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Research article

Endophytic *Pseudomonas* induces metabolic flux changes that enhance medicinal sesquiterpenoid accumulation in *Atractylodes lancea*

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ABSTRACT

The bacterial endophyte *Pseudomonas fluorescens* ALEB7B significantly enhances photosynthate accumulations in *Atractylodes lancea*. These carbohydrates are preferentially used by the host plant to synthesize secondary metabolites, rather than to increase plant biomass accumulation. Mechanisms underlying the allocation of endophyte–increased carbohydrate in different plant metabolic processes are largely unknown. We have studied how *P. fluorescens* ALEB7B enhances photosynthate accumulation and how bacterial elicitors regulate metabolic flux and increase medicinal sesquiterpenoid formation in *A. lancea* using the sterile tissue culture plantlets. *P. fluorescens* ALEB7B enhances plant photosynthate accumulation by synthesizing and secreting indole–3–acetic acid, which has been demonstrated using high–performance liquid chromatography analysis. The increased endogenous indole–3–acetic acid promotes plant root development and then assimilation. Increased carbohydrates provide the material basis for the formations of terpenoid hydrocarbon scaffolds, which has been proved using gas chromatography analysis. Further, protein and polysaccharide elicitors secreted by *P. fluorescens* ALEB7B have been separated and purified from the bacterial fermentation broth, which have been applied to *A. lancea* plantlets. Both elicitors can stimulate the conversions of terpenoid hydrocarbon scaffolds to oxygenous sesquiterpenoids, the active medicinal ingredients in *A. lancea*, by triggering the oxidative burst *in planta*. Moreover, this study separates an ABC transporter substrate–binding protein from protein elicitors secreted by *P. fluorescens* ALEB7B with an ÄKTA Prime Plus Purifier System and firstly shows that this protein is essential to induce oxygenous sesquiterpenoid accumulation in *A. lancea*. This study provides new perspectives for mechanisms of medicinal oxygenous terpenoid synthesis, which has important reference values to the cultivation of medicinal plants that have terpenoids as their active ingredients, such as *Artemisia annua* and *Taxus chinensis*.

1. Introduction

Endophytes are microbes that asymptomatically colonize the internal tissues of almost all plants (Reinhold–Hurek and Hurek, 2011). During long–term co–evolution, the host plants and endophytes adapt to each other. Host plants provide stable growth environments and rich photosynthates for endophytes. Simultaneously, some endophytes can promote plant growth and improve plant resistance to compensate the material and energy consumed by microbial growth *in vivo*, which will enhance the competitiveness of host plants (Compant et al., 2010; Dupont et al., 2015; Zhang et al., 2016). Endophyte–induced plant biomass accumulation is usually correlated with endophyte–secreted auxin and other plant growth regulating factors (da Silveira et al., 2016; Li et al., 2018). Recently, plant primary metabolism promoted by endophytes in agriculture is a research hotspot (Montanez et al., 2012; Johnson et al., 2013). Plant primary metabolism includes several processes, such as photosynthesis, sucrose and starch synthesis, glycolysis, respiration and so on (Plaxton and Mcmanus, 2006). It can affect plant growth and development by providing energy, reducing power and carbon skeletons (Berger et al., 2007; Bolton, 2009).

In the interactions with medicinal plants, endophytes can not only promote plant primary metabolism but also enhance the accumulations

Abbreviations: ABA, abscisic acid; acetyl CoA, acetyl coenzyme A; ANOVA, a one–way analysis of variance; ET, ethylene; GA, gibberellin; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; H_2O_2, hydrogen peroxide; HPLC, high–performance liquid chromatography; IAA, indole–3–acetic acid; IAM, indole–3–acetamide; MVA, mevalonate; NAA, naphthaleneacetic acid; PCIB, *p*–chlorophenoxy isobutyric acid; $_{SE}$, standard errors; TTC, triphenyltetrazolium chloride

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of secondary metabolites that are related to the plant defense via triggering corresponding responses in host plants (Zhou et al., 2016c; Zhai et al., 2017). For example, the fungal endophyte *Trichoderma atroviride* significantly promotes tanshinone synthesis in *Salvia miltiorrhiza* (Ming et al., 2013). The fungal endophyte *Penicillium oxalicum* significantly enhances artemisinin accumulation in *Artemisia annua* (Zheng et al., 2016). Several endophytes isolated from *Atractylodes lancea* significantly enhance sesquiterpenoid accumulation in the host plant, some of which can enhance plant photosynthate accumulation but have no effect on plant biomass (Wang et al., 2012, 2015c; Zhou et al., 2016a). This indicates that the endophyte–increased photosynthate is preferentially used by *A. lancea* to synthesize secondary metabolites instead of increasing plant biomass.

Terpenoid synthesis consumes a substantial amount of energy (Croteau et al., 1972) and reducing power (Selmar and Kleinwachter, 2012). Moreover, terpenoids are hydrocarbons, whose synthesis is positively correlated with the high carbon/nitrogen ratio *in planta* according to the carbon/nutrient balance hypothesis of plant secondary metabolite synthesis (Bryant et al., 1983). Our previous study has isolated a bacterial endophyte *Pseudomonas fluorescens* ALEB7B from *A. lancea* and has demonstrated that this strain can significantly increase sesquiterpenoid accumulation in the host plant (77.34% higher than the control) (Zhou et al., 2016c). Although *P. fluorescens* ALEB7B can enhance the photosynthesis and carbohydrate accumulation *in planta*, it has little effect on plant biomass (Zhou et al., 2016c). The mechanisms of bacterial endophyte–enhanced carbohydrate accumulation and its influences on sesquiterpenoid synthesis are largely unknown. Furthermore, the secondary metabolite synthesis is related to plant defense responses (Mauch–Mani et al., 2017). Elicitors from endophytes are widely reported to trigger plant defenses and enhance secondary metabolite synthesis in host plants (Jisha et al., 2018; Zhao et al., 2005). Whether elicitors from *P. fluorescens* ALEB7B functioning in increasing the sesquiterpenoid accumulation in *A. lancea* and their action mechanisms merit further study. This study elaborates on how *P. fluorescens* ALEB7B promotes plant primary metabolism and sesquiterpenoid synthesis using high–performance liquid chromatography (HPLC) and gas chromatography (GC). Moreover, protein and polysaccharide elicitors secreted by *P. fluorescens* ALEB7B have been separated and purified by ammonium sulfate precipitation and ethanol extraction, respectively. Then, the effects of these bacterial elicitors on sesquiterpenoid synthesis in *A. lancea* were studied. Further, the mechanisms of bacterial elicitors inducing sesquiterpenoid synthesis were explored as well.

2. Materials and methods

2.1. Plant tissue culture conditions

A. lancea aseptic tissue culture plantlets were established as previously described (Wang et al., 2015c). Buds were collected from cultivated *A. lancea* and washed under running water, after which all procedures were conducted aseptically. Buds were surface–sterilized by immersion in 75% (v/v) ethanol for 30 s, soaking in 1% (w/v) mercury chloride for 10 min and thoroughly rinsing 5 times in sterile distilled water. Several buds were randomly selected, homogenized and inoculated on potato dextrose agar to confirm the absence of culturable endophytes. The intact surface–sterilized buds were then transplanted to 50 mL Murashige–Skoog medium supplemented with 0.3 mg L^{-1} naphthaleneacetic acid (NAA) and 2.0 mg L^{-1} 6–benzyladenine in sealed 100–mL Erlenmeyer flasks.

When sufficient buds had germinated, they were separated and transplanted into 50 mL Murashige–Skoog rooting medium supplemented with 0.1 mg L^{-1} NAA in sealed 100–mL Erlenmeyer flasks to develop into plantlets. All aseptic tissue culture plantlets were kept in a growth chamber with a photoperiod of 12 h, a light density of 3400 lm m^{-2} and a temperature cycle of 25/18 °C day/night. All plantlets used in this study were 30 days old.

2.2. Bacterial endophyte and inoculation

P. fluorescens ALEB7B was isolated from the geo–authentic *A. lancea* grown in the Maoshan area (Zhou et al., 2013). Geo–authentic drugs refer to medicinal plants grown in particular regions, which contain higher contents of medicinal ingredients than ones grown in other regions (Zhang et al., 2003). Maoshan area is the geo–authentic producing area of *A. lancea*. *P. fluorescens* ALEB7B was preserved at the China Center for Type Culture Collection (CCTCC AB 2013331). The molecular identification of *P. fluorescens* ALEB7B and its colonization in *A. lancea* were confirmed in our previous study (Zhou et al., 2014). Bacteria were grown in Luria–Bertani broth at 30 °C with agitation (220 rpm) for 24 h, and the bacterial cells were collected and resuspended in sterile double–distilled water with the concentration adjusted to 10^6 cells mL^{-1}.

A. lancea aseptic tissue culture plantlets were sprayed with 200 μL bacterial suspension, which was allowed to flow from the leaf surfaces to the roots. Totally, there were 75 plantlets inoculated with *P. fluorescens* ALEB7B used for different experiments in this study. Plantlets sprayed with equal volume of sterile double–distilled water were used as the controls. Each time point in different experiments included three bacterium inoculated plantlets and another three sterile double–distilled water treated plantlets. There were 4 time points of collection (5, 10, 15 and 20 days after treatments) in the measurements of plant root number and vigor. And there were 5 time points of collection (0, 5, 10, 15 and 20 days after treatments) in the measurement of indole–3–acetic acid (IAA) content *in planta*. All inoculated and control plantlets were randomized in the growth chamber. Plantlets were harvested at different time points after inoculation according to different experimental designs.

2.3. Bacterial IAA measurement and exogenous IAA treatment

The extraction and detection of IAA produced by *P. fluorescens* ALEB7B were conducted as described by Castillo et al. (2013) with some modifications. Bacteria were cultured in Luria–Bertani broth with 25 mM L–tryptophan at 30 °C with agitation (220 rpm) for 48 h, and the bacterial cells were precipitated by centrifugation at 8000 g for 15 min. The pH of the supernatant was adjusted to 3.0 by adding 1 M HCl and then extracted with an equal volume of ethyl acetate at 30 °C with agitation (220 rpm) for 3 h. The organic phase was then evaporated under vacuum to dryness, dissolved in 1 mL methanol and filtered through a 0.22–μm sterile filter prior to analysis. IAA was quantified by HPLC using a reverse–phase column (Hedera Packing Material Lichrospher 5–C18 250 × 4.6 mm). The mobile phase was methanol and 2% acetic acid in water whose ratio was 35/65 (v/v). The flow rate was 0.8 mL min^{-1} and the column was maintained at 25 °C with detection at 220 nm.

To detect the influences of IAA on plant growth and sesquiterpenoid accumulation, newborn *A. lancea* aseptic tissue culture plantlets were transplanted to 50 mL of Murashige–Skoog medium supplemented with different concentrations of IAA (0.1 mg L^{-1}, 0.3 mg L^{-1}, 0.5 mg L^{-1} and 1.0 mg L^{-1}). Each treatment of certain IAA concentration includes three plantlets. All treated and control plantlets were randomized in the growth chamber. Plantlets were harvested 20 days after transplantation.

In order to verify the source of IAA *in planta*, *p*–chlorophenoxy isobutyric acid (PCIB) (0.5, 1 or 2 mM), an inhibitor of plant IAA synthesis, was applied to bacterium inoculated plantlets. Each treatment included three plantlets, which were harvested after 15 days, because *P. fluorescens* ALEB7B–induced sesquiterpenoid accumulation *in planta* peaked at this time point (Zhou et al., 2016c).