dark versus 16 h light d⁻¹) were analyzed by GC-MS. The effect of seedlings on DMDS concentration in the headspace was analyzed by cocultivating B55 with different amounts of seedlings ($n = 3, 5, 10, 15,$ or 25 seeds) for 12 d on two-partite Petri dishes (GB5|0.5 \times YPDA).

ET Measurements

Sterilized wild-type and 35S-*etr1* seeds were sown in sterile three-necked flasks containing 25 mL MM2 medium, supplemented with different MgSO₄ \times 7H₂O concentrations (0, 50, or 1000 μ M), tightly sealed, and cultivated in a Percival growth chamber (16/8-h day/night cycle, 155 μ mol m⁻² s⁻¹, 30/28°C). After 17 d, flasks containing seedlings were subjected to ET measurements using a photoacoustic spectrometer (INVIVO; Sankt Augustin) as described by von Dahl et al. (2007); flasks containing only medium served as blanks. The experiment was performed twice, with $n = 4$ flasks containing \sim 20 seeds.

CO₂ Experiments

In order to test whether elevated CO₂ levels arising from bacterial growth affect wild-type seedling growth, CO₂ was trapped with Ba(OH)₂ by the formation of BaCO₃ as described by Kai and Piechulla (2009). Trapping experiments were performed in tripartite Petri dishes. Seedlings were grown as described above, except that the GB5 medium was amended with 1.2% Suc. The bacterial CO₂ production was indirectly assessed by the formation of BaCO₃. Five microliters of B55 was applied onto 0.5 \times YPDA, while wild-type seeds were cocultivated or not. Seven milliliters of fresh 0.1 M Ba(OH)₂ solution was added in the third compartment, and the plate was twice around with Parafilm. BaCO₃ formation was determined every day (d1-d6). We found that the seedling affinity for CO₂ was much higher than that for the scavenging agent Ba(OH)₂ (see Supplemental Figure 13B online). Thus, it is not possible at this time to entirely exclude PGP effects resulting from CO₂ in the split plate assays.

To confine these effects in consecutive CO₂-trapping experiments, 5 μ L B55 suspension was applied on less nutritious medium (0.25 \times YPDA) and 6 d after sowing only. Leaf area was measured after 12 d, and BaCO₃ dry mass was determined to assess the Ba(OH)₂ saturation level. Five replicates with at least nine seedlings were performed. These experiments revealed that even under reduced bacterial CO₂ exposure, B55 VOCs still exerted positive effects on seedling growth: Seedling surface area of seedlings exposed to B55 VOCs and Ba(OH)₂ was as large as of those exposed only to the CO₂-reduced VOCs (see Supplemental Figure 13C online).

Qualitative Hydrogen Sulfide Test

H₂S production by B55 was tested qualitatively using lead acetate test strips (Sigma-Aldrich). B55 cultures were set up according to the manual and color change from white to black, indicating the formation of lead sulfite, was checked after 36 h.

Statistical Analysis

Data analysis was performed with the StatView software package (SAS Institute) with a completely randomized ANOVA. One-way and multifactorial

ANOVAs followed by Fisher's PLSD test or *t* tests were used to compare differences among treatments. Correlation analysis was performed with simple regression tests.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: sulfate transporter (Na-SULTR 1321), KF321741; 5'-adenylyl sulfate reductase (Na-APR 1047), KF321740; 5'-adenylyl sulfate reductase (Na-APR 5452), KF321745; sulfite reductase (Na-SIR 682), KF321738; O-acetylserine (thiol) lyase (Na-OAS-TL 694), KF321739; O-acetylserine (thiol) lyase (Na-OAS-TL 1656) KF321742; cystathionine- γ -synthetase (Na-CGS 542), KF321737; Met synthase (Na-MS 90), KF321734; S-adenosylmethionine synthetase (Na-SAMS 113), KF321735; S-adenosylmethionine synthetase (Na-SAMS 140), KF321736; 5'-methylthioadenosine nucleoside (Na-MTN 3411), KF321743; and 5'-methylthioribose kinase (Na-MTK 3797), KF321744.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Effect of B55 Volatile Organic Compounds on Seedling Chlorophyll Content.

Supplemental Figure 2. Chromatographic Profile of B55 Volatile Organic Compounds.

Supplemental Figure 3. Dimethyl Disulfide Emission by B55 as Affected by Seedlings.

Supplemental Figure 4. Characteristics of DMDS Production.

Supplemental Figure 5. Protein Loading Gel Related to Figure 3.

Supplemental Figure 6. Sulfate Effects on in Vitro Plant Growth.

Supplemental Figure 7. Effect of Genotype and Sulfate Supply on Ethylene Production.

Supplemental Figure 8. Serial Application of DMDS.

Supplemental Figure 9. B55 VOC and DMDS Effects on *N. attenuata* Seedlings Grown under 22.5 μ M Sulfate Supply.

Supplemental Figure 10. Effect of B55 VOCs and DMDS on *Arabidopsis thaliana* Col-0 Seedlings.

Supplemental Figure 11. Effect of Genotype and DMDS on Chlorophyll, Free Met, and Glutathione Concentration (Independent Repetition Experiment).

Supplemental Figure 12. Hydrogen Sulfide (H₂S) Production by B55.

Supplemental Figure 13. Effect of Carbon Dioxide on Seedling Growth.

Supplemental Table 1. Transcript Rates of S Metabolism Genes as Affected by Genotype, Sulfate Concentration, and DMDS (ANOVA Table Related to Figure 8).

Supplemental Table 2. Volatile Compounds Exclusively Emitted by B55 as Identified by Library Search.

Supplemental Table 3. List of *N. attenuata* Primers Used in This Study.

Supplemental Table 4. List of Bacterial Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

D.G.M. designed experiments, performed research, analyzed data, drafted figures, and wrote the article. S.M. designed and performed experiments and revised the article. L.H.H. designed and performed initial experiments. S.U. performed research. H.W. developed the method for glutathione measurements. I.T.B. designed the study and revised the article.

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