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A multiplex-PCR assay for identification of the quarantine plant pathogen *Xanthomonas axonopodis* pv. *phaseoli*

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ABSTRACT

In this study we developed an algorithm to screen for all exact molecular signatures of the quarantine pathogen *Xanthomonas axonopodis* pv. *phaseoli* (*Xap*), based on available data of the presence or absence of virulence-associated genes. The simultaneous presence of genes *avrBsT* and *xopL* is specific to *Xap*. Therefore we developed a multiplex PCR assay targeting *avrBsT* and *xopL* for the molecular identification of *Xap*. The specificity of this multiplex was validated by comparison to that of other molecular identification assays aimed at *Xap*, on a wide collection of reference strains. This multiplex was further validated on a blind collection of *Xanthomonas* isolates for which pathogenicity was assayed by stem wounding and by dipping leaves into calibrated inocula. This multiplex was combined to the previously described X4c/X4e molecular identification assay for *Xap*. Such a combination enables the molecular identification of all strains of *Xanthomonas* pathogenic on bean. Results also show that assay by stem wounding does not give reliable results in the case of *Xap*, and that pathogenicity assays by dipping should be preferred.

1. Introduction

Most bacteria belonging to the genus *Xanthomonas* are responsible for diseases on a large range of economically important crops, including monocots and dicots. The pathovar *phaseoli* of *Xanthomonas axonopodis* groups all strains of *Xanthomonas* identified as pathogenic on bean (Vauterin et al., 1995). *X. axonopodis* pv. *phaseoli* (*Xap*) induces yield losses and loss of seed marketability, and has therefore an important economic impact (Kennedy and Alcorn, 1980; Karavina et al., 2011). *Xap* can be transmitted to seeds, and then to seedlings (Darsonval et al., 2008, 2009; Darrasse et al., 2010). Only a few contaminated seeds are sufficient for primary inoculum source and subsequent widespread dissemination (Darrasse et al., 2007). *Xap* is not endemic in Europe and to limit its introduction, it is registered on lists of quarantine pathogens. Use of pathogen free seeds is the major means for limiting the spread of the pathogen (Gitaitis and Walcott, 2007).

The taxonomy of the genus *Xanthomonas* is still not fully solved, and delineation of some species within this genus is still under debate (Schaad et al., 2005). In the present paper we will follow the nomenclature proposed by Vauterin et al. (1995), as this nomenclature was used to define quarantine pathovars in the genus *Xanthomonas* by the European and Mediterranean Plant Protection Organization (EPPO). The pathovar *phaseoli* of *X. axonopodis* is genetically and geographically diverse (Mkandawire et al., 2004), and is divided into four distinct genetic lineages (Alavi et al., 2008). Fuscous strains group into one genetic lineage (GL), referred to as GL *fuscans*; these strains produce a brown pigment on a tyrosine containing medium. Therefore they are called fuscous strains and are usually considered as highly aggressive on bean (Birch et al., 1997; Toth et al., 1998). The remaining three genetic lineages contain non-fuscous strains and are referred to as GL1, GL2 and GL3 (Alavi et al., 2008; Hajri et al., 2009). Phylogenetic studies revealed that GL2 and GL3 are closely related to GL *fuscans*, whereas GL1 strains remain very distant from the other three genetic lineages (Alavi et al., 2008; Hajri et al., 2009; Mhedbi-Hajri et al., submitted for publication).

Powerful identification and detection procedures for *Xap* are of crucial importance, as this pathogen has a quarantine status. Currently, the

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identification of seedborne *Xap* relies on dilution plating on a semi-selective media associated to a pathogenicity test by stem wounding with the suspect isolates (ISTA method 7-021). However, such a method leads to conflicting results. Numerous false positive results were observed in the past years worldwide (ISTA method 7-021). Indeed, bean seeds may harbor *Xanthomonas* strains pathogenic on other plants (Darsonval et al., 2008; Darrasse et al., 2010). Such non-host carriage renders mandatory the identification of *Xanthomonas*-like colonies isolated from bean seeds. Several primer sets were developed for the molecular identification of strains of *Xap*. In particular, Audy et al. (1994) developed primers X4c and X4e for the identification of *Xap*. Although the primers set X4c/X4e seems specific, it is targeted towards a sequence located on a plasmid, which may be misleading in cases where the plasmid is lost or transferred. Primers Xpha1 and Xpha2 were designed to target the insertion sequence ISXax1 that was shown to be restricted to *Xap* and *X. axonopodis* pv. *vesicatoria* strains (Alavi et al., 2007). Primers Xf1 and Xf2 only target *Xap* GL *fuscans* strains (Toth et al., 1998). The latter sets of primers thus cannot be used for routine identification of *Xap* on seed lots. Recently Albuquerque et al. (2011) reported the development of a dot-blot assay for the identification of *Xap*. However dot-blot analysis is much time consuming, and large numbers of samples are difficult to perform when no access to dedicated platforms is available.

The development of a molecular test that can detect all strains of *Xap* cannot rely only on the amplification of neutral markers, as pv. *phaseoli* is composed of several genetic lineages that may be distant phylogenetically. Alternatively, a molecular test aimed at the amplification of markers linked to the host specificity of strains of *X. axonopodis* would allow identification of all strains of *Xap*, whatever GL the strains belong to. Among candidate determinants for host specificity in *Xanthomonas* are repertoires of virulence-associated genes. These repertoires of genes include bacterial sensors, chemotactic proteins, adhesins and Type 3 effectors (T3Es) (Hajri et al., 2009; Mhedbi-Hajri et al., 2011; Hajri et al., 2012a, 2012b). A correlation between pathovars and the composition of repertoires of T3Es and adhesins could be observed in *X. axonopodis* (Hajri et al., 2009; Mhedbi-Hajri et al., 2011). However, neither single T3E nor single adhesin was found to be specific to *Xap*.

In the present paper, we report the development of an algorithm aimed at finding the smallest combination of virulence associated genes specific to *Xap*. This combination was then used to design a multiplex PCR assay for the identification of *Xap*. The specificity of this multiplex PCR assay was compared to that of primers X4c/X4e on collections of well known strains. A blind collection of strains of *Xanthomonas* isolated from bean seeds or bean seedlings was assembled, and the pathogenicity of these strains was tested on bean by wounding and dipping assays. Strains of the blind collection were tested with both molecular tests. Altogether, the results obtained show that the combination of both molecular tests provides a very powerful rapid screen for the identification of all strains of *Xanthomonas* pathogenic on bean. Such a combination provides a useful alternative approach to testing the pathogenicity on bean.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Two sets of bacterial strains were assembled. The first set is made of bacterial strains (n = 184) obtained from the French Collection of Plant-associated Bacteria (CFBP; Table 1.). These strains are well characterized phylogenetically and pathologically. This first set contains strains of *X. axonopodis* representative of 16 pathovars, strains representative of other *Xanthomonas* species, as well as strains of plant pathogenic bacteria belonging to other genera. This first set is used to test the specificity (i.e. occurrence of false positive or false negative results) of molecular identification procedures.

Table 1
List of strains tested in this study with molecular reagents designed for detection of *X. axonopodis pv. phaseoli*.

Strain tested		CFBP	Molecular detection	
Species	Pathovar/ subspecies		X4c–X4e	Multiplex AvrBst–XopL
Acidovorax avenae	subsp. *avenae*	1201	nt	–
Acidovorax anthurii		3232	nt	–
Acidovorax avenae	subsp. *cattleyae*	2423	nt	–
Acidovorax avenae	subsp. *citrulli*	4459	nt	–
Acidovorax avenae	subsp. *avenae*	2425	nt	–
Acidovorax delafieldii		2442	nt	–
Acidovorax konjaci		4460	nt	–
Acidovorax temperans		3610	nt	–
Acidovorax valerianellae		4730	nt	–
Agrobacterium tumefaciens		2413	nt	–
Clavibacter michiganensis	subsp. *michiganensis*	4999	nt	–
Curtobacterium flacuumfaciens	pv. *betae*	3509	nt	–
Dickeya diffenbachiae		2051	nt	–
Dickeya sp.		1537	nt	–
Herbaspirilium rubrisubalbicans		1202	nt	–
Herbaspirilium rubrisubalbicans		1295	nt	–
Pectobacterium atrosepticum		1526	nt	–
Pseudomonas brassicacearum		5593	nt	–
Pseudomonas cichorii		2101	nt	–
Pseudomonas corrugata		2431	nt	–
Pseudomonas syringae	pv. *syringae*	1392	nt	–
Xanthomonas alblineans		7087	nt	–
Xanthomonas arboricola	pv. *pruni*	3894	nt	–
Xanthomonas arboricola pv.corylina		1159	nt	–
Xanthomonas axonopodis	pv. *citri*	306	–	–
Xanthomonas axonopodis	pv. *alfalfae*	3835	–	–
Xanthomonas axonopodis	pv. *alfalfae*	3836	–	–
Xanthomonas axonopodis	pv. *alfalfae*	3837	–	–
Xanthomonas axonopodis	pv. *alfalfae*	7120	–	–
Xanthomonas axonopodis	pv. *alfalfae*	7121	–	–
Xanthomonas axonopodis	pv. *allii*	6107	–	–
Xanthomonas axonopodis	pv. *allii*	6358	–	–
Xanthomonas axonopodis	pv. *allii*	6359	–	–
Xanthomonas axonopodis	pv. *allii*	6362	–	–
Xanthomonas axonopodis	pv. *allii*	6364	–	–
Xanthomonas axonopodis	pv. *allii*	6367	–	–
Xanthomonas axonopodis	pv. *allii*	6369	–	–
Xanthomonas axonopodis	pv. *allii*	6376	–	–
Xanthomonas axonopodis	pv. *allii*	6383	–	–
Xanthomonas axonopodis	pv. *allii*	6385	–	–
Xanthomonas axonopodis	pv. *anacardii*	2913	–	–
Xanthomonas axonopodis	pv. *anacardii*	2914	–	–
Xanthomonas axonopodis	pv. *anacardii*	7240	–	–
Xanthomonas axonopodis	pv. *anacardii*	7241	–	–
Xanthomonas axonopodis	pv. *anacardii*	7242	–	–
Xanthomonas axonopodis	pv. *anacardii*	7243	–	–
Xanthomonas axonopodis	pv. *aurantifolii*	2866	–	–
Xanthomonas axonopodis	pv. *aurantifolii*	2901	–	–
Xanthomonas axonopodis	pv. *aurantifolii*	3528	–	–
Xanthomonas axonopodis	pv. *aurantifolii*	3529	–	–
Xanthomonas axonopodis	pv. *aurantifolii*	3530	–	–
Xanthomonas axonopodis	pv. *axonopodis*	5141	–	–
Xanthomonas axonopodis	pv. *axonopodis (type strain)*	4924	–	–
Xanthomonas axonopodis	pv. *begoniae*	1421	–	–
Xanthomonas axonopodis	pv. *begoniae*	2524	–	–
Xanthomonas axonopodis	pv. *begoniae*	5676	–	–
Xanthomonas axonopodis	pv. *begoniae*	5677	–	–
Xanthomonas axonopodis	pv. *begoniae*	5678	–	–
Xanthomonas axonopodis	pv. *citri*	1209	–	–
Xanthomonas axonopodis	pv. *citri*	1814	–	–
Xanthomonas axonopodis	pv. *citri*	2525	–	–
Xanthomonas axonopodis	pv. *citri*	2900	–	–
Xanthomonas axonopodis	pv. *citri*	3369	–	–
Xanthomonas axonopodis	pv. *citri*	5280	–	–
Xanthomonas axonopodis	pv. *citri*	5284	–	–

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