



# Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection

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Accepted 2 May 2003

## Abstract

Plants develop an enhanced defensive capacity against a broad spectrum of plant pathogens after colonization of the roots by selected strains of non-pathogenic, fluorescent *Pseudomonas* spp. In *Arabidopsis thaliana*, this rhizobacteria-induced systemic resistance (ISR) functions independently of salicylic acid but requires responsiveness to the plant hormones jasmonic acid and ethylene. Leaves of plants of which the roots are colonized by ISR-inducing *Pseudomonas fluorescens* WCS417r bacteria show an enhanced capacity to convert the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) to ethylene. Here we show that this enhanced ACC-converting capacity leads to a potentiated expression of the ethylene-responsive genes *PDF1-2* and *HEL* after treatment of the leaves with 1 mM ACC, and a significantly higher level of ethylene emission after challenge inoculation with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000/*avrRpt2*. *P. fluorescens* WCS374r bacteria that are unable to induce ISR against *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* likewise enhanced the *in vivo* ACC oxidase activity in Col-0 plants. Moreover, the ISR-compromised mutants *jar1-1* and *npr1-1* also showed a significant increase in their ability to convert ACC to ethylene after treatment of the roots with *P. fluorescens* WCS417r. These results suggest that the induction of an enhanced ACC-converting capacity is a general response of plants to *P. fluorescens* bacteria and that this response does not contribute to ISR against *P. syringae* pv. *tomato* DC3000 in *Arabidopsis*. Nevertheless, *P. fluorescens* strains clearly prime the plant to produce more ethylene upon pathogen infection. The increased capacity for ethylene production might contribute to an enhanced defensive capacity against pathogens that are sensitive to ethylene-dependent defense responses.

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**Keywords:** ACC; *Arabidopsis thaliana*; Ethylene; Induced systemic resistance; *Pseudomonas fluorescens*; *Pseudomonas syringae* pv. *tomato*

## 1. Introduction

Selected root-colonizing *Pseudomonas* spp. strains have been shown to trigger a plant-mediated resistance response in aboveground plant parts. This type of induced resistance is often referred to as rhizobacteria-mediated induced systemic resistance (ISR) [36]. Phenotypically,

rhizobacteria-mediated ISR resembles classic pathogen-induced systemic acquired resistance (SAR), in which non-infected parts of locally infected plants become more resistant to further infection [25]. *Pseudomonas fluorescens* strain WCS417r has been shown to trigger ISR in several plant species, e.g. carnation [37], radish [16], tomato [6], bean [1] and *Arabidopsis thaliana* [21,22]. Colonization of *Arabidopsis* roots by WCS417r protects the plant systemically 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* f.sp. *raphani*, the fungal leaf pathogen *Alternaria brassicicola* and the oomycete leaf pathogen *Peronospora parasitica* [21,34,40]. The ability to develop ISR in response to selected strains of rhizosphere bacteria has been documented for several plant species [36]

**Abbreviations:** ACC, 1-aminocyclopropane-1-carboxylate; CFU, colony-forming units; FW, fresh weight; ISR, induced systemic resistance; JA, jasmonic acid; SA, salicylic acid; SAR, systemic acquired resistance; TMV, tobacco mosaic virus.

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and is dependent on the host/rhizobacterium combination. For example, *P. fluorescens* WCS374r is able to trigger ISR in radish against *F. oxysporum* [16] but is unable to do so in *Arabidopsis* [40]. This suggests that a specific recognition between the plant and the ISR-inducing rhizobacterium is required for the induction of ISR.

Both rhizobacteria-mediated ISR and pathogen-induced SAR are effective against a broad spectrum of pathogens, but are regulated by distinct signaling pathways. Pathogen-induced SAR requires salicylic acid (SA) whereas WCS417r-mediated ISR functions independently of SA [7,19,21]. Analysis of the jasmonic acid (JA)-response mutant *jar1-1*, the SAR-compromised mutant *npr1-1*, and a range of ethylene-response mutants of *Arabidopsis*, revealed that components of the JA and the ethylene-response are required for triggering ISR and that this induced resistance response, like SAR, depends on the defense regulatory protein NPR1 [13,23,38]. In *Arabidopsis*, both JA and ethylene activate specific sets of defense-related genes and, when applied exogenously, they can confer resistance against certain pathogens, such as *P. syringae* pv. *tomato* DC3000 [23,39]. To investigate whether ISR is associated with changes in JA/ethylene-responsive gene expression, Van Wees *et al.* [39] monitored the expression of a set of well-characterized JA- and/or ethylene-responsive genes (i.e. *LOX1*, *LOX2*, *VSP*, *PDF1-2*, *HEL*, *CHI-B*, and *PAL1*) 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 suggests that the resistance attained was not associated with major increases in the levels of either JA or ethylene. Indeed, analysis of JA levels in, and ethylene production by, leaves treated with WCS417r and untreated plant parts expressing ISR, revealed no changes in the production of these signal molecules [20]. Therefore, it is assumed that the JA and ethylene dependency of ISR is based on enhanced sensitivity to these hormones, rather than on an increase in their production.

Ethylene has repeatedly been implicated in the regulation of primary resistance responses. In many cases, infection by microbial pathogens is associated with enhanced production of this hormone [2] and a concomitant activation of a large set of defense-related genes [27]. In higher plants, ethylene is produced from methionine via *S*-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylate (Met → SAM → ACC → ethylene) [10,11,30]. The last two steps of this biosynthetic pathway are catalysed by ACC synthase and ACC oxidase, respectively. Pathogen infections leading to chlorotic or necrotic symptoms cause an increase in ethylene production with ACC synthase and ACC oxidase activity being increased sequentially [28]. Whereas under normal conditions the conversion of SAM to ACC by ACC synthase is the rate-limiting step, during infections ACC accumulates transiently, indicating that ACC oxidase activity restricts ethylene production. Previously, it was

demonstrated that the capacity for converting ACC to ethylene was increased systemically in SAR-expressing tobacco and *Arabidopsis* plants [5,20], providing a greater capacity for producing ethylene after challenge inoculation. Interestingly, a similar systemic response was observed in plants after treatment of the roots with ISR-inducing WCS417r bacteria [20]. Exogenous application of the ethylene precursor ACC has been shown to induce resistance against *P. syringae* pv. *tomato* in *Arabidopsis* [20,23,39]. Therefore, a faster or greater production of ethylene in the initial phase of infection might contribute to enhanced resistance against this pathogen.

To investigate the role of the WCS417r-induced enhanced capacity to convert ACC to ethylene in ISR, we analysed the expression of the ethylene-responsive genes *HEL*, encoding an hevein-like protein [24], and *PDF1-2*, encoding a plant defensin with antimicrobial properties [18], after exogenous application of ACC to leaves of plants that were grown in soil with or without WCS417r bacteria. In addition, we monitored ethylene emission after challenge inoculation with the necrosis-inducing avirulent pathogen *P. syringae* pv. *tomato* DC3000/*avrRpt2*. To assess the significance of the enhanced ACC-converting capacity to ISR, we tested the effect of an ISR-inducing and an ISR-noninducing strain of *P. fluorescens* on this response using wild-type Col-0 plants and the ISR-response mutants *jar1-1* and *npr1-1*.

## 2. Materials and methods

### 2.1. Growth conditions of rhizobacteria and plants

Non-pathogenic *Pseudomonas fluorescens* WCS417r was used for induction of ISR, whereas *P. fluorescens* WCS374r was used as a non-inducing control strain. Both strains were grown on King's medium B (KB) agar Plate [12] for 24 h at 28°C. Subsequently, bacterial cells were collected and resuspended in 10 mM MgSO<sub>4</sub> to a final density of 10<sup>9</sup> cfu ml<sup>-1</sup> (OD<sub>600</sub> = 1.0), before being mixed through the soil.

Seeds of wild-type *Arabidopsis thaliana* accession Col-0 and Col-0 mutants *jar1-1* [29] and *npr1-1* [3] 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 with a 24 h interval. Before transfer of the seedlings, a suspension of *P. fluorescens* bacteria (10<sup>9</sup> cfu ml<sup>-1</sup>) was mixed through the soil to a final density of 5 × 10<sup>7</sup> cfu g<sup>-1</sup>. Control soil was supplemented with an equal volume of 10 mM MgSO<sub>4</sub>. Plants were cultivated in a growth chamber with a 8 h day (200 μE m<sup>-2</sup> s<sup>-1</sup> at 24°C) and 16 h night (20°C) cycle at 70% relative humidity. Plants were watered on alternate days, and once a week supplied with a modified half-strength Hoagland's nutrient solution, as described [21].

## 2.2. Determination of ACC-converting capacity

To determine the capacity of leaf tissues to convert ACC to ethylene, rosettes of 5-week-old plants were detached from the roots, weighed, and dipped in a solution containing 1 mM ACC and 0.015% (v/v) of the surfactant Silwet L-77 (Van Meeuwen Chemical BV, Weesp, The Netherlands). Control plants were dipped in 0.015% (v/v) Silwet L-77 only. For the determination of the ACC-converting capacity of young and old leaves, rosettes were dissected into two equal parts, thereby separating the oldest leaves from the younger leaves higher up in the rosette. Subsequently, both parts were weighed separately before dipping in 1 mM ACC, 0.015% (v/v) Silwet L-77. After ACC treatment, excess moisture was removed from the leaves with paper towels. Subsequently, each rosette (part) was placed in a gas-tight serum flask that was incubated under climate chamber conditions. At specific time points, 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 [5].

## 2.3. Pathogen inoculation

The avirulent pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 with the plasmid pV288 carrying avirulence gene *avrRpt2* [14] was used for challenge inoculation. *P. syringae* pv. *tomato* DC3000/*avrRpt2* bacteria were cultured overnight at 28°C in liquid King's medium B, supplemented with 25 mg l<sup>-1</sup> kanamycin to select for the plasmid. Subsequently, bacterial cells were collected by centrifugation and resuspended in 10 mM MgSO<sub>4</sub> to a final density of 10<sup>7</sup> cfu ml<sup>-1</sup>. Plants were inoculated by pressure infiltrating a suspension of *P. syringae* pv. *tomato* DC3000/*avrRpt2* at 10<sup>7</sup> cfu ml<sup>-1</sup> into the leaves of 5-week-old plants (12 leaves per plant).

## 2.4. RNA blot analysis

For RNA blot analysis, leaf tissues were collected and frozen in liquid nitrogen. Total RNA was extracted by homogenizing 0.5 g of frozen leaf tissue in 0.5 ml of extraction buffer (0.35 M glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS). The homogenates were extracted with phenol and chloroform, and the RNA was precipitated using LiCl, as described [26]. Fifteen µg of RNA was denatured using glyoxal and dimethyl sulfoxide [26]. Subsequently, samples were electrophoretically separated on 1.5%-agarose gels and blotted onto Hybond-N<sup>+</sup> membranes (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis buffer and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA blots were hybridized with *ACO*, *HEL*, and *PDF1-2* gene-specific probes, and a probe for the constitutively expressed β-tubulin (*TUB*) gene as described previously [39]. Probes for *HEL*, *PDF1-2* and *TUB*

were labeled with α-<sup>32</sup>P-dCTP by random-primer labeling using templates that were generated by PCR as described [39]. Probes to detect *ACO* transcripts were derived from an *Arabidopsis ACO* cDNA clone [9].

## 3. Results

### 3.1. Enhanced ACC-converting capacity in *P. fluorescens* WCS417r-treated *Arabidopsis*

Non-infected leaves of tobacco plants that are induced to express SAR after predisposal infection with tobacco mosaic virus (TMV) show an enhanced capacity to convert ACC to ethylene. The magnitude of the induced increase in the ACC-converting capacity was shown to decrease with leaf age [5]. A similar enhancement of the in vivo ACC oxidase activity has been observed in *Arabidopsis* after colonization of the roots by ISR-inducing WCS417r bacteria [20]. To further investigate this we determined the ACC-converting capacity in the young upper parts and the older lower parts of the rosettes of 5-week-old control and WCS417r-treated Col-0 plants. In control plants grown in soil without rhizobacteria, the constitutive level of ethylene production did not significantly differ between young and old leaves (Fig. 1; -/- ACC). This suggests that the ACC synthase activity, which is the rate-limiting step in ethylene biosynthesis in non-infected plants [4], does not

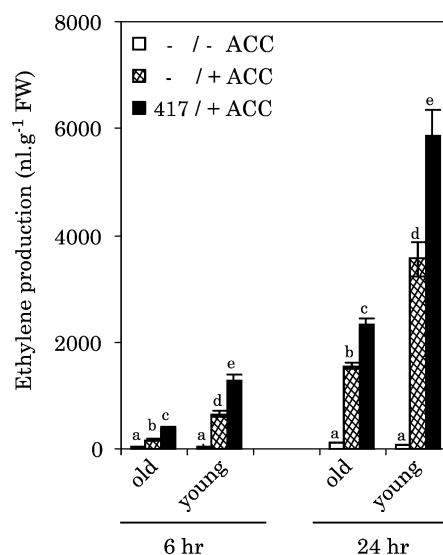


Fig. 1. ACC-converting capacity of young and old leaves of control- and *P. fluorescens* WCS417r-treated *Arabidopsis* Col-0 plants. Ethylene emission by young and old leaves was measured for wild-type Col-0 plants grown in soil with (417) or without (-) ISR-inducing WCS417r bacteria. The ACC-converting capacity was determined over the 6 and 24 h period subsequent to exogenous application of a saturating dose of 1 mM ACC (+ACC). Values are means ( $\pm$ SE) for 10 plants that received the same treatment. Different letters indicate statistically significant differences at the time points indicated (Fisher's LSD test;  $\alpha = 0.05$ ). The data presented are from a representative experiment that was repeated twice with similar results. FW, fresh weight.

differ between young and old leaves. After exogenous application of a saturating dose of 1 mM ACC, young leaves of control plants showed a 12- and 45-fold increase in ethylene production at 6 and 24 h after ACC application, respectively (Fig. 1: -/+ACC). In older leaves, this increase ethylene production was significantly lower (4- and 14-fold at 6 and 24 h after ACC application, respectively), indicating that the capacity to convert ACC to ethylene is higher in young leaves than in old leaves.

In Col-0 plants grown in soil with ISR-inducing WCS417r bacteria, the constitutive level of ethylene production in young and old leaves did not differ from that in control plants (data not shown), confirming previous findings showing that constitutive ethylene emission in WCS417r-treated plants is not enhanced [20]. As in control plants, application of 1 mM ACC to the leaves of WCS417r-treated plants resulted in a considerable increase in the production of ethylene in both young and older leaves (Fig. 1: 417/ + ACC). Compared to ACC-treated control plants, this increase in ethylene emission was significantly higher in both young and older leaves. At 6 h after ACC application, ethylene emission was 91% higher in young leaves and 132% higher in old leaves of WCS417r-treated plants in comparison to similarly treated leaves of control plants. At 24 h after ACC application, these differences were 64 and 49%, respectively.

### 3.2. *ACO* gene expression

To investigate whether the enhanced ACC-converting capacity observed in WCS417r-treated plants is based on increased expression of this gene, we analysed *ACO* transcript levels in young and old leaves of control and WCS417r-treated plants. Fig. 2 shows that *ACO* mRNA levels in control and WCS417r-treated plants were similar. Moreover, when normalized for equal levels of *TUB* mRNA, *ACO* transcript levels did not differ between

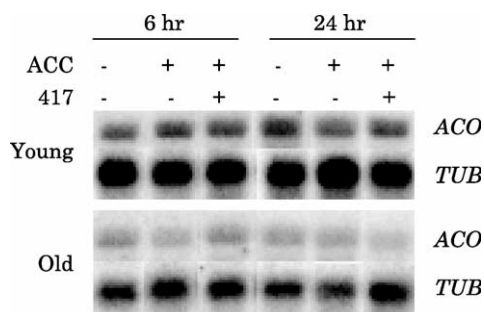


Fig. 2. RNA blot analysis of *ACO* gene expression in young and old leaves of control- and *P. fluorescens* WCS417r-treated *Arabidopsis* Col-0 plants. At the time points indicated, leaf tissue was harvested from ACC-treated and non-treated plants that were grown in soil with or without ISR-inducing WCS417r bacteria (417). Plants were grown in parallel with those used for the experiment of which the results are depicted in Fig. 1. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for  $\beta$ -tubulin (*TUB*).

young and old leaves. This suggests that the higher ACC-converting capacity of young leaves, and the enhanced ACC-converting capacity of *P. fluorescens*-treated plants is regulated post-translationally.

### 3.3. Ethylene-responsive gene expression

To investigate whether the enhanced ACC-converting capacity in WCS417r-treated plants results in a potentiated expression of ethylene-responsive genes, we analysed the expression of the defense-related genes *PDF1-2* and *HEL*. Fig. 3 shows that both *PDF1-2* and *HEL* mRNA accumulated after treatment of the leaves with ACC. At 6 h after ACC application, *PDF1-2* mRNA was detectable in WCS417r-treated plants, but not yet in control plants. At 24 h after ACC application, *PDF1-2* transcript levels were clearly higher in WCS417r-treated plants. Similarly, the expression of the *HEL* gene was considerably higher in rhizobacteria-treated plants at 24 h after ACC application. These results indicate that the enhanced ACC-converting capacity of WCS417r-treated plants leads to an augmented level of ethylene-responsive gene expression in response to treatment with ACC.

### 3.4. Pathogen-induced ethylene production

Upon infection by necrotizing pathogens, ethylene emission rises due to a sequential increase in the activity of ACC synthase and ACC oxidase, respectively [4,28]. The pathogen-induced increase in ACC synthase activity leads to elevated levels of ACC in the initial phases of the infection process. Due to their enhanced ACC-converting

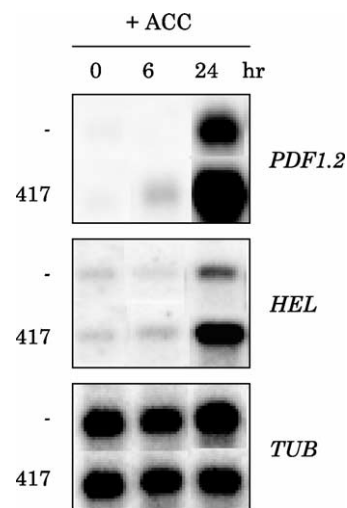


Fig. 3. RNA blot analysis of the expression of the ethylene-responsive genes *PDF1-2* and *HEL*. At the time points indicated, leaf tissues were harvested from the young leaf tissue of ACC-treated plants that were grown in soil with or without ISR-inducing *P. fluorescens* WCS417r bacteria (417). Plants were grown in parallel with those used for the experiment of which the results are depicted in Fig. 1. To check for equal loading, the blots were stripped and hybridized with a gene-specific probe for  $\beta$ -tubulin (*TUB*).

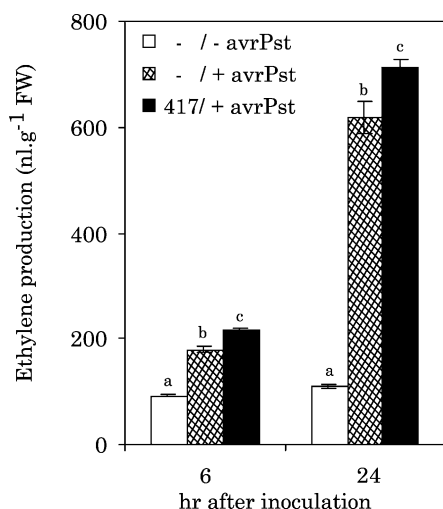


Fig. 4. Ethylene production of control- and *P. fluorescens* WCS417r-treated *Arabidopsis* Col-0 plants after challenge inoculation with *P. syringae* pv. *tomato* DC3000/avrPst2. Ethylene emission was determined at 6 and 24 h after inoculation of wild-type Col-0 plants grown in soil with (417) or without (-) ISR-inducing WCS417r bacteria. Values are means ( $\pm$  SE) for 10 plants that received the same treatment. Different letters indicate statistically significant differences at the time points indicated (Fisher's LSD test;  $\alpha = 0.05$ ). The data presented are from a representative experiment that was repeated twice with similar results. FW, fresh weight.

capacity, WCS417r-treated plants are, therefore, likely to produce more ethylene in the initial phases of infection. To test this hypothesis, we measured ethylene production in control and WCS417r-treated plants at different time points after inoculation with the avirulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000/avrPst2. Fig. 4 shows that WCS417r-treated plants, indeed, produced significantly more ethylene at 6 and 24 h after pathogen infection than similarly inoculated control plants. At 6 h after inoculation,

WCS417r-treated plants produced 20% more ethylene than did control plants. At 24 h after inoculation this difference was still 15%. These results indicate that the enhanced ACC-converting capacity of WCS417r-treated plants leads to an enhanced production of ethylene after pathogen infection.

### 3.5. ACC-converting capacity in plants treated with ISR-noninducing rhizobacteria

To investigate whether the elevated capacity to produce ethylene after pathogen infection in WCS417r-treated plants is associated with ISR, we analysed the ACC-converting capacity of wild-type Col-0 plants after colonization of the roots with *P. fluorescens* WCS374r. WCS374r is a well-characterized rhizobacterial strain that has been shown to induce ISR in radish [16] but is unable to do so in *Arabidopsis* against *P. syringae* pv. *tomato* DC3000 [40]. In similarity to the effect of WCS417r, treatment of the roots with WCS374r bacteria resulted in an enhanced capacity to convert ACC to ethylene (Fig. 5(a)), and a significant increase in ethylene production in the first 24 h after inoculation with *P. syringae* pv. *tomato* DC3000/avrPst2 (Fig. 5(b)). Furthermore, like in WCS417r-treated plants, ACC-induced expression of the ethylene-responsive genes *PDF1-2* and *HEL* was augmented in plants treated with WCS374r bacteria (data not shown). Altogether, these results indicate that the ability of *P. fluorescens* bacteria to induce an enhanced ACC-converting capacity in above-ground plant parts is not related to the ability of these rhizobacteria to induce ISR against *P. syringae* pv. *tomato* DC3000 in *Arabidopsis*.

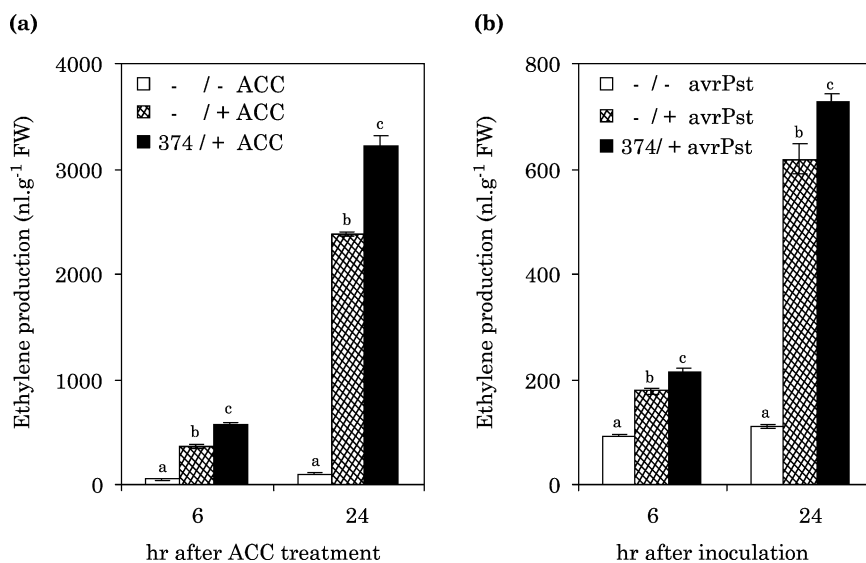


Fig. 5. Ethylene production in *Arabidopsis* Col-0 plants grown in soil with (374) or without (-) ISR-noninducing *P. fluorescens* WCS374r bacteria. Ethylene emission was determined after (a) exogenous application of 1 mM ACC, and (b) after inoculation with *P. syringae* pv. *tomato* DC3000/avrPst2 as described in the legends to Figs. 1 and 4.

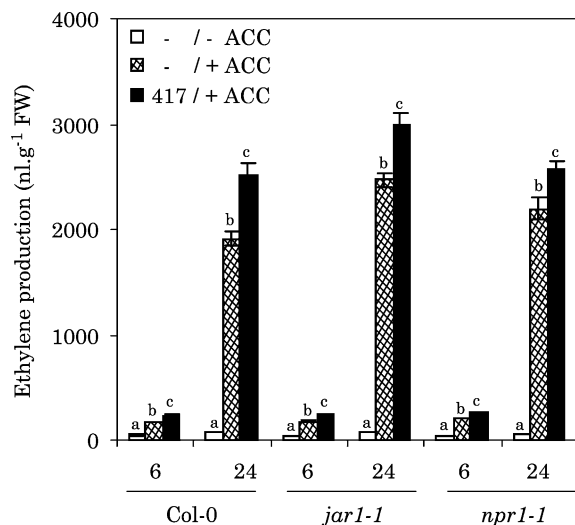


Fig. 6. Ethylene production in wild-type Col-0 plants and the ISR-compromised mutants *jar1-1*, and *npr1-1* grown in soil with (417) or without (-) ISR-inducing *P. fluorescens* WCS417r bacteria. Ethylene emission was determined at the time points indicated as described in the legend to Fig. 1.

### 3.6. ACC-converting capacity in ISR non-expressing mutants *jar1-1* and *npr1-1*

To further investigate the role of the enhanced ACC-converting capacity in ISR, we examined this response in mutant *jar1-1* and *npr1-1* plants that are both unable to express WCS417r-mediated ISR against *P. syringae* pv. *tomato* DC3000 [20,23,38]. Fig. 6 shows that colonization of the roots by WCS417r results in a similar enhancement of the capacity of the leaves to convert ACC to ethylene in Col-0, *jar1-1* and *npr1-1* plants. At 6 h after ACC treatment, WCS417r-treated Col-0, *jar1-1*, and *npr1-1* plants emitted 40, 39, and 25% more ethylene, respectively, than did ACC-treated control plants. At 24 h after ACC treatment, these percentages were 32, 21, and 17%, respectively. Evidently, the enhanced ACC-converting capacity triggered by *P. fluorescens* rhizobacteria develops independently of ISR against *P. syringae* pv. *tomato* DC3000.

## 4. Discussion

*Arabidopsis* plants of which the roots are colonized by ISR-inducing WCS417r bacteria possessed an enhanced capacity to convert ACC into ethylene, confirming previous preliminary observations [20]. As shown, the enhanced *in vivo* ACC oxidase activity in WCS417r-treated *Arabidopsis* is most pronounced in young leaf tissues (Fig. 1) and leads to augmented expression of the ethylene-responsive genes *PDF1-2* and *HEL* after treatment of the leaves with ACC (Fig. 2). The conversion of ACC to ethylene is catalysed by the enzyme ACC oxidase. The expression of the *ACO* gene in the leaves did not change in response to treatment of the roots with WCS417r (Fig. 2), suggesting that the observed

increase in the *in vivo* ACC oxidase activity is regulated post-translationally.

In response to infection by necrosis-inducing pathogens, plants accumulate ACC as a result of enhanced ACC synthase activity [4,28]. Therefore, the enhanced ACC-converting capacity observed in WCS417r-treated plants provides a greater potential for producing ethylene upon pathogen attack. Indeed, WCS417r-treated plants emitted significantly more ethylene after inoculation with *P. syringae* pv. *tomato* DC3000/*avrRpt2* (Fig. 4). Ethylene has been shown to play a crucial role in ISR signal transduction. This is evidenced by the fact that ethylene-insensitive mutants are blocked in their ability to express ISR against *P. syringae* pv. *tomato* DC3000 [13,23]. Moreover, the *Arabidopsis* *ISR1* locus, which is essential for expression of ISR against several pathogens [33,35], is involved in ethylene signaling as well [32]. Thus, a faster or greater production of ethylene in the initial phase of infection upon challenge inoculation of ISR-expressing plants might contribute to the enhanced resistance attained. Therefore, we attempted to correlate the ability of WCS417r to enhance the ACC-converting capacity in the leaves with its capacity to induce ISR against *P. syringae* pv. *tomato* DC3000. Surprisingly, wild-type Col-0 plants and *Arabidopsis* mutants *jar1-1* and *npr1-1*, that are both compromised in their ability to express ISR against *P. syringae* pv. *tomato* DC3000 [20,23,38], were equally able to enhance their ACC-converting capacity in response to colonization of the roots by WCS417r (Fig. 6). Moreover, strain WCS374r, which is unable to induce ISR against *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* [40], induced an enhanced ACC-converting capacity in the leaves as well, resulting in an increase in ethylene production after challenge inoculation with *P. syringae* pv. *tomato* DC3000/*avrRpt2* that was comparable to that observed in WCS417r-treated plants (Fig. 5). Thus, it can be concluded that the enhanced ACC-converting capacity observed in WCS417r- and WCS374r-treated plants is a general response of plants to these *P. fluorescens* bacteria and that this response does not contribute to ISR against *P. syringae* pv. *tomato* DC3000 in *Arabidopsis*.

Our conclusion that the WCS417r-induced enhancement of the ACC-converting capacity does not contribute to the level of ISR against *P. syringae* pv. *tomato* DC3000 is strengthened by another observation in this study. In the absence of WCS417r bacteria, 5-week-old wild-type Col-0 plants showed a significantly higher ACC-converting capacity in the young leaves as compared to the older ones (Fig. 1). Assuming that the resulting elevated levels of ethylene emission in response to *P. syringae* pv. *tomato* DC3000 infection would contribute to resistance against this pathogen, one would expect that young leaves are more resistant to *P. syringae* pv. *tomato* DC3000 than older leaves. However, the opposite is true, because recently Kus et al. [15] demonstrated that resistance to *P. syringae* pathogens increases with leaf age.

Notwithstanding the fact that the enhanced ACC-converting capacity does not contribute ISR against *P. syringae* pv. *tomato* DC3000, our results clearly show that *P. fluorescens* strains prime the plant to produce more ethylene upon infection and to express ethylene-dependent defense-related genes to a higher level upon stimulation with saturating doses of ACC. Therefore, we can assume that this trait can contribute to enhanced resistance against pathogens that are resisted particularly through ethylene-dependent defense responses. In *Arabidopsis*, ethylene has been implicated to function as an important regulator of resistance against necrotrophic pathogens such as *Botrytis cinerea* [31], *Erwinia carotovora* [17], and various *Pythium* spp. [8]. Whether the *P. fluorescens*-mediated enhanced ACC-converting capacity contributes to resistance against these pathogens needs to be investigated.

### Acknowledgements

Drs Paul Staswick and Xinnian Dong are acknowledged for kindly providing seeds of the *Arabidopsis* genotypes *jar1-1*, and *npr1-1*, respectively, and Dr Andrew Bent for providing *P. syringae* pv. *tomato* DC3000 strains. Part of this work was financed by a 12-young-32 grant to S.H. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

### References

- [1] Bigirimana J, Höfte M. Induction of systemic resistance to *Colletotrichum lindemuthianum* in bean by a benzothiadiazole derivative and rhizobacteria. *Phytoparasitica* 2002;30:159–68.
- [2] Boller T. Ethylene in pathogenesis and disease resistance. In: Mattoo AK, Suttle JC, editors. *The Plant Hormone Ethylene*. Boca Raton: CRC Press; 1991. p. 293–314.
- [3] Cao H, Bowling SA, Gordon AS, Dong X. Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. *The Plant Cell* 1994;6:1583–92.
- [4] De Laat AMM, Van Loon LC. Regulation of ethylene biosynthesis in virus-infected tobacco leaves. II. Time course of levels of intermediates and *in vivo* conversion rates. *Plant Physiology* 1982;69:240–5.
- [5] De Laat AMM, Van Loon LC. The relationship between stimulated ethylene production and symptom expression in virus-infected tobacco leaves. *Physiological Plant Pathology* 1983;22:261–73.
- [6] Duijff BJ, Pouhair D, Olivain C, Alabouvette C, Lemanceau P. Implication of systemic induced resistance in the suppression of fusarium wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47. *European Journal of Plant Pathology* 1998;104:903–10.
- [7] Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 1993;261:754–6.
- [8] Geraats BPJ, Bakker PAHM, Van Loon LC. Ethylene insensitivity impairs resistance to soilborne pathogens in tobacco and *Arabidopsis thaliana*. *Molecular Plant Microbe-Interactions* 2002;15:1078–85.
- [9] Gómez-Lim MA, Valdés-López V, Cruz-Hernández A, Saucedo-Arias LJ. Isolation and characterization of a gene involved in ethylene biosynthesis from *Arabidopsis thaliana*. *Gene* 1993;134:217–21.
- [10] Kende H. Ethylene biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 1993;44:283–307.
- [11] Kende H, Zeevaart JAD. The five ‘classical’ plant hormones. *The Plant Cell* 1997;9:1197–210.
- [12] King EO, Ward MK, Raney DE. Two simple media for the demonstration of phycocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* 1954;44:301–7.
- [13] Knoester M, Pieterse CMJ, Bol JF, Van Loon LC. Systemic resistance in *Arabidopsis* induced by rhizobacteria requires ethylene-dependent signaling at the site of application. *Molecular Plant-Microbe Interactions* 1999;12:720–7.
- [14] Kunkel BN, Bent AF, Dahlbeck D, Innes RW, Staskawicz BJ. *RPS2*, an *Arabidopsis* disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *AvrPto2*. *The Plant Cell* 1993;5:865–75.
- [15] Kus JV, Zaton K, Sarkar R, Cameron RK. Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. *The Plant Cell* 2002;14:479–90.
- [16] Leeman M, Van Pelt JA, Den Ouden FM, Heinsbroek M, Bakker PAHM, Schippers B. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. *European Journal of Plant Pathology* 1995;101:655–64.
- [17] Norman-Setterblad C, Vidal S, Palva TE. Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell wall-degrading enzymes from *Erwinia carotovora*. *Molecular Plant-Microbe Interactions* 2000;13:430–8.
- [18] Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Métraux J-P, Manners JM, Broekaert WF. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *The Plant Cell* 1996;8:2309–23.
- [19] Pieterse CMJ, Van Loon LC. Salicylic acid-independent plant defence pathways. *Trends in Plant Science* 1999;4:52–8.
- [20] Pieterse CMJ, Van Pelt JA, Ton J, Parchmann S, Mueller MJ, Buchala AJ, Métraux J-P, Van Loon LC. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiological and Molecular Plant Pathology* 2000; 57:123–34.
- [21] Pieterse CMJ, Van Wees SCM, Hoffland E, Van Pelt JA, Van Loon LC. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *The Plant Cell* 1996;8:1225–37.
- [22] Pieterse CMJ, Van Wees SCM, Ton J, Van Pelt JA, Van Loon LC. Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *Plant Biology* 2002;4:535–44.
- [23] Pieterse CMJ, Van Wees SCM, Van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, Van Loon LC. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *The Plant Cell* 1998;10:1571–80.
- [24] Potter S, Uknes S, Lawton K, Winter AM, Chandler D, DiMaio J, Novitzky R, Ward E, Ryals J. Regulation of a hevein-like gene in *Arabidopsis*. *Molecular Plant-Microbe Interactions* 1993;6:680–5.
- [25] Ross AF. Systemic acquired resistance induced by localized virus infections in plants. *Virology* 1961;14:340–58.
- [26] Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press; 1989.
- [27] Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM. Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:11655–60.
- [28] Spanu P, Boller T. Ethylene biosynthesis in tomato plants infected by *Phytophthora infestans*. *Journal of Plant Physiology* 1989;134: 533–7.

- [29] Staswick PE, Yuen GY, Lehman CC. Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proceedings of the National Academy of Sciences of the United States of America* 1992;89:6837–40.
- [30] Theologis A. One rotten apple spoils the whole bushel: the role of ethylene in fruit ripening. *Cell* 1992;70:181–4.
- [31] Thomma BPHJ, Eggermont K, Tierens KFM, Broekaert WF. Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. *Plant Physiology* 1999;121:1093–102.
- [32] Ton J, Davison S, Van Wees SCM, Van Loon LC, Pieterse CMJ. The *Arabidopsis ISR1* locus controlling rhizobacteria-mediated induced systemic resistance is involved in ethylene signaling. *Plant Physiology* 2001;125:652–61.
- [33] Ton J, Pieterse CMJ, Van Loon LC. Identification of a locus in *Arabidopsis* controlling both the expression of rhizobacteria-mediated induced systemic resistance (ISR) and basal resistance against *Pseudomonas syringae* pv. *tomato*. *Molecular Plant-Microbe Interactions* 1999;12:911–8.
- [34] Ton J, Van Pelt JA, Van Loon LC, Pieterse CMJ. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. *Molecular Plant Microbe-Interactions* 2002;15:27–34.
- [35] Ton J, Van Pelt JA, Van Loon LC, Pieterse CMJ. The *Arabidopsis ISR1* locus is required for rhizobacteria-mediated induced systemic resistance against different pathogens. *Plant Biology* 2002;4:224–7.
- [36] Van Loon LC, Bakker PAHM, Pieterse CMJ. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 1998;36:453–83.
- [37] Van Peer R, Niemann GJ, Schippers B. Induced resistance and phytoalexin accumulation in biological control of fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. *Phytopathology* 1991;91:728–34.
- [38] Van Wees SCM, De Swart EAM, Van Pelt JA, Van Loon LC, Pieterse CMJ. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:8711–6.
- [39] Van Wees SCM, Luijendijk M, Smoorenburg I, Van Loon LC, Pieterse CMJ. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. *Plant Molecular Biology* 1999;41:537–49.
- [40] Van Wees SCM, Pieterse CMJ, Trijssenaar A, Van 't Westende YAM, Hartog F, Van Loon LC. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Molecular Plant-Microbe Interactions* 1997;10:716–24.