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## The ethylene pathway contributes to root hair elongation induced by the beneficial bacteria *Phyllobacterium brassicacearum* STM196

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### ABSTRACT

In *Arabidopsis* roots, some epidermal cells differentiate into root hair cells. Auxin regulates root hair positioning, while ethylene controls cell elongation. *Phyllobacterium brassicacearum* STM196, a beneficial strain of plant growth promoting rhizobacteria (PGPR) isolated from the roots of field-grown oilseed rape, stimulates root hair elongation in *Arabidopsis thaliana* seedlings. We investigated the role of ethylene in the response of root hair cells to STM196 inoculation. While we could not detect a significant increase in ethylene biosynthesis, we could detect a slight activation of the ethylene signalling pathway. Consistent with this, an exhaustive survey of the root hair elongation response of mutants and transgenic lines affected in the ethylene pathway showed contrasting root hair sensitivities to STM196. We propose that local ethylene emission contributes to STM196-induced root hair elongation.

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### 1. Introduction

Plant growth promotion by rhizobacteria can be achieved by several mechanisms [1]. Plant growth promoting rhizobacteria (PGPR) can improve resistance to pathogens by triggering an induced systemic resistance (ISR) [2], stimulate plant mineral nutrition [3,4], and finally, elicit changes in root architecture and root hair development (for reviews, see [4–7]).

The root development of inoculated plants can be modified by the presence of large amounts of auxin produced by bacteria such as *Azospirillum brasilense*, *Pseudomonas putida* UW4 or *Pseudomonas fluorescens* HP72 [8–11]. Alternatively, root development can also be modified when PGPR affect the host's hormonal balance. For example, the genomes of many PGPR strains contain the ACC deaminase gene (*acdS*) [12,13], the activity of which modifies the ethylene balance in the root thereby affecting root development [13–15]. Root hair development is also affected by PGPR inoculation in oilseed rape [3], *Arabidopsis thaliana* [13,16], tomato [17] and wheat [18,19]. Root hair development in response to PGPR involves ethylene, as shown by tomato root hair elongation in

response to *Azospirillum* sp. [17]. However, this may not be the general rule because *Arabidopsis* root hair elongation promoted by *Bacillus megaterium* sp. appears to occur independent of ethylene signalling [16]. Root hair development may also involve auxin, as inoculation of wheat plants with an auxin-deficient mutant of the beneficial *A. brasilense* Sp245 strain results in significantly less root hair elongation than that which was observed upon inoculation with the wild type strain [19].

Auxin plays an essential role in root hair development. The positioning of the emerging tip on hair-forming cells, referred to as planar polarity, is dependent on the local accumulation of auxin [20]. Auxin is locally synthesized and transported in non-hair-forming cells [21,22]. This auxin gradient also aids in maintaining root hair elongation [23]. Consistent with this, some auxin-resistant or -insensitive mutants are impaired in root hair elongation [23–26]. In addition to auxin, ethylene also stimulate root hair elongation [25]. Additionally, ethylene plays a more important role in controlling root hair elongation compared to auxin. Indeed, ten times more NAA is required to restore a wild type root hair length in the *ein2* mutant compared to the *aux1-17* and *aux1-22* mutants [26]. That primacy can also be extended to root hair positioning, as an ethylene signalling intermediate (*i.e.*, CTR1) controls auxin-dependent planar polarity [21]. Ethylene also plays a key role during beneficial plant–microbe interactions, particularly with respect to root development and the induction of systemic resistance (ISR) to pathogens [17,27–30].

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Several rhizobacteria strains have been isolated from the rhizosphere of field-grown oilseed rape (*Brassica napus* L.) [3,31]. One of these strains is a novel subspecies of *Phyllobacterium*, namely *Phyllobacterium brassicacearum* STM196 (STM196) [32]. The STM196 strain is beneficial to the growth of both oilseed rape and Arabidopsis [31,33]. Upon inoculation, STM196 also stimulates Arabidopsis root hair elongation [13]. In this work, we focus on the implication of ethylene in the promotion of root hair elongation in Arabidopsis seedlings via the inoculation of the beneficial strain *P. brassicacearum* STM196. Here, we report that STM196 affects Arabidopsis root hair elongation in a dose-dependent manner. We propose that the underlying mechanism would not depend on a measurable increase in ethylene biosynthesis but would instead result in the activation of the ethylene signalling pathway, which would in turn contribute to root hair elongation.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

*A. thaliana* seeds of wild type Col-0 and ethylene mutants (*etr1-1*, *ein3-1*, *ebf2-3*, *wei2wei7*) were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds of the *ein2*, *eto1* and *ctr1* mutants were kindly provided by Dr. Liam Dolan (John Innes Centre, Norwich, UK). Seeds of the *ein3eil1* double mutant were kindly provided by Dr. Brad Binder (University of Tennessee, Knoxville, USA). Seeds of the *35S::EIN3-FLAG* and the *35S::EBF1* transgenic lines were generous gifts of Dr. Jen Sheen (Massachusetts General Hospital, Boston, USA) and Dr. T. Potuschak and Dr. P. Genschick (IBMP, Strasbourg, France), respectively. All lines used in this study are of the Col-0 genetic background.

The Arabidopsis seeds were surface-sterilised by immersion in 2% bleach supplemented with 0.1% Tween 20 for 15 min. Seeds were subsequently washed five times with sterile distilled water and sown onto solid 1% (w/v) agar (A1296, Sigma, St Louis, MO, USA) mineral medium [33]. Plates were sealed with micropore tape (3M™, St Paul, MN, USA). After 2 days in the cold room (4 °C) in the dark, plates were placed in a nearly vertical position to allow the primary roots to grow on the surface of the agar medium. The growth chamber light and environmental conditions were identical to those previously described [33].

### 2.2. Bacterial growth conditions and plant inoculation

The PGPR bacterial strain used in this study was *P. brassicacearum* strain STM196 [32]. The strain was regularly streaked from the glycerol stock on solid E' medium and allowed to grow for 3 days at 27 °C [33].

The STM196 inoculum was prepared from a liquid culture in exponential growth phase. Bacteria were pelleted by centrifugation at 7000 rpm for 5 min. Pellets were resuspended in sterile liquid plant medium. The amount of bacteria present in the inoculum was determined by the measurement of the optical density at 595 nm (WPA UV 1101, Biotech Photometer, Cambridge, UK). Using a previously established correlation between optical density and CFU per ml (Mantelin, unpublished data), the exact volume of inoculum was added to melted sterile plant media kept at 52 °C to obtain the required final bacterial concentration. After inoculation, the media was immediately poured and allowed to solidify. Typically, seven- to ten-day-old seedlings that were uniform in appearance were aseptically transferred onto the inoculated solid plant medium. After transfer, plates were sealed with micropore tape (3M™, St Paul, MN, USA) and again laid vertically in the growth chamber. Plantlets were allowed to grow for a week before being either measured or harvested.

### 2.3. Root hair length analysis

The length of fully elongated root hairs was assessed using a binocular microscope (Olympus SZH10, Olympus, Tokyo, Japan) equipped with a CCD camera (JVC TK-C1381, JVC, Yokohama, Japan). Images of elongated root hairs were digitalised, and the length was measured using AnalySiS Pro 3.00 software (Soft Imaging System GmbH, Münster, Germany). Typically, 30 root hairs were measured per plant on the primary root between the end of the elongation zone and the site of emergence of the first lateral root.

To calculate the sensitivity of root hairs to STM196 inoculation for the various genotypes, we first calculated the average increase in root hair length in response to STM196 using Col-0 (STM196 effect). Next, we added this average to the average root hair length measured in each independent experiment for each genotype grown under control conditions. The resulting value corresponds to the expected root hair length in each experiment for each genotype. Finally, the root hair sensitivity to STM196 in each experiment and for each genotype was obtained by subtracting the expected root hair length from the observed root hair length.

### 2.4. Measurement of ethylene emission

To assay ethylene emission, the various wild type and mutant lines were sown on mineral media in square Petri dishes. After a cold treatment (2 days at 4 °C in the dark), plates were moved to the growth chamber. Two days after germination, two plantlets were transferred into a small sterile gas chromatograph (GC) vial (VAG M549/551, Chromacol, Welwyn Garden City, UK) containing 500 µl of solid plant media. The glass vials were closed with micropore tape and the vials containing the seedlings were placed in the growth chamber. After a week, the vials were then closed hermetically using a screw cap fitted with a septum. The plantlets were left to grow for an additional week. For ethylene measurements, 600 µl of air was taken from the vial with a syringe and injected into a GC (6850 series GC system equipped with a capillary column HP-AL/S, Agilent, Palo Alto, CA, USA) coupled to a flame ionisation detector. Ethylene measurement was performed at least 3 times independently per condition. Results were given as peak area per fresh weight and expressed relative to the wild type under control conditions.

### 2.5. RNA extraction, cDNA synthesis and real-time quantitative PCR

For quantitative real-time RT-PCR amplification, total RNA was isolated from 14-day-old plants inoculated with STM196 for seven days and uninoculated controls. Roots and shoots were harvested separately, frozen in liquid N<sub>2</sub> and stored at –80 °C. For each treatment, about 100 mg of frozen tissue was collected in 2 ml Eppendorf tubes. The frozen plant material was ground in a mixing mill (Retsch MM200, Haan, Germany) using a 5 mm stainless steel bead. Total RNA was isolated from crushed plant tissue using the SV Total RNA isolation system (Promega, Madison, WI, USA). DNase I treatment was performed during the extraction according to the manufacturer's instructions. The quality and quantity of the recovered total RNA was assayed by UV spectrophotometry with a Nanodrop (ND1000, Thermo Fisher Scientific, MA, USA). 500 ng of RNA were used to synthesise first strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Eugene, OR, USA), and a quality control was done as previously described [33].

Real-time PCR was performed on the Mx3005P QPCR System using Brilliant II SYBR Green QPCR Master Mix (Agilent-Stratagene, Cedar Creek, TX, USA). Each QPCR reaction is a mix of 0.1 µl cDNA, 4.9 µl H<sub>2</sub>O, 10 µl Brilliant II SYBR Green QPCR Master Mix and 5 µl primers at 1 µM. The reactions were incubated at 95 °C for 10 min,

followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 1 min. At the end of the 40 cycles, all reactions were heated to 95 °C for 1 min, cooled to 60 °C and then warmed up regularly from 60 °C to 95 °C for determination of melting temperature.

The expression level of a gene of interest was standardised to the *ubiquitin11* (At4g05320) and *actin2* (At3g18780) reference genes [34] using the following formula:  $\Delta Ct = Ct_x - Ct_{ref}$ . The efficiency of amplification for a cDNA template was evaluated by a dilution series using the *ubiquitin11* and *actin2* primers. QPCRs were performed with cDNA templates characterised by similar amplification efficiency. Because the difference in expression levels calculated using the two reference genes was minimal, we present only results obtained using *actin2* as a reference. All results are expressed as  $2^{-\Delta Ct}$  against a logarithmic scale.

A biological repeat is defined as RNA extraction, cDNA synthesis and finally gene expression level measurement from a unique plant tissue sample harvested on the same date from eight plants growing simultaneously. The expression level for each gene was determined at least three times.

### 2.6. Plant soluble protein extraction, protein electrophoresis and western blotting

Seven days after inoculation, wild type and *35S::EIN3::FLAG* seedlings were harvested. Soluble proteins were extracted from whole plants (1 g fresh weight) ground in 1 ml of a protein extraction buffer (Saccharose 0.5 M, EDTA-Na<sup>2</sup> 10 mM, EGTA-Na<sup>2</sup> 10 mM, DTT 10 mM, Ascorbate 5 mM, PMSF 1 mM, Tris-MES 0.2 M pH 8.0) using a Potter device. Extracts were stored on ice and later centrifuged at 4 °C for 20 min at 15,000 rpm. Supernatants were carefully collected, aliquoted and stored at –20 °C.

Protein amounts were determined using the Bradford method [35] (Sigma, St. Louis, MO, USA). BSA (Sigma, St. Louis, MO, USA) was used as a protein standard.

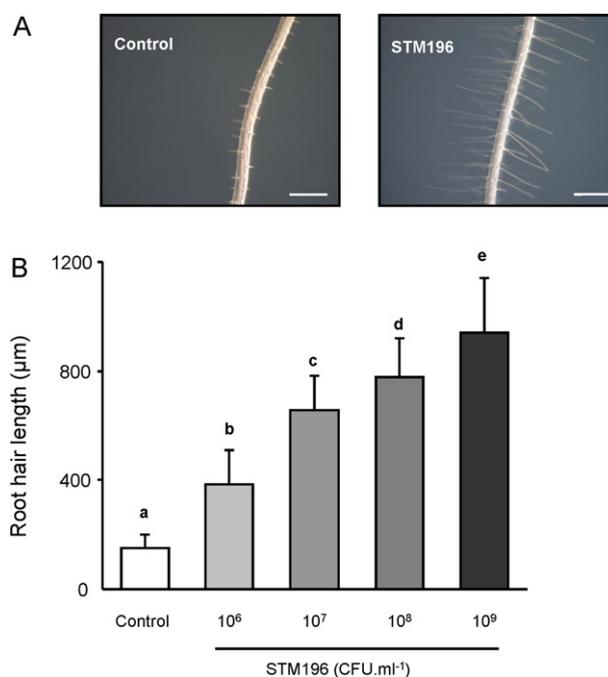
Prior to electrophoresis, identical amounts of soluble proteins were pipetted and mixed in Laemmli denaturation buffer and subsequently boiled for 1 min [36]. Soluble proteins were then separated on a SDS electrophoresis gel (4% stacking; 10% running [36]) in a mini proteanII electrophoresis system (Biorad, Hercules, CA, USA) using a current field of 15 mA per gel. We always ran a gel for western blotting as well as a gel for coomassie staining simultaneously in order to verify the amount of protein loaded onto the gel (EZBlue™ Gel Staining Reagent, Sigma, St. Louis, MO, USA).

For western blotting, separated proteins were electroblotted onto PVDF membrane (Hybond-P, Amersham, Little Chalfort, HP, UK) according to the manufacturers' recommendations. For immunodetection, the membrane was washed in PBS buffer and blocked overnight at 4 °C in blocking buffer (1× PBS, 1% (w/v) BSA, 0.1% Tween 20). The anti-flag antibody (F-3165, Sigma, St. Louis, MO, USA) was diluted 15,000 times in blocking buffer and incubated with the membrane for 1.5 h. The membrane was then extensively washed using fresh blocking buffer and incubated with the secondary antibody (anti-mouse alkaline phosphatase labelled, A3562, Sigma, St. Louis, MO, USA) for 1 h. Finally, the membrane was thoroughly washed again in fresh blocking buffer.

Prior to detection, the membrane was successively rinsed first in 1× PBS followed by alkaline phosphatase buffer (1 mM MgCl<sub>2</sub>, 20 mM Tris, pH 9.8). Alkaline phosphatase activity was monitored by chemiluminescence (Immobilon Western chemiluminescence AP substrate, Millipore, Billerica, MA, USA) using a LAS3000 imaging system (Fujifilm, Tokyo, Japan) according to the manufacturers' recommendations.

### 2.7. Statistical analysis

ANOVA tests were performed with the Statistica 6.0 software (StatSoft, Maisons-Alfort, France) using the Fisher Least Significant



**Fig. 1.** Inoculation with STM196 increases Col-0 seedling root hair length in a dose-dependent manner. (A) Images of representative Col-0 root hairs inoculated with STM196 at 10<sup>8</sup> CFU ml<sup>-1</sup> along with uninoculated controls. The scale bars represent 500 μm. (B) Each bar represents the mean (+SD) root hair length of Col-0 seedlings inoculated with STM196 at 10<sup>6</sup>, 10<sup>7</sup>, 10<sup>8</sup> or 10<sup>9</sup> CFU ml<sup>-1</sup> along with uninoculated controls. Means were calculated from 224 to 770 measurements taken from at least three biological replicates. Different letters indicate significantly different means according to Fisher's LSD test ( $P=0.05$ ).

Difference test at the significance threshold indicated in the figure legends.

## 3. Results

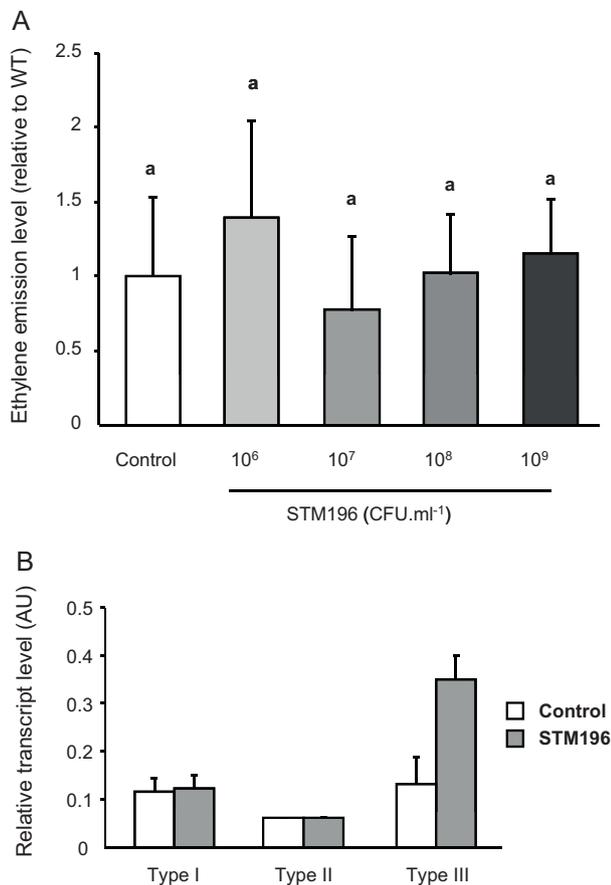
### 3.1. *P. brassicacearum* STM196 promotes *Arabidopsis* root hair elongation without a significant increase in ethylene emission

One of the most notable effects of STM196 inoculation on *Arabidopsis* is the stimulation of root hair elongation [13]. Indeed, seven days after inoculation, the average root hair length of inoculated seedlings was two to five times longer compared to control plants (Fig. 1A and B). Additionally, the resulting root hair length increased in parallel with increasing STM196 inoculum concentrations (Fig. 1B).

Given the key role of ethylene in root hair elongation, we measured ethylene emission in two-week-old *Arabidopsis* seedlings inoculated with STM196 at various concentrations (Fig. 2A). At the whole plant level, STM196 inoculation did not significantly alter ethylene emission. We further tested the implication of ethylene biosynthesis by measuring the transcript levels of the functional *aminocyclopropane synthase* (ACS) gene family members in the roots and shoots of *Arabidopsis* seedlings inoculated with STM196 at 10<sup>8</sup> CFU ml<sup>-1</sup>. We observed that the accumulation of subtype III ACS transcripts (*ACS7* and *ACS11*) increased in the roots of inoculated plants as compared to non-inoculated plants (Fig. 2B).

### 3.2. STM196 inoculation would stabilise the EIN3 protein

Although we observed transcript accumulation of subtype III ACS genes in inoculated plants, we failed to detect significant changes in ethylene emission. However, the ethylene signalling



**Fig. 2.** STM196 does not increase Arabidopsis ethylene emission but does increase subtype 3 transcript abundance. (A) Each bar represents the mean ( $\pm$ SD) ethylene emission of Col-0 seedlings inoculated with STM196 at  $10^6$ ,  $10^7$ ,  $10^8$  or  $10^9$  CFU ml<sup>-1</sup> along with uninoculated controls. No statistical differences could be observed based on Fisher's LSD test ( $P=0.05$ ). (B) Each bar corresponds to the average expression level ( $\pm$ SD) of the *ACC synthase* genes (*ACS*) in the roots presented by *ACS* subtype (type I: *ACS2* and *ACS6*; type II: *ACS4*, *ACS5*, *ACS8*, *ACS9*; type III: *ACS7* and *ACS11*). The expression level of the *ACS* gene family members relative to the *actin2* gene was measured in the root of two-week-old Arabidopsis Col-0 seedlings inoculated with STM196 at  $10^8$  CFU ml<sup>-1</sup> along with uninoculated controls. For each biological repeat ( $n=3$ ), the expression levels of *ACS* genes belonging to the same subtype were added together.

pathway could be activated in the presence of STM196, thereby promoting root hair elongation.

Treatment with ethylene reduced the EIN3 and EIN3::FLAG chimeric protein turnover, and these proteins subsequently accumulated [37]. To measure EIN3 abundance in the presence of STM196, we monitored the abundance of the EIN3::FLAG polypeptide. We observed a small but reproducible increase in the abundance of the EIN3::FLAG protein in the shoots of inoculated seedlings (Fig. 3A), but this was not observable in the roots (Fig. 3B). The increase in abundance of EIN3::FLAG in the presence of STM196 in the Arabidopsis transgenic line suggests that STM196 could stabilise EIN3 in the wild type line.

*EIN3* encodes a transcription factor that regulates the expression of *EBF2*, and to a lesser extent, *EBF1* [38]. The increased abundance of the *EBF2* transcript could reflect an increase in EIN3 protein abundance. We measured the *EBF2* and *EBF1* transcript levels in the roots and shoots of control and inoculated seedlings, and we recorded a small but significant (3 fold) increase in *EBF1* and *EBF2* transcript levels in both the roots and the shoots of inoculated seedlings (Fig. 3C and D).

Taken together, these results suggest that STM196 could stabilise a small but significant portion of the EIN3 transcription factor

pool in the shoot; this would in turn increase transcription of *EBF1* and *EBF2*.

### 3.3. Root hair elongation in ethylene signalling mutants is sensitive to STM196 inoculation

If ethylene is involved in the root hair response to STM196 inoculation, we would expect ethylene signalling mutants to be affected in response to the bacteria. We first tested the root hair response of the *etr1* and *ein2* mutants [39] together with the *ctr1* mutant [40]. Under non-inoculated conditions, the *etr1* and *ein2* mutants have shorter root hairs than the wild type, while the *ctr1* mutant has longer root hairs (Fig. 4A). Upon inoculation with STM196, the root hairs of *etr1* and *ein2* mutants were longer than in control conditions (1.9 and 2.5 fold, respectively) but remained shorter than the wild type root hairs under the same conditions, which were 3.7 fold longer (Fig. 4A). Similarly, the root hairs of the *ctr1* mutant were 1.6 fold longer than in control conditions and remained longer than the wild type root hairs under the same conditions (Fig. 4A).

We then measured the root hair lengths of *EIN3* mutants and transgenic lines. In the absence of STM196, the root hair length of *ein3* was slightly shorter than the wild type, whereas that of *eil1* and, more surprisingly, of *ein3eil1* was not different from the wild type (Fig. 4A). Upon inoculation with STM196, the *ein3*, *eil1* and *ein3eil1* mutants all exhibited longer root hairs (3, 3.4 and 3.3 fold increases, respectively) (Fig. 4A). The root hair length of *35S::EIN3::FLAG* seedlings also increased 2.6 fold after STM196 inoculation (Fig. 4A).

Finally, we measured the root hair response of *EBF1* and *EBF2* mutants and transgenic lines. We observed longer root hairs in the *ebf1* mutant compared to the wild type under control conditions, while the *ebf2* mutant had a similar root hair length (Fig. 4A). Upon inoculation, the *ebf1* and *ebf2* root hair lengths increased; however, the *ebf1* fold increase was smaller than that of *ebf2* (2.5 vs. 4.7, respectively) (Fig. 4A). Under control conditions, *35S::EBF1* seedlings have shorter root hairs compared to the wild type (Fig. 4A). Upon inoculation, the *35S::EBF1* line showed a 3.3 fold increase in root hair length (3.3 fold) (Fig. 4A).

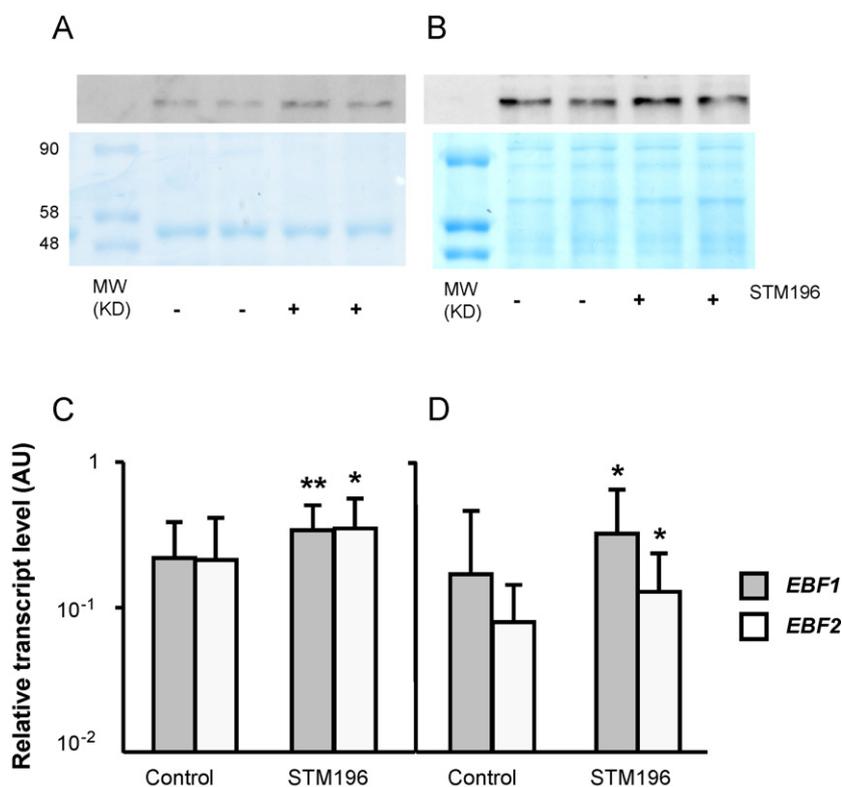
Taken together, our data indicate that the root hairs of all ethylene mutants and transgenic lines tested are still able to elongate to various degrees in response to STM196. Thus, the ethylene signalling pathway is not directly required for this response.

### 3.4. Genotypes affected in ethylene signalling show contrasting root hair elongation types in response to STM196

Interestingly, some ethylene mutants show differential fold increases in root hair length. This could indicate an interaction between the genotype and STM196 that affects root hair elongation. To gain further insight into this issue, we calculated an expected root hair length for each genotype upon inoculation and evaluated how similar the expected value was to the actual (measured) value (Fig. 4B). It was apparent that the root hairs of *etr1*, *ctr1*, *ein2* and *ein3* mutants, as well as the *35S::EBF1* transgenic lines, have reduced sensitivities to STM196 compared to the wild type. However, the *ebf2* mutant showed an increased sensitivity. This indicates that the ethylene signalling pathway (*ETR1*, *CTR1*, *EIN2* and *EIN3*) and some of its negative regulators (*EBF1*) might contribute to the regulation of root hair elongation in response to STM196.

### 3.5. Local auxin biosynthesis is unlikely to contribute to root hair elongation by STM196

Previous work conducted in our laboratory showed that STM196 inoculation (i) affects auxin distribution in the root tip with



**Fig. 3.** STM196 inoculation stabilises EIN3 and slightly induces *EBF1* and *EBF2* transcript accumulation. (A and B) The amount of EIN3::FLAG protein was determined by western blotting followed by immunodetection with a monoclonal anti-FLAG antibody. Total soluble proteins were extracted from the shoot (A) or root (B) of transgenic *35S::EIN3::FLAG* seedlings inoculated with STM196  $10^8$  CFU ml<sup>-1</sup> along with uninoculated controls. Total soluble proteins separated on a 12% SDS-PAGE (Western blot with anti-FLAG antibody, upper panel; Coomassie staining of total proteins, lower panel). These data represent two biologically independent experiments. (C and D) The relative transcript levels of *EBF1* and *EBF2* were measured in the roots (C) and in the shoots (D) of two-week-old Arabidopsis Col-0 seedlings inoculated with STM196 at  $10^8$  CFU ml<sup>-1</sup> along with uninoculated controls. Each mean (+SD) gene expression value was calculated from at least three biological replicates and normalised relative to the transcript level of the *actin2* gene. Statistically different means were tested at \*\* $P=0.05$  and \* $P=0.1$  with the Student's *t*-test.

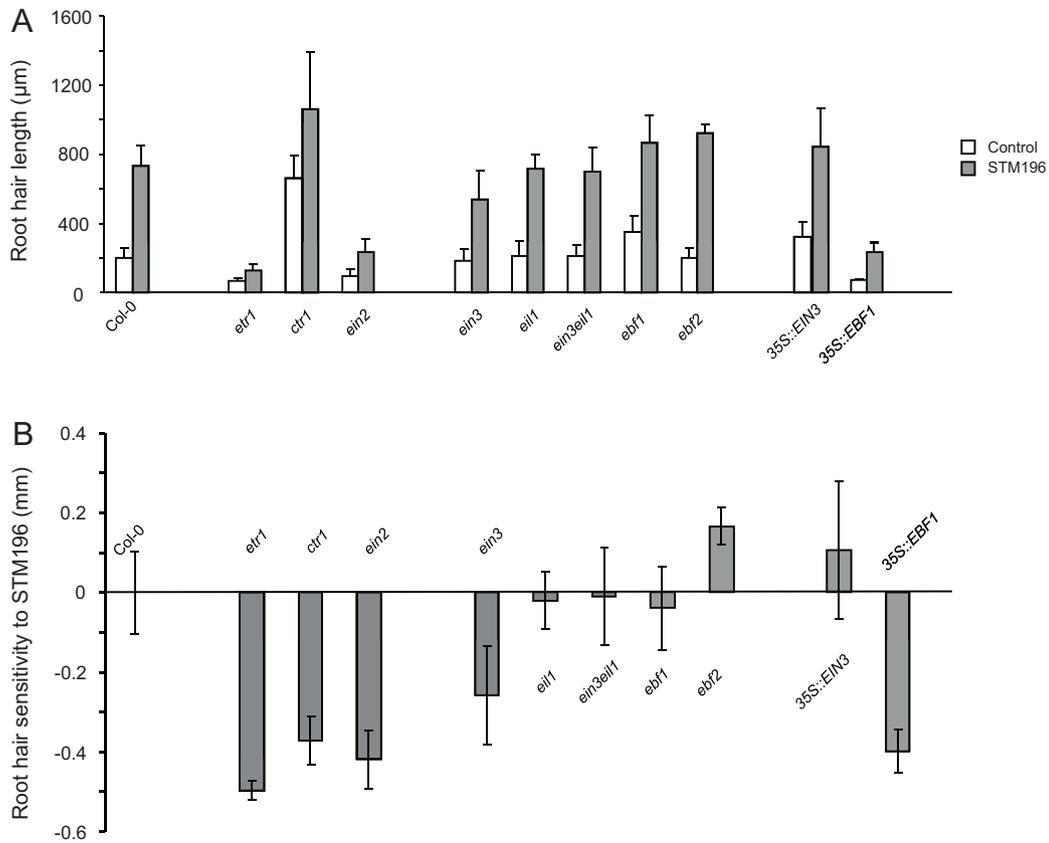
increased accumulation in the vasculature and, at the same time, (ii) increases the transcript abundance of two key auxin biosynthesis genes (*ASA1* and *ASB1*) [41]. These two genes encode different subunits of the anthranilate synthase enzyme, which catalyses the rate-limiting conversion of chorismate into anthranilate in the tryptophan pathway, which in turn serves as a precursor for auxin biosynthesis [42,43]. Auxin is also known to increase the expression of ethylene biosynthesis genes such as *ASC4* and *ACS11* [44,45]. It is possible that STM196 triggers local auxin production, which consequently promotes local ethylene biosynthesis. We obtained a double loss-of-function mutant affected simultaneously in *ASA1* (*wei2*) and *ASB1* (*wei7*) [43]. Under control conditions, the *wei2wei7* double mutant showed a slight increase in root hair length compared to the wild type (Fig. 5). However, upon inoculation, the root hair length was almost indistinguishable from the wild type (Fig. 5). This indicates that *ASA1* and *ASB1* do not contribute to STM196-dependent root hair elongation.

#### 4. Discussion

Inoculation with plant growth promoting rhizobacteria (PGPR) remarkably alters root and root hair development as reported for oilseed rape [3], *A. thaliana* [13,16], tomato [17] and wheat [18,19]. This involves multiple complex mechanisms and eventually results in growth promotion [1]. To understand how a beneficial rhizobacteria is able to modify plant development, we focused mainly on root hair elongation induced by bacteria. Root hairs are single epidermal cells [46], and the relative simplicity of this cellular model is likely to enhance studies of the beneficial effects of PGPR.

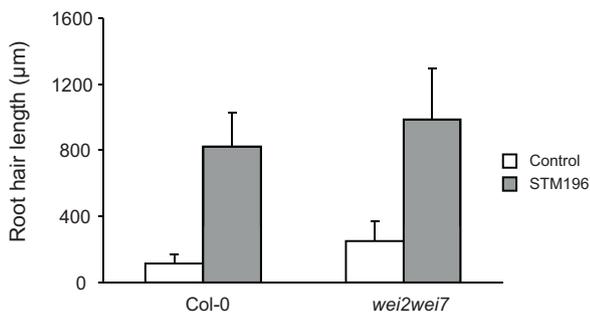
The ethylene emission level remained unchanged after treatment with inocula of STM196 at various concentrations (Fig. 2A). Nevertheless, we observed a small but significant accumulation of both *EBF1* and *EBF2* transcripts (Fig. 3C and D). This could result from a decreased activity of the *XRN4* ribonuclease that negatively regulates *EBF1* and *EBF2* transcript levels [47,48]. However, upon inoculation, the *XRN4* transcript abundance remained unaffected (data not shown). This may suggest that *EBF1* and *EBF2* transcript accumulation does not result from a decrease in *XRN4* activity. The two F-box proteins *EBF1* and *EBF2* negatively regulate EIN3 abundance [49–51], while EIN3 and EIL1 positively regulate *EBF1* and *EBF2* transcription [38]. The observed increases in *EBF1* and *EBF2* transcripts may thus result from the increase in the abundance of the EIN3 polypeptide. We have indirectly estimated the effect of STM196 inoculation on EIN3 abundance by measuring the abundance of the EIN3::FLAG chimeric protein (Fig. 3A and B) [37]. Upon inoculation, EIN3::FLAG appeared more abundant in the shoot. This suggests that STM196 increases EIN3 abundance. As the EIN3 transcript level is not affected by STM196 (data not shown), this would indicate that the rhizobacteria stabilises EIN3. This is consistent with the activation of the ethylene pathway and the accumulation of *EBF1* and *EBF2* transcripts. Interestingly, recent transcriptomic studies on model legume plants also showed transcript accumulation for some Arabidopsis *EBF1* and *EBF2* homologues during the early and late stages of nodulation [52,53].

We analysed the root hair response of ethylene signalling mutants (*ein2*, *ein3*, *etr1*, *ctr1*, *eil1*, *ein3eil1*) [39,40,47,54–58] as well as mutants and transgenic plants affected in their ethylene signalling regulation (*ebf1*, *ebf2*, *35S::EIN3::FLAG*, and *35S::EBF1*) [37,47,50]. These various mutants and transgenic lines have



**Fig. 4.** The ethylene signalling pathway indirectly regulates STM196-triggered root hair elongation. (A) The mean (+SD) root hair length of Col0, *etr1*, *ctr1*, *ein2*, *ein3*, *eil1*, *ein3eil1*, *ebf1*, *ebf2*, *35S::EIN3*, and *35S::EBF1* seedlings inoculated with STM196 at  $10^8$  CFU ml<sup>-1</sup> along with uninoculated controls are indicated. Means are the result of at least 243 measurements obtained from three biological replicates. (B) Root hair sensitivity to STM196 inoculation in various ethylene signalling mutants. The difference between the expected root hair length and the observed root hair length for each genotype including the wild type were calculated as describe in Section 2.

alterations in the ethylene signalling pathway (*etr1*, *ctr1*, *ein2*, *ein3*; *35S::EIN3::FLAG*) and the mechanism that controls the ethylene signalling pathway (*ebf1*, *ebf2*, and *35S::EBF1*). For each genotype tested, we were still able to observe root hair elongation upon STM196 inoculation (Fig. 4A). The ethylene signalling pathway is therefore not directly involved in this response. However, root hairs of *etr1*, *ctr1*, *ein2*, *ein3* and *35S::EBF1* were less sensitive to STM196 whereas root hairs of *ebf2* were more sensitive (Fig. 4B). The *etr1*, *ctr1*, *ein2* and *ein3* mutants are impaired in the direct ethylene signalling pathway whereas the *ebf2* mutant is impaired in the negative regulation of the ethylene signalling pathway. This indicates that the ethylene signalling pathway is contributing to root hair elongation in response to STM196. We propose that



**Fig. 5.** The double mutation *wei2wei7*, which affects auxin biosynthesis in the root tip, does not interfere with the root hair elongation triggered by STM196. Root hair length (mean +SD, four independent biological repeats) of Col-0 wild type plants and the *wei2wei7* double mutant was determined as described in Section 2.

inoculation with STM196 causes minimal ethylene emission, the amount of which is too low to be recorded by standard gas chromatography. This ethylene emission could stabilise EIN3 at the whole plant level and consequently would result in *EBF2* transcript accumulation. The resulting activation of the ethylene pathway could participate in the regulation of root hair elongation. Although in a different biological system, i.e. nitrogen fixing endosymbiosis, our model is consistent with ethylene emission in response to rhizobia inoculation as observed in *Medicago truncatula* [59] and soybean [60].

One possible explanation for the low level of ethylene emission could be that ethylene emission happens at a very local scale and would involve too few root cells. However, this does not imply a minimal impact on overall plant root development. In legumes for example, only the root elongation zone is susceptible to inoculation by rhizobacteria that will result in nodule development and other broader changes in root architecture [61]. Alternatively, the involvement of ethylene could be secondary to the activation of another hormonal pathway such as the jasmonate or auxin pathways.

During plant/microbe interactions, there is crosstalk between the ethylene and jasmonic acid signalling pathways. Both hormones act synergistically to promote the expression of the transcription factor *ERF1* that in turn activates the expression of defence genes such as *PDF1.2* [62,63]. Jasmonate is also a positive regulator of root hair development as increasing jasmonate concentrations progressively increase root hair density [64]. However, this effect is almost completely abolished in *etr1* mutants and in the *ein3eil1* double mutant [64,65]. Therefore, at the root hair elongation level, the ethylene signalling

pathway is likely to play a more important role than jasmonate.

There are well-established connections between the auxin and ethylene pathways in the root (for a review, see [66]). *In vitro* inoculation of *Arabidopsis* with STM196 modifies the DR5::GUS staining pattern in the root tip, indicating that auxin accumulates more strongly along the vasculature upon inoculation [41]. We have observed an accumulation of AS7 and of ACS11 transcript in the root (Fig. 2B). In addition, it is well known that auxin strongly stimulates ACS11 expression [45]. This suggests that local auxin accumulation resulting from STM196 inoculation could activate the root ACS11 gene expression in the root expansion zone. This would promote local ethylene biosynthesis in the root tip that may be too small to be detectable by standard gas chromatography. However, the sensitivity of the *wei2wei7* double mutant root hairs (*i.e.* the difference between expected root hair length and observed root hair length as described in Fig. 4B) is indistinguishable from the wild type (data not shown). This indicates that  $\alpha$  and  $\beta$  anthranilate synthase subunits, and by extension, local auxin biosynthesis do not contribute to root hair elongation. Local ethylene production could be triggered by a mechanism independent of local auxin biosynthesis.

Our results indicate that the ethylene pathway contributes to the STM196 root hair elongation response, although it is not a central player. Additionally, activation does not depend on local auxin biosynthesis. It is possible that ethylene biosynthesis would result from the local accumulation of auxin that results from impaired auxin transport as opposed to auxin biosynthesis. However, an AUX1 loss-of-function mutant retains the ability to elongate root hairs in response to STM196 inoculation [67].

The inoculation of *Arabidopsis* by STM196 affects root hair elongation. This has also been observed when *Arabidopsis* is inoculated by other beneficial rhizobacteria such as *A. brasilense* [17,68] and *B. megaterium* [13,16]. This indicates that different PGPR have similar effects on root hair elongation, suggesting that this phenotype is a typical response to beneficial rhizobacteria. However conflicting results on the implication of the ethylene signalling pathway into this response have been reported [16,17]. That contradiction would only be apparent if the various bacterial strains have different ability to trigger local ethylene emission. Alternatively, we could be looking at several mechanisms resulting in the same phenotype. The activation of one of these mechanisms may be specific to a bacterial strain. While some of these mechanisms may depend on local ethylene emission, some other may depend on molecules secreted by bacteria, such as N-acyl-L-homoserine lactones (AHL) or small cyclopeptides [69,70]. They could as suspected for the small cyclopeptides, interfere with the plant auxin pathway resulting in modification of root development [69,70]. However, it remains unclear whether bacteria will produce AHL or small peptides while colonising the root surface in quantities large enough to affect root-hair elongation. New information regarding this process could emerge from studies of model legumes during nitrogen fixation symbiosis. A transcription factor controlling root hair elongation in the model legume *Lotus japonicus* has been identified [71]. There are three homologues of these genes in *Arabidopsis*, and the transcript level of one of these is affected by STM196 (Desbrosses, unpublished result). This raises the possibility that root hair elongation in response to bacteria could be controlled by a subclass of transcription factors. Insight into the mechanism involved in STM196-promoted root hair elongation can be obtained by simultaneously studying the response of loss-of-function mutants in these genes together with the regulation of their expression by phytohormones.

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