Journal of Biotechnology 238 (2016) 22–29

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Expression levels of antimicrobial peptide tachyplesin I in transgenic *Ornithogalum* lines affect the resistance to *Pectobacterium* infection

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ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form 8 September 2016
Accepted 13 September 2016
Available online 14 September 2016

Keywords:
Antimicrobial activity
Ornithogalum dubium
Ornithogalum thyrsoides
Pectobacterium
Tachyplesin
Transgenic plants

ABSTRACT

The genus *Ornithogalum* includes several ornamental species that suffer substantial losses from bacterial soft rot caused by *Pectobacteria*. The absence of effective control measures for use against soft rot bacteria led to the initiation of a project in which a small antimicrobial peptide from an Asian horseshoe crab, tachyplesin (*tpn*I), was introduced into two commercial cultivars: *O. dubium* and *O. thyrsoides*. Disease severity and bacterial colonization were examined in transgenic lines expressing this peptide. Disease resistance was evaluated in six lines of each species by measuring bacterial proliferation in the plant tissue. Three transgenic lines of each species were subjected to further analysis in which the expression level of the transgene was evaluated using RT-PCR and qRT-PCR. The development of disease symptoms and bacterial colonization of the plant tissue were also examined using GFP-expressing strain of *P. carotovorum* subsp. *brasiliense Pcb*3. Confocal-microscopy imaging revealed significantly reduced quantities of bacterial cells in the transgenic plant lines that had been challenged with the bacterium. The results clearly demonstrate that *tpn*I expression reduces bacterial proliferation, colonization and disease symptom (reduced by 95–100%) in the transgenic plant tissues. The quantity of *tpn*I transcripts, as measured by qRT-PCR, was negatively correlated with the protection afforded to the plants, as measured by the reduced severity of disease symptoms in the tissue.

1. Introduction

The genus *Ornithogalum* is comprised of bulbous perennial plants of the family Asparagaceae. It includes about 200 species spread across Asia, Europe and Africa (Tun et al., 2013). The South African species *O. dubium* and *O. thyrsoides* are popular cut flowers that are also cultivated as potted plants and for use in gardening, with relatively high commercial value. These species are becoming increasingly popular for their extremely long vase life (with or without water), pure bright colors ranging from white through yellow to deep orange, and flexibility in use within different mixed bouquets (Luria et al., 2002). The demand for *O. dubium* is increasing steadily in Europe and North America, requiring a high level of plant adaptability to different climates and environmental conditions. Under these circumstances, sensitivity to bacterial soft rot caused by *Pectobacterium* spp. is clearly a limiting factor for *Ornithogalum* production.

These soft rot-causing agents are necrotrophic, Gram-negative, facultative anaerobic, non-sporing, motile, straight rods with peritrichous flagellae (Charkowski, 2006; Toth et al., 2003). They are clustered in the Enterobacteriaceae family and produce a variety of plant cell wall-degrading enzymes that allow the maceration of the plant host tissues on which they feed (Charkowski, 2006). Pectobacteria are well recognized for their ability to cause soft rot in economically important tuberous food crops, as well as ornamentals. Currently, there are no effective means of control available (Byther and Chastanger, 1993; Czajkowski et al., 2011; Ma et al., 2007; Mansfield et al., 2012).

Many of the hosts of these pathogens are characterized by a geophytic lifestyle represented by a fleshy underground storage organ (tuber or bulb) that is commonly used for propagation. Examples include potato, onion, leek, carrot and *Allium* (edible crops), as well as some striking ornamental flowers such as *Ornithogalum*, *Lillium*, *Zantedeschia*, *Hyacinth*, *Freesia* and *Crocus* (Byther and Chastanger, 1993; Wright, 1998; Yishay et al., 2008). Soft rot disease of *Ornithogalum* is a major cause of loss of cut flowers, with

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http://dx.doi.org/10.1016/j.jbiotec.2016.09.008
0168-1656/

losses reaching up to 30% of total yield, and this disease is a limiting factor for the cultivation of potted *Ornithogalum* plants, among which infection may result in total yield losses (Littlejohn, 2006).

The production of soft rot-resistant plants through the introduction of foreign genes with antimicrobial properties has been demonstrated previously in potato (*Solanum tuberosum*), as well as ornamentals including *Ornithogalum* (Allefs et al., 1996; Lipsky et al., 2014; Yip et al., 2007; Yu-Jin and Kwon-Kyoo, 2014).

Several organisms bear genes that code for proteins or peptides with broad-spectrum antimicrobial activity (Brogden, 2005; Izadpanah and Gallo, 2005). Among these, tachyplesin I polypeptide (*tpnI*), isolated from Japanese horseshoe crab, has been extensively characterized, including its structure and mechanism of action (Laederach et al., 2002; Nakamura et al., 1988). This peptide has strong effects against Gram-negative and Gram-positive bacteria (Brogden, 2005; Imura et al., 2007; Kushibiki et al., 2014). Recently, our lab demonstrated that transgenic plants that express tachyplesin I gene (*tpn*I) display higher levels of resistance upon infection with *Pectobacterium* (Lipsky et al., 2014). The protection that the transgene provides to the transformed plants was demonstrated in leaf-segment assays, in which the presence of the transgene was confirmed.

Two *Pectobacterium* spp. are the major cause of ornamental soft rot in Israel: the recently described *P. aroidearum* (Nabhan et al., 2013), and the broad host-range pathogen *P. carotovorum* subsp. *carotovorum* (Yishay et al., 2008). Another subspecies, *P. carotovorum* subsp. *brasiliense* (*Pcb*), has been reported to be a causal agent of soft rot and blackleg disease in potatoes and ornamentals in Israel. *Pcb* is a subject of increasing concern in warmer climates (Ma et al., 2007; Nabhan et al., 2012). This has made *Pcb* our preferred choice for challenging transgenic *Ornithogalum* plants expressing the antimicrobial peptide *tpn*I. To date, no attempts have been made to quantify and visualize bacterial colonization in the transgenic plants or to measure the level of expression of the transgene. Here, an effort was made to: (i) quantitatively measure *tpn*I expression in the control and transgenic plants; (ii) evaluate the colonization of *Pcb* in the host tissues; and (iii) correlate gene expression levels with bacterial colonization rates in the tissues of the transgenic plant hosts.

2. Material and methods

2.1. Plant material and growing conditions

Transgenic *O. dubium* and *O. thyrsoides* plants expressing *tpnI* were produced, in an effort to decrease their susceptibility to bacterial soft rot as described previously (Lipsky et al., 2014). Arabidopsis constitutive polyubiquitin promoter (UBQ3) and the constitutive strawberry vein-banding virus-deleted promoter (ΔSVB) were used to drive the expression of *tpnI* and cloned into a pCAMBIA2301 vector (Cohen and Krens, 2012). The choice of promoter is often species or even cultivar specific. Thus, the efficiency of both promoters for the transformation was tested showing no effect on the frequencies of the transformation, or the expression of the target gene (Lipsky et al., 2014). Accordingly, the transgenic lines were chosen based on their resistance to the bacterium and characterized independently of the promoter used. The final construct also contained kanamycin resistance gene (*npt*II) and β-glucuronidase; GUS reporter gene (*uid*A), both driven by *Cauliflower Mosaic Virus* CaMV35S promoter independently (Lipsky et al., 2014). The transgenic species (obtained by particle bombardment of the construct), as well as wild-type plants, were grown under tissue-culture conditions and under greenhouse conditions throughout the experiment. The in vitro cultures were grown on agar-solidified Murashige and Skoog (MS) medium (supplemented with 3% w/v sucrose) with pH 5.7. Uniform leaf segments from in vitro cultures of transgenic and non-transgenic plants (as shown in Table 1) were used for the evaluations of bacterial colonization and the level of transgene expression in the tissue. Similarly, uniform leaf discs from fully developed plants grown under greenhouse conditions were used to evaluate bacterial colonization, as well as transgene expression.

Table 1
List of the plants used in the experiment.

Plant ID	Background	Description
Odc	*Ornithogalum dubium*	Wild type
Od94	*O. dubium*	Transgenic
Od168	*O. dubium*	Transgenic
Od245	*O. dubium*	Transgenic
Otc	*O. thyrsoides*	Wild type
Ot1d11	*O. thyrsoides*	Transgenic
Ot2u20	*O. thyrsoides*	Transgenic
Ot3u22	*O. thyrsoides*	Without target gene

2.2. Bacterial strains

Pectobacterium carotovorum subsp. *brasiliense* (*Pcb*3) is considered an emerging pathogen in potato fields in Israel, as well as other warm countries such as South Africa (van der Merwe et al., 2010). This strain was used for pathogenesis assays throughout the study. *Pcb*3 proliferation was assessed either by using a reporter strain *Pcb*3+ that contained green fluorescent protein (GFP) to allow the observation of bacterial colonization of the tested plant material, by direct counting from infected tissues, or by evaluating the development of symptoms. The development of symptoms was measured as the level of decay in the transgenic and wild-type host plants.

The GFP-expressing strain of *Pcb3* was generated as described by Golan et al. (2010). In short, a plasmid pPROBE-AT (Miller et al., 2000) containing a 131-base pair *npt*II promoter fragment from Tn5 fused to GFP and a replicon from pBBR1 carrying ampicillin resistance was introduced into *Pcb*3 cells by electroporation.

2.3. Antimicrobial activity of tpnI

Pcb3 was grown overnight at 28 °C in 4 ml of liquid LB medium under continuous shaking at 150 rpm in a TU-400 incubator shaker (MRC, Holon, Israel). The suspensions were then diluted to a final concentration of 1×10^6 colony-forming units (CFU) in 200 μl of fresh LB supplemented with 250 or 500 ng/ml synthetic *tpn*I (Entelechon GmbH, Regensburg, Germany) or without the peptide (controls), in Bradford 96-well microtiter plates (Bar-Naor, Ramat Gan, Israel). Plates were incubated at 28 °C with continuous shaking and growth was determined by measuring optical density at 600 nm (OD_{600}) at 1-h intervals for 14 h, using a micro-plate reader (Spectra MR, Dynext Technologies, VA, USA).

2.4. Bacterial colonization assay

*Pcb*3+ (expressing GFP) was used to monitor bacterial colonization in transgenic and wild-type plants. Bacteria were cultured overnight in LB medium supplemented with 100 μg/ml ampicillin, centrifuged and washed twice in PBS buffer (pH 7.2) before being re-suspended in PBS to a final concentration of 10^8 CFU/ml. Two-ml aliquots of the bacterial suspension were mixed with 1 ml of 2% warm agar and poured over Petri dishes containing half-strength solid MS medium to fully cover the plate with a thin layer of bacteria in soft agar. Then, uniform 2-cm-long leaf segments or whole bulblets were excised from in vitro-grown *Ornithogalum* plants and immersed in the agar. The plates were then transferred to a growth chamber, where they were kept at 24 °C with a 16 h light/8 h dark photoperiod until analysis. Three days later, the leaf segments or