



Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production

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Plants develop an enhanced defensive capacity against a broad spectrum of plant pathogens after colonization of the roots by selected strains of nonpathogenic biocontrol bacteria. In *Arabidopsis thaliana*, this induced systemic resistance (ISR) functions independently of salicylic acid but requires an intact response to the plant hormones jasmonic acid (JA) and ethylene. To further investigate the roles of JA and ethylene in the ISR signalling pathway, the levels of these signalling molecules were determined in *A. thaliana* upon induction of ISR by *Pseudomonas fluorescens* WCS417r and subsequent challenge inoculation with *Pseudomonas syringae* pv. *tomato* DC3000. Upon treatment of the roots with ISR-inducing WCS417r bacteria, neither the JA content, nor the level of ethylene evolution was altered in systemically resistant leaves. Infiltration of leaves with WCS417r triggered the JA- and ethylene-dependent ISR pathway, but did not cause local changes in the production of either of these signalling molecules. These results indicate that rhizobacteria-mediated ISR is not based on the induction of changes in the biosynthesis of either JA or ethylene. However, in ISR-expressing plants the capacity to convert 1-aminocyclopropane-1-carboxylate (ACC) to ethylene was significantly enhanced, providing a greater potential to produce ethylene upon pathogen attack. © 2000 Academic Press

Keywords: *Arabidopsis thaliana*; biological control; defense signalling; ethylene; induced systemic resistance (ISR); jasmonic acid; *Pseudomonas fluorescens*; *Pseudomonas putida*; *Pseudomonas syringae* pv. *tomato*; salicylic acid-independent; systemic acquired resistance (SAR).

INTRODUCTION

Rhizosphere bacteria are present in large numbers on root surfaces, where plant exudates and lysates provide nutrients [25]. Certain strains of rhizosphere bacteria stimulate plant growth and are therefore called plant growth-promoting rhizobacteria (PGPR). Strains that were isolated from naturally disease-suppressive soils, mainly fluorescent *Pseudomonas* spp., have been demonstrated to reduce plant diseases by suppressing soil-borne pathogens. This biological control activity is effective under field conditions [48, 58] and in commercial greenhouses [24], and can be the result of competition for nutrients, siderophore-mediated competition for iron, or antibiosis [1]. Some of these biological control strains are also able to reduce disease caused by foliar pathogens by triggering a plant-mediated resistance

mechanism called induced systemic resistance (ISR) [50]. Rhizobacteria-mediated ISR has been reported for bean, carnation, cucumber, radish, tobacco, tomato and the model plant *Arabidopsis thaliana*, and is effective against different types of plant pathogens. In this respect, ISR resembles pathogen-induced systemic acquired resistance (SAR), which renders uninfected plant parts more resistant towards a broad spectrum of pathogens [40, 44]. Previously, we developed an *Arabidopsis*-based model system to study the molecular basis underlying rhizobacteria-mediated ISR [37]. Colonization of *Arabidopsis* roots by ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria protects the plants against different types of pathogens, including the bacterial leaf pathogens *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *armoraciae*, the fungal root pathogen *Fusarium oxysporum* and the fungal leaf pathogen *Peronospora parasitica* [37, 53] (J. Ton and C. M. J. Pieterse, unpublished work).

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The ISR signalling pathway clearly differs from the one controlling pathogen-induced SAR. The state of SAR is characterized by an early increase in endogenously synthesized salicylic acid (SA) [26, 30] and the concomitant activation of a set of so-called SAR genes [56]. Many SAR genes encode pathogenesis-related proteins (PRs) [49], some of which have been shown to possess anti-fungal activity and are thought to contribute to induced resistance. Transgenic NahG plants that express the bacterial salicylate hydroxylase (*nahG*) gene and thus cannot accumulate SA, are incapable of developing SAR and do not show SAR gene activation upon pathogen infection, indicating that SA is a necessary intermediate in the SAR signalling pathway [14]. Some rhizobacteria trigger the SA-dependent SAR pathway by producing SA at the root surface [11–13, 28]. However, *P. fluorescens* WCS417r induces normal levels of protection against *P. syringae* pv. *tomato* DC3000 in NahG plants that do not accumulate SA, demonstrating that WCS417r-mediated ISR is SA-independent [37]. Instead, the WCS417r-mediated ISR pathway requires components from the jasmonic acid (JA) and the ethylene response, because the JA response mutant *jar1-1* [39] and a range of ethylene response mutants are unable to express WCS417r-mediated ISR [21, 39]. Interestingly, the level of induced resistance can be enhanced further when ISR and SAR are activated simultaneously, indicating that ISR and SAR are additive [51].

Increased production of JA and ethylene is an early symptom of active defense in plants [7, 16, 27]. Blocking the response to either of these signal molecules renders plants more susceptible to pathogens [17, 22, 43, 46, 55] and even insects [29]. Both JA and ethylene co-ordinate the activation of a large set of defense responses, and when applied exogenously can induce resistance themselves [4, 6, 39]. In *Arabidopsis*, both JA and ethylene have been shown to activate specific sets of defense-related genes and resistance against *P. syringae* pv. *tomato* DC3000 [39, 52]. Recently, Van Wees *et al.* [52] monitored the expression of a set of well-characterized JA- and/or ethylene-responsive genes (i.e. *Lox1*, *Lox2*, *Atvsp*, *Pdf1.2*, *Hel*, *ChiB* and *Pall1*) in *Arabidopsis* plants expressing WCS417r-mediated ISR. None of the genes tested were up-regulated in induced plants, neither locally in the roots, nor systemically in the leaves. This suggested that ISR is not accompanied by major changes in the production of either JA or ethylene, but rather seems to be the result of sensitization of the tissue to these regulators. This latter hypothesis is supported by our recent finding that the JA-inducible gene *Atvsp* shows a potentiated expression in ISR-expressing leaves after challenge with virulent *P. syringae* pv. *tomato* DC3000 [52]. In other plant systems similar phenomena have been reported. For instance, Schweizer *et al.* [41]

demonstrated that during infection of rice with the fungal pathogen *Magnaporthe grisea*, jasmonate-inducible genes are activated without an increase in endogenous JA levels. Moreover, Tsai *et al.* [47] provided evidence that an increase in ethylene sensitivity rather than ethylene production is the initial event in triggering JA-enhanced senescence in detached rice leaves. These results demonstrate that ethylene- and JA-dependent plant responses can be triggered without a concomitant increase in the levels of these phytohormones.

Recently, Van Wees *et al.* [52] tested a concentration range of the resistance-inducing agents methyl jasmonate (MeJA) and the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) for their effect on both the level of induced protection against *P. syringae* pv. *tomato* DC3000 and the level of expression of JA-, and ethylene-responsive marker genes. A maximal level of resistance was already achieved at five- to 100-fold lower concentrations than needed for the induction of the expression of the marker genes. Thus, JA- or ethylene-dependent resistance can be elicited in the absence of detectable changes in JA- or ethylene-responsive gene expression. For this reason, it cannot be excluded that subtle changes in the production of either JA or ethylene may be sufficient to trigger the ISR pathway without directly activating the expression of the JA- and ethylene-responsive genes.

To further investigate the role of JA and ethylene in the ISR signalling pathway, we tested whether the state of WCS417r-mediated ISR is associated with changes in the production of JA and/or ethylene. To this end, we monitored the local and systemic levels of these signalling molecules in ISR-expressing plants. Moreover, we tested transgenic *Arabidopsis* S-12 plants, that lack the lipoxygenase isozyme LOX2 required for wound-induced synthesis of JA, for their ability to express WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000 infection. Finally, we investigated whether ISR-expressing plants have a higher capacity to produce ethylene by determining ethylene evolution after application of the ethylene precursor ACC. Our results demonstrate that the JA and ethylene dependency of WCS417r-mediated ISR is not based on changes in the production of either JA or ethylene. However, *Arabidopsis* plants develop a higher ACC-converting capacity upon induction of ISR. The possible roles of JA and ethylene in the ISR pathway are discussed.

MATERIALS AND METHODS

Bacterial cultures

Nonpathogenic rhizobacterial strains *P. fluorescens* WCS417r, *P. fluorescens* WCS374r and *P. putida* WCS358r were grown on King's medium B agar plates

[20] for 24 h at 28°C. Subsequently, bacterial cells were collected and resuspended in 10 mM MgSO₄ to a final density of 10⁹ cfu ml⁻¹ (OD₆₀₀ = 1.0) before being mixed through the soil. For leaf infiltration experiments, the rhizobacterial suspensions were diluted 100-fold in 10 mM MgSO₄ to a final density of 10⁷ cfu ml⁻¹.

The avirulent pathogen *P. syringae* pv. *tomato* strain DC3000 with the plasmid pV288 carrying avirulence gene *avrRpt2* [23] was used for induction of SAR. *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) bacteria were cultured overnight at 28°C in liquid King's medium B, supplemented with 25 mg l⁻¹ kanamycin to select for the plasmid. Bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO₄ to a final density of 10⁷ cfu ml⁻¹.

The virulent pathogen *P. syringae* pv. *tomato* strain DC3000 [59], used for challenge inoculations, was grown overnight in liquid King's medium B at 28°C. After centrifugation, the bacterial cells were resuspended in 10 mM MgSO₄ containing 0.01 % (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands) to a final density of 2.5 × 10⁷ cfu ml⁻¹.

Cultivation of plants

Seeds of wild-type *A. thaliana* ecotype Col-0 plants, transgenic S-12 plants harbouring a sense construct of *Lox2* cDNA behind the constitutive 35S-CaMV promoter [2], transgenic NahG plants harbouring the bacterial *nahG* gene [10] and mutant *jar1-1* [42], *etr1-1* [3] and *npr1-1* [5] plants were sown in quartz sand. Two week old seedlings were transferred to 60 ml pots containing a sand-potting soil mixture that had been autoclaved twice for 20 min at 121°C with a 24 h interval. Before transfer of the seedlings, the potting soil was supplemented with either a suspension of rhizobacteria or an equal volume of a solution of 10 mM MgSO₄. Plants were cultivated in a growth chamber with a 9 h day (200 μE m⁻² s⁻¹ at 24°C) and a 15 h night (20°C) cycle at 70 % RH. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland's nutrient solution, as described [37].

Induction treatments

ISR-inducing and noninducing rhizobacteria were applied in either of the two ways: (1) plant roots were treated by transferring two-week-old seedlings to soil that was mixed with a suspension of rhizobacteria to a final density of 5 × 10⁷ cfu g⁻¹ of soil; or (2) three lower leaves of 5 week old plants were pressure infiltrated with a suspension of rhizobacteria at 10⁷ cfu ml⁻¹ using a 1 ml syringe without a needle. Pathogen-induced SAR was triggered in a similar manner by pressure infiltrating a suspension of the avirulent pathogen *P. syringae* pv. *tomato*

DC3000 (*avrRpt2*) at 10⁷ cfu ml⁻¹ into three lower leaves of 5 week old plants. Of the control plants, three lower leaves were pressure infiltrated with 10 mM MgSO₄. In the figures, root treatments are indicated with the extension "(R)", whereas leaf treatments are indicated with the extension "(L)".

To relate ISR to levels of JA, SA and ethylene, nontreated leaves of treated plants were harvested. Just before harvest, the treated plant parts (either the root system or the pressure-infiltrated leaves) were removed. Subsequently, rosettes were either frozen in liquid nitrogen (for JA and SA extraction), or transferred to gas-tight serum flasks (for ethylene measurement).

For the determination of local production of JA and ethylene, all fully expanded leaves of 5 week old plants were pressure infiltrated with rhizobacteria, the avirulent pathogen *P. syringae* pv. *tomato* DC3000 (*avrRpt2*), or 10 mM MgSO₄ as described above. At intervals, rosettes were detached from the root system and either frozen in liquid nitrogen (for JA extraction), or transferred to gas-tight vials (for ethylene measurement).

Challenge inoculation and disease assessment

Before challenge inoculation, root-treated plants were allowed to grow for 3 weeks in soil supplemented with rhizobacteria. Leaf-treated plants were challenged 3 days after pressure infiltration. One day before challenge, the plants were placed at 100 % RH. Nonflowering plants were challenge inoculated by dipping the rosettes in a suspension of the virulent pathogen *P. syringae* pv. *tomato* DC3000 at 2.5 × 10⁷ cfu ml⁻¹ in 10 mM MgSO₄, 0.01 % (v/v) Silwet L-77. Four days after challenge, disease severity was assessed by determining the percentage of leaves with symptoms per plant (*n* = 20). Leaves were scored as diseased when showing necrotic or water-soaked lesions surrounded by chlorosis. Pressure-infiltrated leaves were ignored during disease assessment.

Extraction and quantification of JA

Leaves were frozen in liquid nitrogen and pulverized with mortar and pestle. One g of fresh weight was used to extract JA. One hundred ng of 9,10-dihydrojasmonic acid were added per gram of fresh weight as an internal standard. Extraction and GC-MS quantification of JA was carried out as described by Mueller and Brodschelm [31].

Ethylene measurement

Individual rosettes were detached from the roots, weighed and placed in 25 ml gas-tight serum flasks that were subsequently incubated under climate chamber conditions. At intervals, 1 ml gas samples were withdrawn

through the rubber seal. The concentration of ethylene was determined by GC as described by De Laat and Van Loon [9]. To determine the capacity of leaf tissue to convert ACC to ethylene, rosettes were dipped in a solution containing 1 mM ACC and 0.01 % (v/v) Silwet L-77. Control plants were dipped in 0.01 % (v/v) Silwet L-77 only. Excess moisture was removed from the leaves with paper towels. The leaves were weighed, placed in 25 ml gas-tight serum flasks, and ethylene production was measured as described above.

Extraction and quantification of SA

Leaves were frozen in liquid nitrogen and pulverized with mortar and pestle. For the analysis of free and conjugated SA, 200 ng of the internal standard *ortho*-anisic acid was added per gram of fresh weight. Subsequently, extraction and quantification of free and conjugated SA were carried out as described by Meuwly and Métraux [32].

RESULTS

ISR is not associated with increased levels of JA, ethylene or SA in systemic tissues

To assess whether colonization of the roots with ISR-inducing WCS417r bacteria is associated with systemic changes in JA or ethylene production, hormone levels were monitored in leaves of *Arabidopsis* ecotype Col-0

plants that were grown for 3 weeks in soil with or without WCS417r bacteria. Expression of ISR against virulent *P. syringae* pv. *tomato* DC3000 was verified in a subset of plants using our standard ISR bioassay [37] (data not shown). As a measure of induction by pathogenic bacteria, JA content and ethylene evolution were measured in leaves that were pressure infiltrated with the avirulent pathogen *P. syringae* pv. *tomato* DC3000 (*avrRpt2*). The production of both JA and ethylene was stimulated after infiltration of the leaves with avirulent bacteria as expected [Fig. 1(a) and (b)]. However, in noninfected leaves of ISR-expressing plants the JA level was similar to that observed in noninfected control plants. Also the production of ethylene was comparable in control and ISR-expressing plants. The latter confirms previous findings by Knoester *et al.* [21]. Thus, it can be concluded that induction of ISR through colonization of the rhizosphere with ISR-inducing WCS417r bacteria, does not result in an overall increase in the production of either JA or ethylene in the aerial plant parts.

WCS417r-mediated ISR functions independently of SA and is not associated with SA-inducible *PR* gene activation [37]. Therefore it can be expected that WCS417r-mediated ISR is not associated with changes in SA levels. Indeed, ISR-expressing leaves showed no elevated levels of either free or conjugated SA [Fig. 1(c)], this in contrast to systemic tissue expressing pathogen-induced SAR.

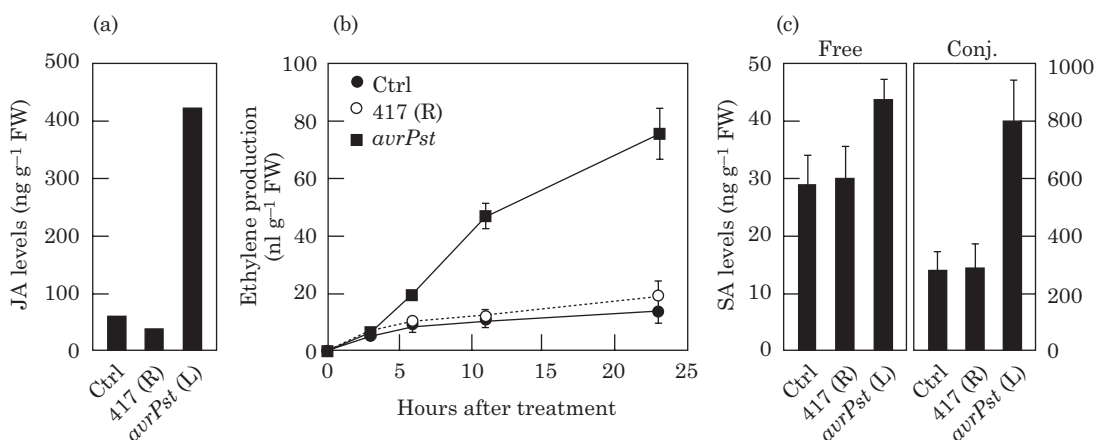


FIG. 1. Systemic production of JA, ethylene, and SA in *Arabidopsis* leaves expressing rhizobacteria-mediated ISR. (a) Endogenous JA levels in leaves of Col-0 plants that were grown in soil with [417 (R)] or without (Ctrl) ISR-inducing WCS417r bacteria, and in leaves inoculated with *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) 24 h after pressure infiltration [*avrPst* (L)]. The values represented are from 10 pooled plants that received the same treatment. (b) Cumulative ethylene production over a 24 h time period in leaves of Col-0 plants that were grown in soil with or without ISR-inducing WCS417r bacteria, and in leaves inoculated with *P. syringae* pv. *tomato* DC3000 (*avrRpt2*). The represented values are means (\pm S.E.) for 10 plants that received the same treatment. (c) Endogenous levels of free and conjugated SA in leaves of Col-0 plants that were grown in soil with or without ISR-inducing WCS417r bacteria, and in systemic leaf tissue expressing *P. syringae* pv. *tomato* DC3000 (*avrRpt2*)-induced SAR 3 days after induction of SAR. The values presented are means (\pm S.E.) for five plants that received the same treatment. FW, fresh weight. The experiments were repeated with similar results.

Infiltration of leaves with ISR-inducing rhizobacteria triggers the ISR signalling pathway

Previously, we demonstrated that infiltration of leaves with ISR-inducing rhizobacteria induces protection against *P. syringae* pv. *tomato* DC3000 in noninfiltrated leaves [37]. Before we investigated whether elicitation of ISR through leaf infiltration is accompanied by local changes in the production of JA and/or ethylene, we first tested whether infiltration of leaves with ISR-inducing rhizobacteria triggers the same signalling pathway as root application. To this end, the *Arabidopsis* genotypes Col-0, NahG, *jar1-1*, *etr1-1* and *npr1-1* were tested for their ability to express ISR against *P. syringae* pv. *tomato* DC3000 after pressure infiltrating three lower leaves with ISR-inducing WCS417r bacteria. Three days after induction, upper leaves were challenge inoculated with virulent *P. syringae* pv. *tomato* DC3000 bacteria and plants were assessed for disease severity 4 days later. Fig. 2(a) shows that leaf infiltration and root application of WCS417r are similarly effective in eliciting ISR in wild-type Col-0 plants. SA-deficient NahG plants also developed a statistically significant level of ISR after leaf induction, although the magnitude of the effect was somewhat lower than in wild-type Col-0 plants. This can be explained by the fact that the highly susceptible NahG plants were more heavily diseased (87.2% of the leaves of the control NahG plants showing symptoms vs. 61.1% in wild-type Col-0 plants), which usually results in a lower efficacy of ISR induction (C.M.J. Pieterse,

unpublished observations). Mutants *jar1-1*, *etr1-1* and *npr1-1* did not express ISR after infiltration of the leaves with ISR-inducing WCS417r bacteria. These results are in full agreement with the results of a previous study in which we tested the same *Arabidopsis* genotypes on their ability to express ISR against *P. syringae* pv. *tomato* DC3000 after application of WCS417r bacteria to the roots [39].

In *Arabidopsis*, rhizobacteria-mediated ISR depends on the rhizobacterial strain used. Previously, Van Wees *et al.* [53] demonstrated that treatment of the roots with strain WCS417r and *P. putida* WCS358r induces ISR against *P. syringae* pv. *tomato* DC3000, whereas strain *P. fluorescens* WCS374r is ineffective. Consistent with these findings, infiltration of three lower leaves per plant with WCS417r or WCS358r resulted in a significant level of protection against *P. syringae* pv. *tomato* DC3000 in the nontreated leaves, whereas WCS374r did not induce resistance [Fig. 2(b)]. Together, these results demonstrate that ISR-inducing rhizobacteria trigger the same systemic signalling pathway when applied to either roots or leaves.

Induction of ISR is not accompanied by local changes in the production of JA

To determine endogenous JA levels at the site of ISR induction, leaves of *Arabidopsis* Col-0 plants were infiltrated with the ISR-inducing strains WCS417r or

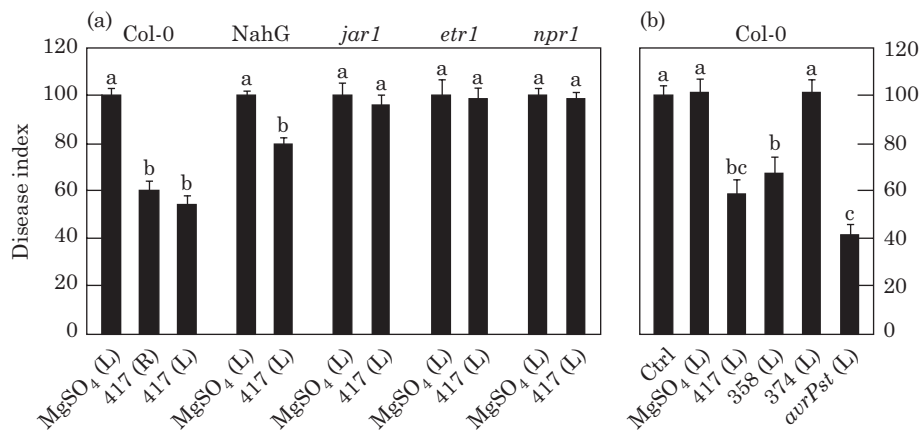


FIG. 2. Quantification of ISR against *P. syringae* pv. *tomato* DC3000 in *Arabidopsis*. In (a) ISR was induced in *Arabidopsis* genotypes Col-0, NahG, *jar1-1*, *etr1-1* and *npr1-1* by either growing the plants in soil containing ISR-inducing WCS417r bacteria [417 (R)], or by infiltrating three lower leaves per plant with WCS417r [417 (L)] 3 days before challenge inoculation. Three lower leaves per plant were pressure infiltrated with 10 mM MgSO₄ [MgSO₄ (L)] as a control. In (b) ISR was quantified in wild-type Col-0 plants after infiltration of three leaves per plant with the rhizobacterial strains WCS417r [417 (L)], WCS358r [358 (L)], WCS374r [374 (L)], or 10 mM MgSO₄ [MgSO₄ (L)]. The disease index is the mean (\pm s.e., $n = 20$) of the percentage of leaves with symptoms per plants 4 days after challenge with virulent *P. syringae* pv. *tomato* DC3000, in comparison to control-treated plants (set at 100%). The absolute proportions of diseased leaves of the control-treated plants were in (a) 61.1% (Col-0), 87.2% (NahG), 63.9% (*jar1-1*), 58.6% (*etr1-1*) and 74.3% (*npr1-1*) and in (b) 58.0% (Ctrl). Within each frame, different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha = 0.05$). The experiments were repeated with similar results.

WCS358r, or with the noninducing strain WCS374r. Leaves were infiltrated with avirulent *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) as a positive control. Fig. 3(a) shows that infiltration of the leaves with *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) resulted in a strong increase in the JA content, which reached a maximum 24 h after infiltration. Upon infiltration with WCS417r bacteria, the JA content in the leaves remained at a low, basal level comparable to the JA content of noninfiltrated leaves. Infiltration of the leaves with the ISR inducer WCS358r or the noninducer WCS374r did not result in elevated JA levels either [Fig. 3(b)]. Evidently, induction of ISR is not accompanied by local changes in JA content of the leaves.

Transgenic Arabidopsis S-12 plants express normal levels of ISR

The enzyme lipoxygenase (LOX) plays an important role in the octadecanoid pathway leading to the biosynthesis of JA [54]. Transgenic *Arabidopsis* S-12 plants have severely reduced levels of the lipoxygenase isozyme LOX2 due to co-suppression of the *Lox2* gene. The reduced LOX2 levels in S-12 plants have no effect on the steady-state JA level, but the wound-induced production of JA is blocked [2]. To verify whether S-12 plants are also impaired in pathogen-induced production of JA, JA levels were determined in water- and *P. syringae* pv. *tomato* DC3000-infiltrated leaves of Col-0 and S-12 plants. Two days after treatment, *P. syringae* pv. *tomato* DC3000-infiltrated Col-0 leaves showed an eight-fold increase in

JA levels compared to the water control (115 vs. 15 ng g⁻¹ FW, respectively). In contrast, water- and *P. syringae* pv. *tomato* DC3000-infiltrated S-12 leaves showed similar, low basal levels of JA (8 vs. 19 ng g⁻¹ FW, respectively), indicating that S-12 plants are also blocked in pathogen-induced production of JA.

ISR induction is not accompanied by changes in JA content, therefore, S-12 plants are expected to express normal levels of ISR. To test this, wild-type Col-0 and transgenic S-12 plants were treated with ISR-inducing WCS417r bacteria, either by application to the roots, or by infiltration into the leaves. Both Col-0 and S-12 plants expressed a significant level of induced protection against *P. syringae* pv. *tomato* DC3000 after treatment of the roots with WCS417r (Fig. 4), indicating that a rise in JA content is not required for the expression of ISR. In S-12 plants, infiltration of three leaves per plant with WCS417r bacteria also resulted in a significant level of systemic resistance. This demonstrates that neither a local nor a systemic increase in JA content is required for expression of ISR.

In contrast to ISR, elicitation of SAR by pre-infecting three leaves per plant with *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) results in a local [Fig. 1(a)] and a systemic (data not shown) increase in JA content. To investigate whether these increased JA levels play a role in the SAR pathway, we tested S-12 plants for their ability to express SAR. Like WCS417r-mediated ISR, SAR was not affected in S-12 plants (Fig. 4), indicating that increased JA levels are also not required for expression of SAR.

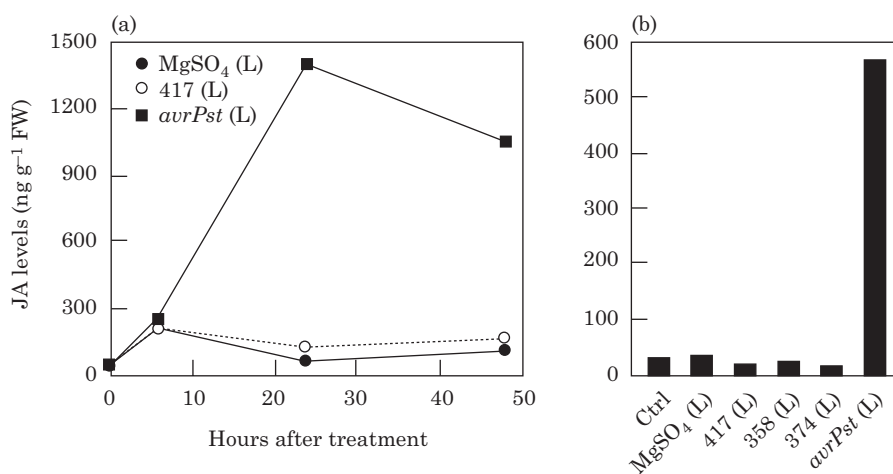


FIG. 3. Local accumulation of JA in *Arabidopsis* leaves after infiltration with rhizobacteria or *P. syringae* pv. *tomato* DC3000 (*avrRpt2*). (a) and (b) The values represent the endogenous JA content in leaves of Col-0 plants that were infiltrated with ISR-inducing WCS417r [417 (L)] or WCS358r bacteria [358 (L)], noninducing WCS374r bacteria [374 (L)], avirulent *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) bacteria [*avrPst* (L)], or 10 mM MgSO₄ [MgSO₄ (L)]. The JA content was measured in noninfiltrated leaves as a control (Ctrl). In (a) leaves were collected 6, 24 and 48 h after infiltration, whereas in (b) leaves were harvested 24 h after infiltration. Data are from 10 pooled plants that received the same treatment. The experiments were repeated with similar results.

FW, fresh weight.

Induction of ISR is not accompanied by local changes in ethylene production

Previously, Knoester *et al.* [21] demonstrated that rhizobacteria-mediated ISR in *Arabidopsis* requires ethylene perception at the site of application of the ISR inducing agent. To determine whether induction of ISR is accompanied by local changes in ethylene production at the site of application of the ISR inducer, ethylene evolution was monitored in leaves that were infiltrated with the ISR-inducing strains WCS417r or WCS358r, the noninducing strain WCS374r, or the SAR-inducing pathogen *P. syringae* pv. *tomato* DC3000 (*avrRpt2*). Infiltration with *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) resulted in a substantial increase in ethylene production over the first 48 h. In contrast, infiltration with WCS417r

led to a slight increase in ethylene production in the first 24 h only. At later time points, ethylene production was comparable to that observed in $MgSO_4$ -infiltrated leaves. A similar small increase in ethylene production was observed in leaves that were infiltrated with either the ISR inducer WCS358r or the noninducer WCS374r [Fig. 5(b)], suggesting that this small increase in ethylene production is a nonspecific reaction of the leaf tissue to bacterial infiltration. It can thus be concluded that induction of ISR, through infiltration of leaves with ISR-inducing rhizobacteria, is not accompanied by local changes in ethylene production. These results are in accord with previous findings of Knoester *et al.* [21], who demonstrated that induction of ISR by application of WCS417r to the roots is not associated with specific changes in the production of ethylene in the treated root tissue.

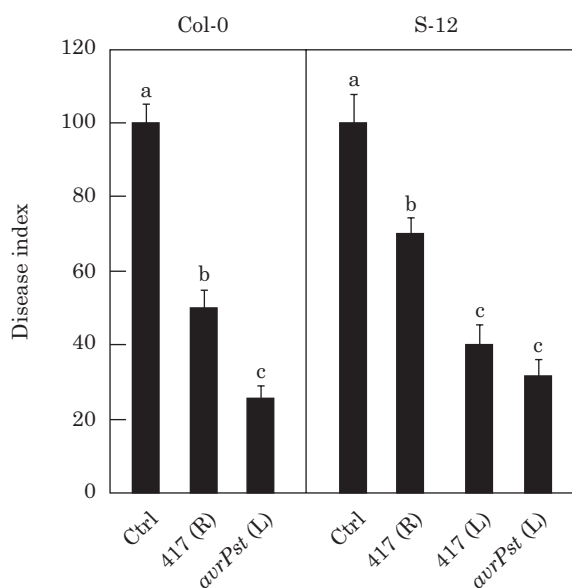


FIG. 4. Quantification of ISR and SAR against *P. syringae* pv. *tomato* DC3000 in Col-0 and S-12 plants. Induced protection against *P. syringae* pv. *tomato* DC3000 was quantified in wild-type Col-0 and in transgenic S-12 plants that are unable to produce elevated levels of JA. ISR was induced by either growing plants in soil containing ISR-inducing WCS417r bacteria [417 (R)], or by infiltrating three lower leaves per plant with WCS417r bacteria [417 (L)] 3 days before challenge inoculation with virulent *P. syringae* pv. *tomato* DC3000. SAR was induced by infiltrating three lower leaves per plant with the avirulent pathogen *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) (*avrPst*) 3 days before challenge inoculation. The disease index is the mean (\pm S.E., $n = 20$) of the percentage of leaves with symptoms per plant 4 days after challenge with virulent *P. syringae* pv. *tomato* DC3000, in comparison to control-treated plants (set at 100%). The absolute proportions of diseased leaves of the control-treated Col-0 and S-12 plants were 64.9 and 67.7%, respectively. Within each frame, different letters indicate statistically significant differences between treatments (Fisher's LSD test, $\alpha = 0.05$). The experiment was repeated with similar results.

ISR is associated with an enhanced capacity for converting ACC to ethylene

In higher plants, ethylene is produced from methionine via *S*-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylate ($Met \rightarrow SAM \rightarrow ACC \rightarrow ethylene$) [18, 19, 45]. The last two steps of this biosynthetic pathway are catalysed by ACC synthase and ACC oxydase, respectively. Pathogen infections leading to chlorotic or necrotic symptoms cause an increase in ethylene production with ACC synthase and ACC oxydase activity being increased sequentially [8]. Whereas under normal conditions the conversion of SAM to ACC is the rate-limiting step, during infections ACC accumulates transiently, indicating that ACC oxydase activity restricts ethylene production. Previously, it was demonstrated that the capacity for converting ACC to ethylene was increased systemically in SAR-expressing tobacco and tomato plants [9], providing a greater capacity for producing ethylene after challenge inoculation. Fig. 6 shows that ethylene production in systemic tissues expressing either ISR or SAR was not raised compared to noninduced plants. To investigate whether WCS417r-mediated ISR in *Arabidopsis* is associated with an enhanced ACC-converting capacity in systemic tissue, ethylene production was measured in control and ISR-expressing plants after treatment of the leaves with a saturating dose of 1 mM ACC. The ACC-converting capacity in SAR-expressing plants was also examined as a check. Indeed, after application of 1 mM ACC, ISR-expressing plants showed a statistically significant higher level of ethylene production than ACC-treated control plants, irrespective of whether ISR was induced by WCS417r-treatment of the roots or by leaf infiltration. The magnitude of the increase in ACC-converting capacity varied from 20 to 50% between experiments and was comparable to that observed in SAR-expressing plants.

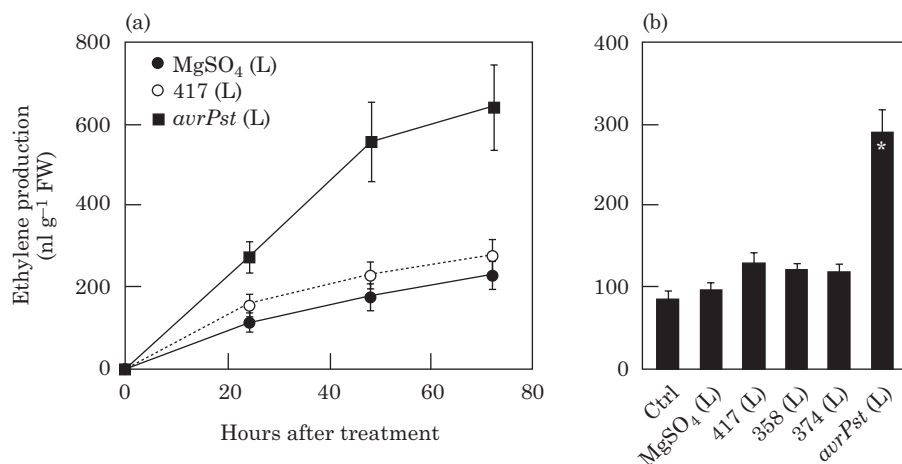


FIG. 5. Local ethylene production in *Arabidopsis* leaves after infiltration with rhizobacteria or *P. syringae* pv. *tomato* DC3000 (*avrRpt2*). (a) and (b) Cumulative ethylene production by leaves of Col-0 plants that were not treated (Ctrl) or infiltrated with either ISR-inducing WCS417r [417 (L)] or WCS358r bacteria [358 (L)], noninducing WCS374r bacteria [374 (L)], avirulent *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) bacteria [*avrPst* (L)], or 10 mM MgSO₄ [MgSO₄ (L)]. Ethylene production was determined by GC analysis at the time points indicated (a), or after 24 h (b). Values are means (\pm s.e.) for 10 plants that received the same treatment. Asterisks indicated statistically significant differences compared to the control (Ctrl) treatment (Fisher's LSD test, $\alpha = 0.05$). The experiments were repeated with similar results. FW, fresh weight.

Evidently, the capacity to convert ACC to ethylene is increased in *Arabidopsis* plants expressing either ISR or SAR.

DISCUSSION

Recent advances in research on plant defense signalling pathways have shown that plants are capable of differentially activating distinct defense pathways depending on the type of invader encountered [36]. Upon pathogen infection, the production of JA and ethylene is stimulated, leading to the activation of specific defense-related genes [4, 57]. In *Arabidopsis*, increased biosynthesis of both JA and ethylene has been implicated in the pathogen-induced expression of the plant defensin gene *Pdf1.2* [35] and in disease resistance signalling in response to the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* [34]. Like WCS417r-mediated ISR, both plant responses are SA-independent and require components from the JA and the ethylene response. To investigate whether ISR is associated with changes in JA and/or ethylene biosynthesis, we monitored local and systemic production of both signal molecules in plants expressing ISR. When plants were grown in soil containing ISR-inducing rhizobacteria, the JA content and the level of ethylene evolution in systemically resistant leaves was similar to that observed in non-induced control plants (Fig. 1). Moreover, infiltration of leaves with ISR-inducing rhizobacteria elicited JA- and ethylene-dependent resistance against *P. syringae* pv.

tomato DC3000 (Fig. 2), but this was not accompanied by a local increase in the production of these hormones (Figs 3 and 5). It can thus be concluded that neither induction, nor expression of WCS417r-mediated ISR is associated with an increase in the production of either JA or ethylene. This conclusion fits with earlier observations that ISR is not accompanied by the activation of JA- or ethylene-inducible genes [39, 52].

By using the *Lox2* co-suppressed transgenic line S-12, we confirmed that an increase in JA production is not required for the induction or expression of ISR. Transgenic S-12 plants, that are affected in the production of JA in response to wounding [2], expressed normal levels of ISR, irrespective of whether ISR was induced via roots or leaves (Fig. 4). This raises the question: how can the JA-dependency of ISR be explained? A plausible explanation is that ISR-expressing plants are potentiated for the expression of JA-inducible, defense-related genes, leading to a faster and greater gene activation after infection with a challenging pathogen. Potentiation of defense-related gene activation has been demonstrated for SA-inducible *PR* genes in SAR-expressing tobacco [33] and *Arabidopsis* plants [52]. Recently, we demonstrated that the expression of the JA-inducible *Atvsp* gene is potentiated in ISR-expressing plants [52], leading to a higher *Atvsp* transcript accumulation after challenge inoculation. However, other JA-inducible genes, i.e. *Pdf1.2*, *Lox2* and *Pal1*, were not potentiated. Thus, the enhanced expression of *Atvsp* cannot be explained by accelerated JA production during infection. Indeed, JA production after infection with *P. syringae* pv. *tomato* DC3000 does not increase faster in

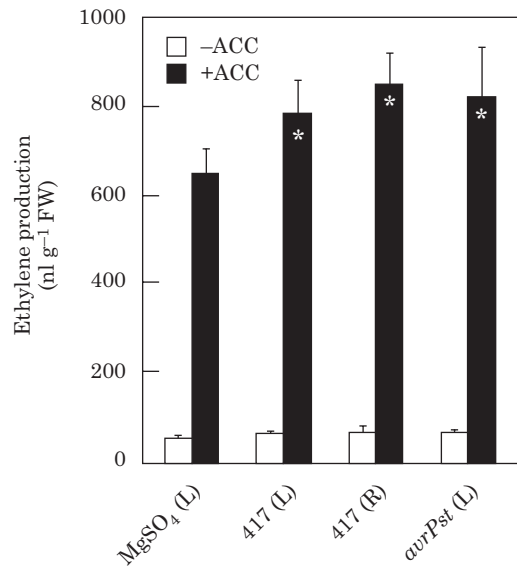


FIG. 6. ACC-converting capacity of ISR- and SAR-expressing plants. Ethylene produced over a 24 h period by leaves from noninduced plants and plants expressing either ISR or SAR. WCS417r-mediated ISR and *P. syringae* pv. *tomato* DC3000 (*avrRpt2*)-induced SAR were triggered as described in the text and legend to Fig. 4. Three days after infiltration of the leaves with either 10 mM MgSO₄, WCS417r or *P. syringae* pv. *tomato* DC3000 (*avrRpt2*), the infiltrated leaves were removed and the remaining leaves were treated with 1 mM ACC. The ACC-converting capacity was determined by measuring the ethylene production in the 24 h period subsequent to the treatment with ACC. Values are means (\pm S.E.) for 10 plants that received the same treatment. Asterisks indicate statistically significant differences compared to the control (MgSO₄) treatment (Fisher's LSD test, $\alpha = 0.05$). The experiment was repeated with similar results. FW, fresh weight.

ISR-expressing plants compared to infected control plants (data not shown). Therefore, potentiation of *Atvsp* gene expression must be the result of sensitization of the tissue for specific JA-responsive genes.

Similarly, WCS417r-mediated ISR is not accompanied by increased ethylene evolution. So how can the ethylene dependency of ISR then be explained? Previously, Knoester *et al.* [21] demonstrated that ethylene sensitivity is required at the site of ISR induction. Mutant *eir1-1*, which is insensitive to ethylene in the roots only, was able to mount ISR against *P. syringae* pv. *tomato* DC3000 when WCS417r was infiltrated in the leaves, but not when these bacteria were applied to the roots. This suggests that ethylene is involved in the generation or translocation of the systemically transported ISR signal. However, this does not rule out the possibility that ethylene responsiveness is required for the expression of ISR in tissues distant from the site of induction.

Previously, De Laat and Van Loon [9] demonstrated that the capacity for converting ACC to ethylene was increased systemically in tobacco leaves expressing

tobacco mosaic virus-induced SAR. The authors postulated that this increase in ACC-converting capacity might be involved in limiting pathogen growth in systemically resistant leaves, because upon challenge inoculation ACC did not accumulate, but was converted faster to ethylene. In *Arabidopsis*, both WCS417r-mediated ISR and pathogen-induced SAR are associated with a significant enhancement of the ACC-converting capacity, indicative of increased ACC oxidase activity. In *Arabidopsis*, ACC oxidase is encoded by a single gene [15] whose expression is not enhanced in ISR- or SAR-expressing leaves [21] (C. M. J. Pieterse, unpublished results). Thus, the increase in ACC oxidase activity in systemically resistant leaves is likely to be regulated post-translationally. The enhanced capacity for converting ACC to ethylene provides a greater potential for producing ethylene upon pathogen attack. With the application of ACC being shown to induce resistance against *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* [39], a faster or greater production of ethylene in the initial phase of infection might contribute to enhanced resistance against this pathogen. However, the significance of this phenomenon needs further investigation.

Fig. 7 combines these results in a model aimed at explaining the role of JA and ethylene in ISR signalling. In summary, ISR is a JA- and ethylene-dependent resistance response [39]. Ethylene responsiveness is required at the site of ISR induction, suggesting that this signal molecule is involved in the generation or translocation of the systemically transported signal [21]. ISR is neither locally, nor systemically accompanied by an increase in JA or ethylene biosynthesis (this study). In agreement with this, *Arabidopsis* plants show no increase in JA- or ethylene-responsive gene expression in response to induction of ISR [39, 52]. ISR expressing plants have a higher capacity to convert ACC to ethylene, providing a greater potential to produce ethylene upon infection (this study). A specific set of JA-responsive genes is potentiated in ISR-expressing plants, leading to a higher level of expression after pathogen infection. For ethylene-responsive genes, such a potentiating effect was not observed [52]. Future research will be focussed on the mechanisms involved in the potentiation of JA-responsive gene expression and the increased ACC-converting capacity in relation to the level of induced resistance.

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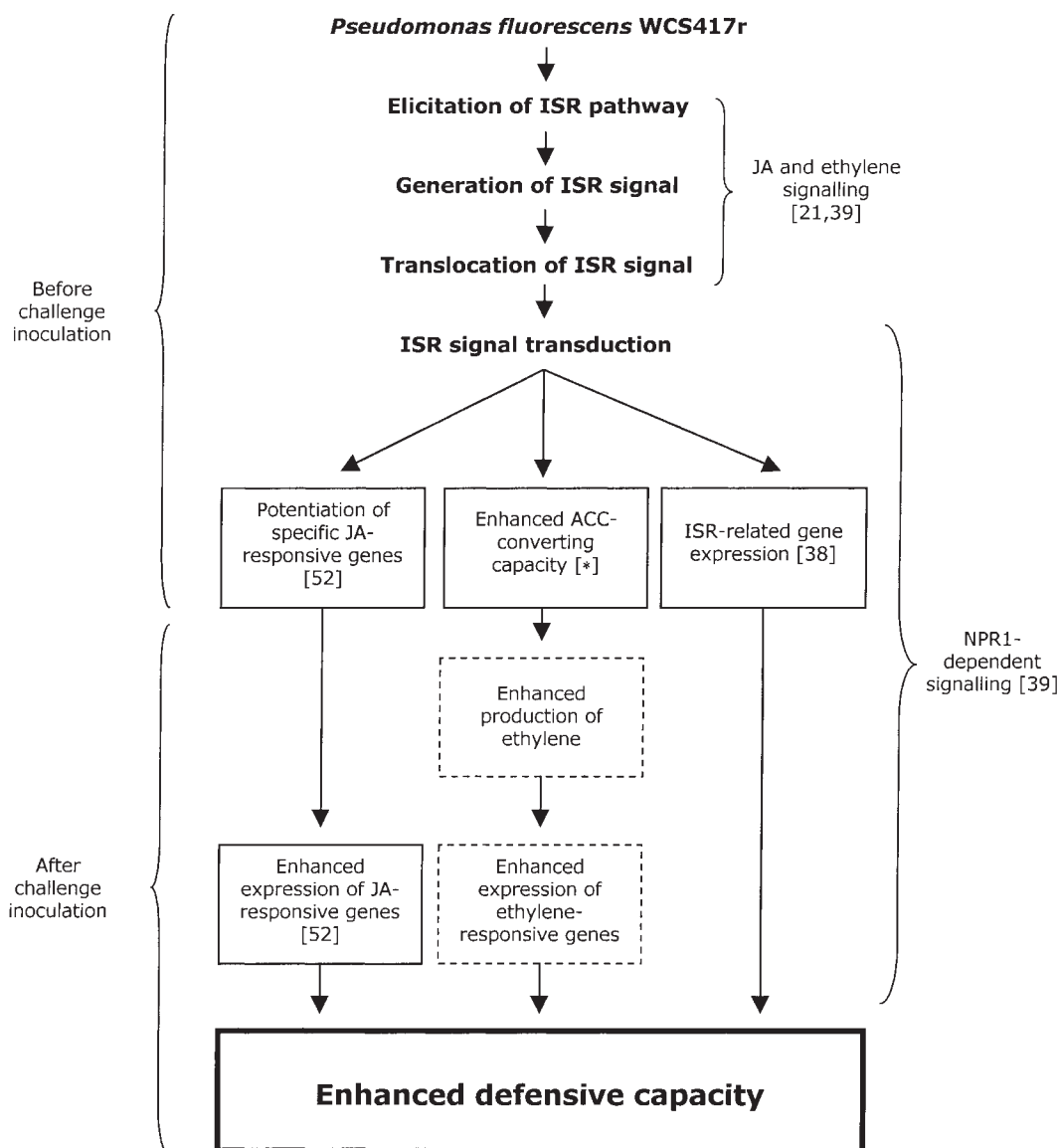


FIG. 7. Working model for the involvement of JA and ethylene in the *Arabidopsis* ISR signalling pathway. Hypothetical steps are in boxes with dotted lines. References are between square brackets. Asterisks indicate experimental evidence presented in this paper.

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