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Transgenic plants expressing the quorum quenching lactonase AttM do not significantly alter root-associated bacterial populations

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Abstract

The possible impact of genetically engineered plants that degrade the quorum sensing (QS) signal of the plant pathogen *Pectobacterium carotovorum* was evaluated on non-target plant-associated bacterial populations and communities using *Nicotiana tabacum* lines expressing the lactonase AttM that degrades QS signals (AttM), and the wild type (WT) parent line. Cell densities of total culturable bacteria and those of selected populations (pseudomonads, agrobacteria) isolated from plant rhizospheres and rhizoplanes were comparable whatever the genotype of the plants (AttM or WT). Similarly, cell densities of members of the bacterial communities relying upon acyl-homoserine-lactones (AHLs) to communicate, or naturally degrading AHL signals, were identical and independent of plant genotype. Bacterial populations isolated from the two plant genotypes were also analyzed irrespective of their culturability status. DGGE analyses targeting the *rrs* gene (16S rRNA gene) did not reveal any significant differences within these populations. All these data indicate that bacterial population changes that could have resulted from the genetic modification of the plants are non-existent or very limited, as no changes linked to the plant genotype were observed.

Keywords: Quorum sensing; Genetically modified plants; Acyl-homoserine lactone; Lactonase; *Pectobacterium*

1. Introduction

Quorum sensing (QS) is a bacterial regulatory process that couples gene expression to cell density. It involves low molecular weight signal molecules that are synthesized by a given bacterial population and accumulate in the environment as a function of the population density. QS signals are sensed by the bacteria that produced it once a threshold concentration (hence a threshold cell density) has been reached. Amongst proteobacteria, the most common signals belong to the N-acyl-homoserine lactone (AHL) class. While the homoserine lactone ring is the same for all AHL molecules, the acyl chain is variable in length (ranging from 4 to 18 carbon atoms), saturation and C3 acyl chain substituents (none, hydroxy or keto group). These three features provide specificity to the signal emitted and perceived by different bacterial populations (recent reviews: Atkinson and Williams, 2009; Boyer and Wisniewski-Dyé, 2009; Ng and Bassler, 2009; Uroz et al., 2009).

Amongst QS-regulated functions are biofilm development, production of antifungal or antibacterial compounds and virulence-related functions. For instance, in the plant pathogen *Pectobacterium carotovorum*, production of the main virulence factors, macerating exoenzymes (e.g. pectate lyase, pectinase, cellulase) that digest plant tissues, is regulated by QS, as are the secreted virulence factors termed harpins in *P. carotovorum* and the related species *P. atrosepticum* (Mukherjee et al., 1997; Smadja et al., 2004). In both cases, the major AHL signals involved are *N*-(3-oxo-hexanoyl)-homoserine lactone (3O,C6-HSL) and *N*-(3-oxo-octanoyl)-homoserine lactone (3O,C8-HSL) depending upon the strain (Andersson et al., 2000; Chatterjee et al., 2005; Burr et al., 2006). The secretion of harpins within plant cells contributes to disease development in host plants, but triggers a characteristic hypersensitive reaction (HR) in non-host plants. Data

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concerning *Pectobacterium* pathogenicity with respect to QS regulation have been recently reviewed (Barnard et al., 2007).

Because QS-regulated virulence functions in *Pectobacterium* are only induced in the presence of sufficient signal concentrations, biocontrol strategies aimed at reducing signal concentrations in the bacterial environment have been developed. These strategies (and others targeting recognition of the QS signal by the bacterial receptor; for a review see Rasmussen and Givskov, 2006) have been termed quorum quenching (QQ; Zhang, 2003). AHL degradation most often results from enzymatic activities, either lactonases that yield *N*-acyl homoserines (not recognized as a QS signal) from AHLs, or acylases (amidohydrolases) that cleave this molecule to homoserine lactone and a fatty acid (reviewed by Uroz et al., 2009). Two approaches have been successfully used in the laboratory to reduce or suppress the aggressiveness of *Pectobacterium* towards its host plant The first involves cloning and transfer of a lactonase gene of bacterial origin (from the firmicute *Bacillus* sp.) to plants (Dong et al., 2000). Potato plants expressing the lactonase gene were clearly protected from *Pectobacterium*-induced maceration (Dong et al., 2001). The second approach relies upon the use of bacteria naturally able to degrade AHLs (Uroz et al., 2003). Several AHL-degrading bacteria have been isolated from soils or waters of various origin over the last 10 years (review Uroz et al., 2009). Amongst these, *Comamonas* and *Rhodococcus* strains have proved to be highly efficient in degrading AHLs and protecting potato plants from *Pectobacterium*-induced macerations (Uroz et al., 2003, 2007). Interestingly, rhodococcal populations can be stimulated in the plant environment by supplementing with compounds such as gamma caprolactone (GCL), or gamma heptalactone (GHL). These two compounds, structurally related to AHLs, are not recognized as QS signals but favor the growth of AHL-degrading populations (Cirou et al., 2007).

Whatever the QQ strategy developed, a need to evaluate ecological consequences of use of GCL, GHL and plants expressing AHL lactonase exists. In this report, the ecological impact of cultivation of AttM-expressing plants on root-associated bacterial populations has been evaluated in soils in terms of both composition (structure) of the bacterioflora and functions with respect to the ability of bacteria to communicate via AHL or to degrade AHL signals.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents, including AHL, were all from commercial sources.

2.2. Production of transgenic plants

attM codes for a lactonase that hydrolyses a broad range of AHLs, with or without C3 substituents. The gene originating from *Agrobacterium tumefaciens* strain C58 was amplified by PCR (polymerase chain reaction) with Promega *Pfu* DNA polymerase according to the manufacturer's protocol, from primers attMgtgNcoI (CAAGGGAGGAACCATGG TGACCGATATCAG) and attMBstEII (AGATTGCATGCAAGGTCACCGTATCTGGCC). The resulting PCR products were digested by NcoI and BsteII and inserted after the CaMV 35S promoter, in place of the *gus* gene in pCAMBIA 3301 (www.cambia.org) to yield plasmid p3301*attM* (Fig. 1). The constructions were verified by double-strand DNA sequencing.

Seeds of *Nicotiana tabacum* cv. Samson were surface-sterilized and deposited onto half-strength Murashige and Skoog (MS/2) medium (Sigma–Aldrich France, ref. M5519) supplemented with Morel's vitamins. Plants that developed were cultivated in a growth chamber at 24 °C for 4 weeks under long daylight conditions. Plasmid p3310*attM* was transformed into *A. tumefaciens* strain EHA105 (Hood et al., 1993) by the freeze-thaw method (Chen et al., 1994), and the resulting *Agrobacterium* strains were used for transformation of tobacco leaf disk by cocultivation (Horsch et al., 1985). The transgenic plants were regenerated under glufosinate (Basta) selection (20 mg/L). Eight transgenic tobacco lines expressing *attM* at various levels were obtained from different experiments and therefore resulted from independent transformation events with strain EHA105(p3301*attM*). "Wild type" plants were regenerated from hormone-induced calli formed on uninfected leaf disks of the same plants used to produce *Agrobacterium*-transformed leaf disks.

Plant genomic DNA was extracted as described by Dellaporta et al. (1983) and the presence of the *attM* gene in the transgenic tobacco plants was verified by PCR (polymerase chain reaction) using the above described primers attMBstEII and attMgtgNcoI.

2.3. Detection of AHL lactonase activity in transformed plants

Proteins were extracted from leaves and roots with a standard extraction buffer (100 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 18% (w/v) sucrose, 40 mM 2-mercaptoethanol). Protein concentration was assessed using various commercial

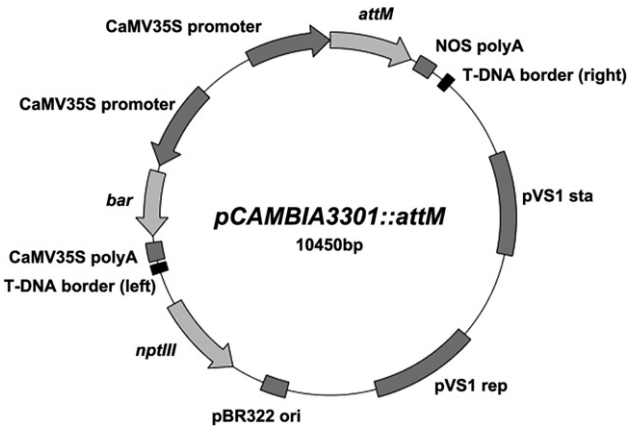

Fig. 1. Genetic map of p3301attM. The *attM* gene encoding AHL lactonase was PCR-amplified from *Agrobacterium* strain C58. The resulting PCR product was inserted in place of the *gus* gene into pCAMBIA 3301 to yield plasmid p3310*attM* (see text for additional details).