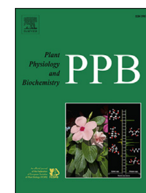
Plant Physiology and Biochemistry 118 (2017) 98–106

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy



Research article

Bacillus subtilis affects miRNAs and flavanoids production in Agrobacterium-Tobacco interaction

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ARTICLE INFO

Article history:
Received 11 April 2017
Received in revised form
9 June 2017
Accepted 9 June 2017
Available online 10 June 2017

Keywords:
Nta-miRNAs
Flavanoids
Agrobacterium tumefaciens
Bacillus subtilis

ABSTRACT

Agrobacterium tumefaciens is a very destructive plant pathogen. Selection of effective biological agents against this pathogen depends on more insight into molecular plant defence responses during the biocontrol agent-pathogen interaction. Auxin as a phytohormone is a key contributor in pathogenesis and plant defence and accumulation of auxin transport carriers are accompanied by increasing in flavonoid and miRNAs concentrations during plant interactions with bacteria. The aim of this research was molecular analysis of *Bacillus subtilis* (ATCC21332) biocontrol effect against *A. tumefaciens* (IBRC-M10701) pathogen interacting with *Nicotiana tabacum* plants. Tobacco plants were either treated with both or one of the challenging bacteria and the expression of miRNAs inside the plants were analysed through qRT-PCR. The results indicated that the bacterial treatments affect expression level of nta-miRNAs. In tobacco plants treated only with *A. tumefaciens* the expression of nta-miR393 was more than that was recorded for nta-miR167 (3.8 folds, $P < 0.05$ in 3dpi). While the expression level of nta-miR167 was more than the expression of nta-miR393 in other treatments including tobacco plants treated only with *B. subtilis* (2.1 folds, $P < 0.05$) and the plants treated with both of the bacteria (3.9 folds, $P < 0.05$) in 3 dpi. Also, the composition and concentration of rutin, myrecetin, daidzein and vitexin flavanoid derivatives were detected using HPLC and analysed according the standard curves. All of the tested flavanoid compounds were highly detected in Tobacco plants which were only challenged with *A. tumefaciens*. The amount of these compounds in the plants which were challenged with the *B. subtilis* alone, was similar to the amount recorded for the plants challenged with the both bacteria. This study suggests a relationship between the upregulation of nta-miR167, nta-miR393 and accumulation of flavanoid compounds. Overall, the expression of these miRNAs as well as flavonoid derivatives has the potential of being used as biomarkers for the interaction of *B. subtilis* and *A. tumefaciens* model system in *N. tabacum*.

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1. Introduction

Agrobacterium tumefaciens as a plant pathogen causes disease by transferring and integrating DNA (T-DNA) of the bacterium into the plant genome (Escobar and Dandekar, 2003; Gelvin, 2003; McCullen and Binns, 2006). Crown gall is a very destructive plant disease that reduces the yield of infected plants by up to 40% (Schroth et al., 1988). The disease has been reported in the Middle East, Japan, North and South America, South Africa, China, and several European countries (Burr et al., 1998). Plant defence against pathogens is complex (Chisholm et al., 2006; Eulgem, 2005; Jones and Dang, 2006) and this complexity of the *A. tumefaciens*–host interaction is reflected in changes of the host gene expression induced by the pathogen (Ditt et al., 2001; Veena et al., 2003).

Multiple strains of *Bacillus* spp. were demonstrated to exploit as biopesticides for the control of plant diseases and to stimulate plant defence responses (Fravel, 2005). The strains of *Bacillus pumilus*, *B. mycoides*, *B. subtilis*, *B. amyloliquefaciens*, *B. pasteurii*, *B. thuringiensis* or *B. cereus* species reported as ISR inducers (Kloepper et al., 2004). *Bacillus cereus* AR156 pre-treatment primes ISR to *Pst* infection by suppressing miR825 and miR825* and

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http://dx.doi.org/10.1016/j.plaphy.2017.06.010
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activating the defence related genes they targeted (Niu et al., 2016). Lipopeptides (Peptide antibiotics) including surfactin, iturin and fengycin could be involved in antibiosis by *B. subtilis* (Peypoux et al., 1999; Marahiel et al., 1997; Kohli et al., 2001). Surfactin is the main elicitor secreted by *B. subtilis* and *B. amyloliquefaciens* S499 displaying consistent induced systemic resistance activity in plants (Ongena et al., 2007; Ongena and Jacques, 2008; Jourdan et al., 2009). These studies have led to the development of this bacterium as a biocontrol agent. Despite more studies on the interactions of *A. tumefaciens* with biocontrol agents in plant defences, there is no clear or complete understanding of host responses to this interaction system. miRNAs and flavanoids as mediators and biomarkers may be useful tools in the perception of microbial signals and changes in plant response.

miRNAs are small (~21-24nt) class of recently discovered endogenous non-coding RNAs (ncRNAs) can regulate the expression of target genes by slicing, with post-transcriptional cleavage of gene expression, and/or translation inhibition in plants (Bartel, 2004; Jones-Rhoades et al., 2006; Voinnet, 2009; Li et al., 2013). They have a fundamental role in all biological processes, including hormone signalling pathways, hormone homeostasis, signal transduction, immunity against pathogens and related developmental processes. miRNAs emerged as molecular links between modulation of plant promoting and auxin signalling during stress (Mallory et al., 2005; Rubio-Somoza et al., 2009; Jin, 2008; Sunkar et al., 2007; Padmanabhan et al., 2009).

Auxin is a key character in pathogenesis and plant defence (Fu and Wang, 2011). Auxin as a phytohormone has diverse and important roles in the formation of organs and response to different environmental plant stress (Palme et al., 1991; Woodward and Bartel, 2005). The interaction of two different protein families, Aux/IAA proteins and auxin response factors (ARFs) cause the complex response of auxin. TIR1 is one of the families that perceives auxin and degrades the Aux/IAA proteins, which has negative regulator roles in auxin signalling by binding and inhibiting ARF transcription factors. ARFs activate or repress transcription of auxin-responsive genes (Sunkar et al., 2012). The targets of miR167 are ARF6 and ARF8 (Rhoades et al., 2002; Mallory et al., 2005; Wu et al., 2006). TIR1 is one of the predicted targets of miR393 (Sunkar and Zhu, 2004). MicroRNAs have potential of being as biomarkers in biotic stress (Bej and Basak, 2014). In Arabidopsis, bacterial PAMP flg22 induces the miR393 which plays an important role in PTI response by silencing auxin receptors and subsequently suppressing auxin signalling (Navarro et al., 2006). miRNAs including miR160, miR167 and miR393 were induced by virulent (DC3000) and non-virulent (DC3000 hrcC) forms of *Pseudomonas syringae* pv. *tomato* (Fahlgren et al., 2007). Expression of miR393 and miR167 was reduced in *A. tumefaciens e* C58-induced tumours in plants (Dunoyer et al., 2006). These studies showed miRNAs that target genes involved in auxin perception and signalling are up regulated during the pathogenesis of bacterial infection.

In addition, secondary metabolites such as flavonoids, with diverse chemical structure, modulate plant development by acting as natural auxin transport inhibitors (Jacobs and Rubery, 1988). They may affect transport by disrupting the complex between ABCB1/TWD1 (TWISTED DWARF1) (Bailly et al., 2008; Wang et al., 2013) and binding BIG, a protein required for PIN cycling (Gil et al., 2001). In addition, they inhibit auxin transport by competing with NPA (1-naphthylphthalamic acid) and TIBA (2, 3, 5-triiodobenzoic acid), synthetic auxin transport inhibitors, for plasma membrane and microsomal binding sites (Jacobs and Rubery, 1988; Bernasconi, 1996; Stenlid, 1976). It was closely associated between Flavonoids and auxin in several developmental responses. Accumulation and expression of auxin transport carriers are accompanied by increasing in flavonoid concentration specifically during plant interactions with bacteria, nematodes and fungi (Hassan and Mathesius, 2012; Dakora and Phillips, 1996; Mathesius et al., 1998; Terasaka et al., 2005; Peer et al., 2001; Grunewald et al., 2012; Noh et al., 2001). There are many reports on the antimicrobial activity of flavonoids against different bacteria, fungi, and viruses (Baez et al., 1999; Xu and Lee, 2001; Ogundipe et al., 2001; Cushnie and Lamb, 2005). Flavanoids including kaempferol, myricetin, naringin, rutin, daidzein and vitexin have antibacterial activity against bacteria (Cushnie and Lamb, 2005; Demetzos et al., 2001; Upadhyay et al., 2013; Das et al., 2016). Rutin could be as an activator to improve plant immunity with abroad range of host. The antibacterial activity of rutin was showed in specific bacteria species, such as *Xanthomonas oryzae* pv. *oryzae*, *A. tumefaciens*, *Ralstonia solanacearum*, *Xylella fastidiosa*, *Pseudomonas syringae* pv.*tomato* strain DC3000 etc. (Taguri et al., 2006; Maddox et al., 2010; Yang et al., 2016). The accumulation of rutin in tobacco tissues that transformed with AK-6b gene of *A. tumefaciens* was higher than the normal ones (Galis et al., 2004). Myricetin has bactericidal effect on *Burkholderia cepacia* by inhibiting protein synthesis (Xu and Lee, 2001). Daidzein and genistein as simple isoflavones have antimicrobial activity, auxin transport regulatory and are precursor of complex isoflavonoids, phytoalexins (Samac and Graham, 2007; Auguy et al., 2011). The antimicrobial and antibiofilm activity of vitexin against *P. aeruginosa* was reported (Upadhyay et al., 2013; Das et al., 2016). These studies confirmed that miRNAs and flavanoids could be ideal mediators and biomarkers between the perception of microbial signals and changes in plant development.

In order to understand the relationship between miRNAs and Flavanoids in interaction of biocontrol-pathogen agents during plant response, we used *B. subtillis* (ATCC21332) and *A. tumefaciens* (IBRC-M10701) interaction system in *Nicotiana tabacum*. Subsequently the accumulation amount of nta-miR167, nta-miR393 and flavanoids compounds was compared according the standards. This working model allows the study of miRNA biomarkers and flavanoids as powerful tools to recognize effective biocontrol agents to protect plants from pathogens.

2. Material and methods

2.1. Plant, bacteria and infection

Tobacco Plants (*N. tabacum* L. var Xanthi) were grown in a growth chamber under 16 h light (200 μmol/m2/s)/8 h dark cycled at 24 °C, with70% relative humidity. The bacteria used in this study were *B. subtilis* ATCC21332, kindly provided by M. A. Marahiel (Department of Chemistry, Biochemistry, Philipps-University Marburg, Hans-Meerwein-Strasse, D-35032 Marburg, Germany) and *A. tumefaciens* strain IBRC-M10701, purchased from the Iranian Biological Research Center. The strains ATCC21332 and IBRC-M10701 were inoculated in 50 ml of Nutrient Broth and Luria Broth media, respectively. Subsequently incubated in an incubator shaker at 180 rpm at 28 °C for 24 h.

The main vein of four-week-old tobacco leaves were scratched and treated with 20 μl of strain ATCC21332 with $OD600 = 0.3$ in water. The plants were inoculated again with strain IBRC-M10701 with the same above concentration, three days after pre-treatment with *B. subtilis*. Water treatment was used as control. Plant samples were collected at three time points including 1, 3 and 6 days after treatment with *A. tumefaciens*. Three plants that were used for each time point were pooled as one replicate. Three biological replicates per treatment (altogether 9 plants) were used in the study.

2.2. Total RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was isolated using Trizol (Invitrogen) according to the

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