Molecular Plant Pathology

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Pathogen profile

Xanthomonas campestris pv. *campestris* (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops

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SUMMARY

Background: *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Pammel) Dowson is a Gram-negative bacterium that causes black rot, the most important disease of vegetable brassica crops worldwide. Intensive molecular investigation of *Xcc* is gaining momentum and several whole genome sequences are available.

Taxonomy: Bacteria; Phylum Proteobacteria; Class Gammaproteobacteria; Order Xanthomonadales; Family Xanthomonadacea; Genus *Xanthomonas*; Species *X. campestris*.

Host range and symptoms: *Xcc* can cause disease in a large number of species of Brassicaceae (ex-Cruciferae), including economically important vegetable *Brassica* crops and a number of other cruciferous crops, ornamentals and weeds, including the model plant *Arabidopsis thaliana*. Black rot is a systemic vascular disease. Typical disease symptoms include V-shaped yellow lesions starting from the leaf margins and blackening of the veins.

Race structure, pathogenesis and epidemiology: Collections of *Xcc* isolates have been differentiated into physiological races based on the response of several brassica species lines. Black rot is a seed-borne disease. The disease is favoured by warm, humid conditions and can spread rapidly from rain dispersal and irrigation water.

Disease control: The control of black rot is difficult and relies on the use of pathogen-free planting material and the elimination of other potential inoculum sources (infected crop debris and cruciferous weeds). Major gene resistance is very rare in *B. oleracea* (brassica C genome). Resistance is more readily available in other species, including potentially useful sources of broad-spectrum resistance in *B. rapa* and *B. carinata* (A and BC genomes, respectively) and in the wild relative *A. thaliana*.

Genome: The reference genomes of three isolates have been released. The genome consists of a single chromosome of approximately 5 100 000 bp, with a GC content of approximately 65% and an average predicted number of coding DNA sequences (CDS) of 4308.

Important genes identified: Three different secretion systems have been identified and studied in *Xcc*. The gene clusters *xps* and *xcs* encode a type II secretion system and *xps* genes have been linked to pathogenicity. The role of the type IV secretion system in pathogenicity is still uncertain. The *hrp* gene cluster encodes a type III secretion system that is associated with pathogenicity. An inventory of candidate effector genes has been assembled based on homology with known effectors. A range of other genes have been associated with virulence and pathogenicity, including the *rpf*, *gum* and *wxc* genes involved in the regulation of the synthesis of extracellular degrading enzymes, xanthan gum and lipopolysaccharides.

Useful website: http://www.xanthomonas.org/

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INTRODUCTION

The genus *Xanthomonas* includes economically important pathogenic bacteria that are generally associated with plants (Hayward, 1993; Vauterin *et al*., 1990). The taxonomy of this genus was initially determined according to host preference (typically the host of origin) and, consequently, a large number of species and pathovars have been defined (Burkholder, 1957). Morphological and other physiological and biochemical characters were subsequently used to classify the *Xanthomonas* isolates into eight phenotypic groups (Van Den Mooter and Swings, 1990). The *Xanthomonas* species were later reclassified on the basis of DNA–DNA hybridization, leading to *X. campestris* being restricted to comprise only the vascular pathogen *X. campestris* pv. *campestris* (Pammel) Dowson (*Xcc*), which causes black rot of brassica species, and additional pathovars that cause vascular or leaf spot diseases in cruciferous hosts, including *X. campestris* pv. *aberrans* (Knösel) Dye, *armoraciae* (McCullock) Dye, *barbareae* (Burkholder) Dye, *incanae* (Kendrick & Baker) Dye and *raphani* (White) Dye (Vauterin *et al*., 1995).

Debate continues with regard to what constitutes different pathovars. For example, some authors, such as Alvarez *et al*. (1994), have considered that *X. campestris* pv. *raphani*, a pathovar originally described by White (1930), which has a broad range of

hosts within the Brassicaceae and Solanaceae, and *X. campestris* pv. *armoraciae*, described one year earlier by McCulloch (1929) as a leaf spot disease of horse radish, are synonymous. Other authors, such as Tamura *et al*. (1994) and Vicente *et al*. (2006), have considered them to be distinct pathovars with a different host range. Other *X. campestris* pathovars have received less attention. Some of these pathovars, such as *X. campestris* pv. *aberrans*, may not be distinct from *Xcc* (Fargier and Manceau, 2007; Fargier *et al*., 2011; Vicente *et al*., 2001). Fargier and Manceau (2007) considered that the species can be restricted to three pathovars (*campestris*, *raphani* and *incanae*), but some isolates from ornamental crucifers, which are currently identified as pv. *campestris* or *incanae*, may still belong to distinct pathovars (Vicente *et al*., 2006).

THE DISEASE

Black rot was first described by Garman (1894) as a disease of cabbage in Kentucky, USA. He isolated two types of bacteria from diseased plants, but could not determine which type of bacterium was causing the disease. In Iowa, USA, Pammel (1895a, b) observed a similar disease in rutabaga and turnip, and showed that the disease was caused by a bacterium (named *Bacillus campestris*) with yellow pigmented colonies in culture. Reports from Wisconsin also attributed the disease of turnips and cabbage to the yellow bacterium (Russell, 1898; Smith, 1898). Since then, the disease has been identified in all continents wherever Brassicaceae crops are grown (Bradbury, 1986), and is considered to be the most important disease of vegetable brassica crops worldwide (Williams, 1980).

Brassica oleracea (including cabbage, cauliflower, broccoli, Brussels sprouts and kale) is economically the most important host of *Xcc*. However, the disease also occurs in other brassica crops, radish, ornamental crucifers and related weed species (Bradbury, 1986). Some accessions of *Arabidopsis thaliana*, the model plant for molecular plant research, are also susceptible when inoculated with *Xcc*.

LIFE CYCLE

Black rot is primarily a seed-borne disease (Cook *et al*., 1952). However, the disease can also be transmitted in infected transplants, infested soil, crop residues and carry-over in related weed species (Schaad and Alvarez, 1993; Walker, 1953). Schaad and White (1974) and Dane and Shaw (1996) showed that *Xcc* can survive in the soil, independent from the host, for approximately 40 days in winter and 20 days in summer. The results of Arias *et al*. (2000) showed that high soil matric potential (saturated soils) can reduce the survival of the pathogen. The pathogen can survive longer in soil within plant tissues than as free living cells. Kocks and Zadoks (1996) showed that crop residues in fresh (2 weeks) refuse piles are more effective in spreading the disease than older (4 months) piles. In some conditions, cruciferous weeds can survive all year round and can provide potential carry-over inoculum for the crops (Schaad and Dianese, 1981). Arias *et al*. (2000) showed that epiphytic survival of the bacteria on the phylloplane is dependent on the plant species, as bacteria survived for 48 days on cabbage, mustard and lettuce, but only for 9 days on rice. In some cases, infected crops have also been shown to provide inoculum for the weeds (Dane and Shaw, 1996), and one study has indicated that weeds do not play an important role in the dissemination of black rot (Schaad and Thaveechai, 1983).

The bacteria can disperse over short distances via wind, insects, aerosols, irrigation water, rain, farm equipment and workers. Commercial vegetable brassica crops are raised from transplants. In plant nurseries that produce module-raised transplants, the overhead irrigation system can increase significantly the dissemination of the bacteria, and can subsequently lead to a high level of disease in the field; changing the irrigation method can therefore limit the spread of the disease (Roberts *et al*., 2007).

The bacteria generally enter the plant through hydathodes on the leaf margins, when droplets of guttation contaminated with bacteria are reabsorbed into the leaf (Russell, 1898). This mode of entry is dependent on a combination of environmental, biological and mechanical factors (Meier, 1934). In contrast, stomata generally do not appear to be important for *Xcc* infection, because the disease generally does not spread into surrounding tissues, even though the bacteria can enter the plant through the stomata and produce small dark spots (Cook *et al*., 1952). This suggests that vascular movement of bacteria is essential for disease development. The bacteria can also enter the plant through wounds caused by machinery, insects, animals, rain, irrigation and wind.

The typical symptom of black rot is the formation of V-shaped, chlorotic yellow lesions with vertices towards the middle vein of the leaves (Fig. 1a,b) and darkened veins that result from bacterial movement in the vascular system. The affected tissues can become necrotic, and leaves can fall prematurely; systemic infections can cause stunted growth (Fig. 1c) and the death of young plants. Secondary infection by other bacterial species can also contribute to further development of severe rotting of vegetable tissue. The infection is often latent when temperatures are low, as the bacteria can persist in the vascular system without producing symptoms and, when the temperature rises, the typical symptoms become evident (Cook *et al*., 1952; Schaad, 1982; Walker, 1953).

In general, *Xcc* thrives as a severe disease agent in warm, humid climates and, consequently, is most serious in tropical, subtropical and humid continental regions (Williams, 1980). Given the global distribution of *Xcc*, black rot will become an increasingly important disease constraint favoured by climate change in more northern latitudes of vegetable production, including the warmer regions of Europe, such as the southern regions of the UK.

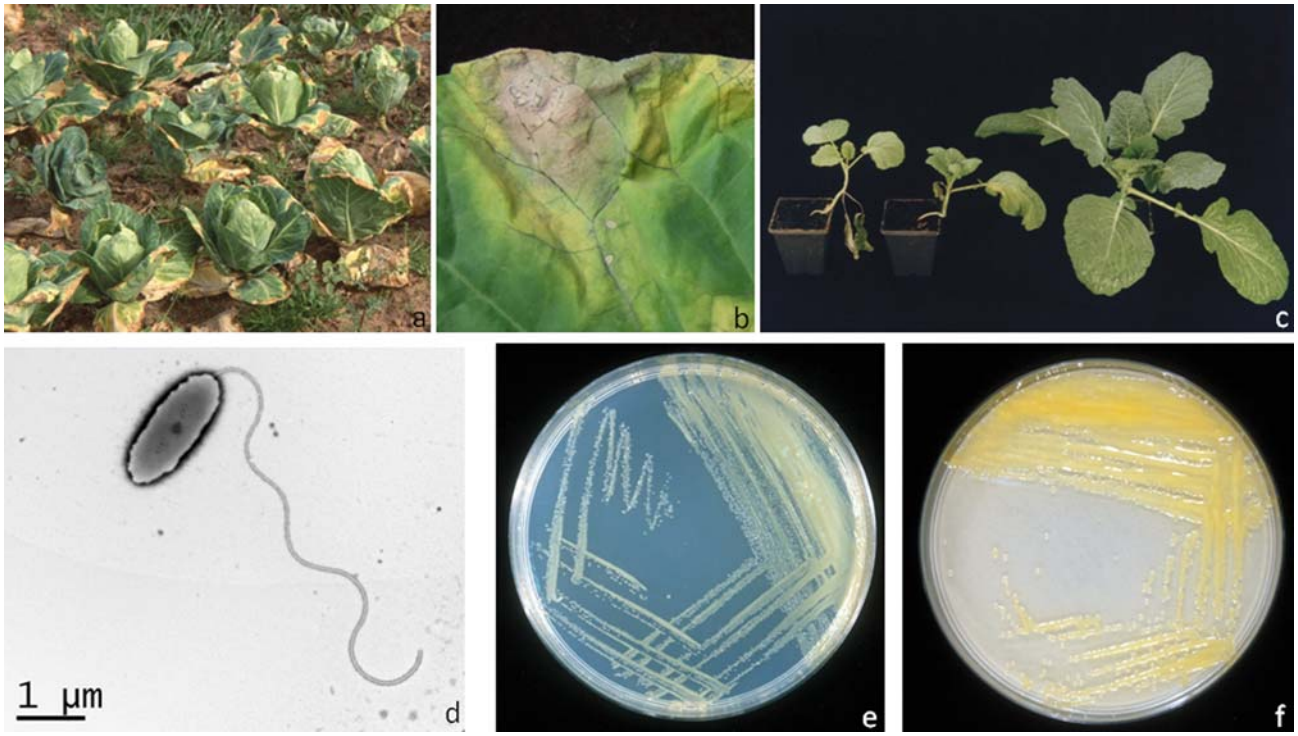

Fig. 1 (a) Symptoms of black rot on a cabbage field. (b) Typical black rot V-shaped lesion on a cabbage leaf. (c) Two plants of Savoy cabbage with symptoms of systemic infection following inoculation of *Xanthomonas campestris* pv. *campestris*, and a healthy control plant. (d) Electron microscopy image of a *X. campestris* pv. *campestris* rod-shaped cell showing a single polar flagellum. (e) *Xanthomonas campestris* pv. *campestris* culture growing on King's medium B. (f) *Xanthomonas campestris* pv. *campestris* culture growing on Yeast Dextrose Calcium Carbonate medium.

BLACK ROT CONTROL

Sanitation and management practices, including crop rotation, weed control and the use of assayed clean seed, can provide significant control of the disease (Schaad and Alvarez, 1993). For example, black rot was a minor disease in the most important production areas in the USA during certain decades, probably because growers followed the recommended practices, including the use of tested, disinfected seed and rotation of seedbeds. However, there was a resurgence of the disease during the 1970s, probably associated with the use of F_1 hybrid seed produced in areas in which the disease was endemic (Williams, 1980).

Standard seed testing methods have been developed (Roberts and Koenraadt, 2006). The tolerance for reliable disease control through seed testing needs to be adjusted according to the system of production, e.g. the number of seeds tested should be higher for transplants raised with overhead irrigation than for direct-drilled crops (Roberts *et al*., 2007). Seed treatments, including hot water, antibiotics, antibiotics and sodium hypochlorite, hydrogen peroxide and hot acidified cupric acetate or zinc sulphate, are available, but no treatment is totally effective. Several methods can be used to reduce the spread of the disease during transplant raising, including the use of web and flow irrigation systems instead of overhead irrigation and of chlorine dioxide in the irrigation water (Krauthausen *et al*., 2011).

The development and use of black rot-resistant cultivars have long been recognized as important methods of control, but, in practice, have had only limited success (Taylor *et al*., 2002). Natural variation and the inheritance of black rot resistance have been studied in several brassica species and, so far, no disease resistance gene has been cloned. Most studies have focused on *B. oleracea* (representing the C genome of brassicas), and a limited number of sources of resistance have been identified, including the cabbage cultivar Early Fuji and the cabbage accession PI 436606 (cv. Heh Yeh da Ping Tou) (Camargo *et al*., 1995; Dickson and Hunter, 1987; Hunter *et al*., 1987; Taylor *et al*., 2002; Vicente *et al*., 2002; Williams *et al*., 1972). Badger Inbred-16, a line derived from Early Fuji, contains quantitative trait loci (QTLs) for black rot resistance which have been genetically mapped (Camargo *et al*., 1995).

The most common and potentially useful sources of black rot resistance occur in the A and B genomes of brassica species, and a number of sources of resistance have been identified in the different species containing these genomes (Bain, 1952; Taylor *et al*., 2002; Westman *et al*., 1999). The inheritance of major gene resistance has been studied in the diploid *B. rapa* (A genome) and

in the tetraploids *B. carinata* (BC genome) and *B. napus* (AC genome) (Guo *et al*., 1991; Ignatov *et al*., 2000; Vicente *et al*., 2002). A single dominant race-specific gene has been mapped to the A genome in *B. napus* (Vicente *et al*., 2002), and QTLs that control resistance to at least two of the most prevalent races of *Xcc* have been mapped in a Chinese cabbage accession of *B. rapa* (Soengas *et al*., 2007).

Genes present in the brassica A and B genomes could potentially provide durable black rot control, especially if strong race-specific genes (matching the most prevalent races) could be combined in a genetic background of race-nonspecific genes (e.g. providing quantitative resistance). To achieve this aim, genes from the wild relative *A. thaliana* could potentially be easier and quicker to characterize molecularly, and either be used directly in transgenic brassica crops, or facilitate the identification and interspecific transfer of homologous black rot resistance genes from A or B genome sources into vegetable crops. Interestingly, most *A. thaliana* accessions are resistant to one or more races of *Xcc*, and more than half exhibit broad-spectrum resistance to all major races of the pathogen (described below), suggesting that this wild relative of brassica crops could indeed provide useful sources of durable black rot resistance (Holub, 2007). Tsuji *et al*. (1991) showed that the resistance to an *Xcc* isolate in the accession Columbia is controlled by a single dominant gene/locus. In addition, Buell and Somerville (1997) described a monogenic and a digenic resistance mechanism in this accession, and mapped the three genes involved. Plant mutants impaired in resistance to *Xcc* have been isolated and a gene involved in the establishment of the hypersensitive response (HR) and defence response has been identified and mapped (Lummerzheim *et al*., 2004). However, although *A. thaliana* and *Xcc* provided one of the earliest experimental models for the investigation of the interactions of *A. thaliana* with a major crop pathogen (Simpson and Johnson, 1990), the molecular basis of natural variation in black rot resistance is largely unexplored in this pathosystem.

PATHOGEN IDENTIFICATION AND DETECTION

The bacterium *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson is a Gram-negative rod, that occurs mostly alone or in pairs and is usually motile by means of a single polar flagellum (Fig. 1d). Most strains form yellow, mucoid, glistening colonies (Fig. 1e,f). The yellow pigments, xanthomonadins (mono- or dibromo-arylpolyene structures), and the exopolysaccharide xanthan, responsible for the mucoid or viscous cultures, are typical of the genus (Vauterin *et al*., 1995), although the existence of atypical pigmented isolates has been reported (Poplawsky and Chun, 1995).

The taxonomy of the genus was mainly based on the hosts of origin and the phenotypic characteristics until the early 1990s. A detailed study of the phenotypic characteristics of the genus was conducted by Van den Mooter and Swings (1990). Vauterin *et al*. (1995) later reclassified the genus on the basis of DNA–DNA hybridization studies. In the new classification, the species *X. campestris* was restricted to strains that cause disease in Brassicaceae (Cruciferae) plants (including *X. campestris* pv. *aberrans*, *armoraciae*, *barbarea*, *campestris*, *incanae*, *raphani* and, possibly, *plantaginis*). The reclassification is mainly supported by data obtained through other molecular techniques, including amplified fragment length polymorphism (AFLP) and polymerase chain reaction (PCR) fingerprinting (Rademaker *et al*., 2000), but there has been some discussion on the shifts in the classification of some groups of isolates (Schaad *et al*., 2000; Vauterin *et al*., 2000).

The DNA–DNA hybridization technique is not suitable for the routine identification of new pathogen isolates, and so other molecular methods have been developed. Studies of the 16S rRNA gene (Hauben *et al*., 1997; Moore *et al*., 1997a, b) and the 16S–23S intergenic region (Gonçalves and Rosato, 2002) can generally only be used to identify the strains at the genus level. Simões *et al*. (2007) differentiated species of *Xanthomonas* by PCR-restriction fragment length polymorphism of the genes *rpfB* and *atpD* involved in the regulation of pathogenicity factors and the synthesis of ATP.

Methods based on DNA sequencing have become more popular as the cost of sequencing has decreased. The sequencing of genes that encode conserved proteins involved in essential cell processes and collectively constitute the 'core genome' has been developed for the identification of pathogens. Parkinson *et al*. (2007, 2009) have shown that sequences of DNA gyrase subunit B (*gyrB*) can be used as an identification tool at the genus, species and, possibly, pathovar level of *Xanthomonas*; this method does not have sufficient resolution to differentiate isolates within each pathovar. Young *et al*. (2008) showed that multilocus sequence analysis (MLSA) based on the partial sequences of four genes, chaperone protein dnaK (*dnaK*), tonB-dependent receptor (*fyuA*), *gyrB* and RNA polymerase sigma factor (*rpoD*), can differentiate *X. campestris* from other species and can possibly also detect differences between some isolates of the same species. The results of MLSA of eight genes, ATP synthase β chain (*atpD*), *dnaK*, elongation factor P (*efP*), glutamine synthetase I (*glnA*), *gyrB*, *rpoD*, triosephosphate isomerase (*tpiA*) and *fyuA*, showed high genetic diversity, with *Xcc* isolates forming two distinct groups; the results also support the existence of two other related pathovars (*raphani* and *incanae*) (Fargier *et al*., 2011).

The identification of *Xcc* at the pathovar level is generally based on the isolation of the pathogen using semi-selective media. The currently used protocol for the detection of the pathogen in seeds uses Fieldhouse-Sasser and mCS20ABN media (Koenraadt *et al*., 2005; Roberts and Koenraadt, 2006). The morphology of the cultures is generally then checked in subcultures on media such as Yeast Dextrose Calcium Carbonate. Classic bacteriological tests, carbon source metabolic fingerprinting (Biolog, Hayward, CA,

USA) (Poplawsky and Chun, 1995), fatty acid analysis (MIDI, Newark, DE, USA) (Massomo *et al*., 2003) and serological tests using polyclonal or monoclonal antibodies (Alvarez *et al*., 1994; Franken, 1992) have been used to speed up the identification of the organisms. All of these methods rely on the availability of databases with the results obtained with representative isolates of different species and pathovars, but frequently problems with the standard isolates used (e.g. misidentification) can complicate the interpretation of new results. The inoculation of susceptible brassica seedlings is still the most reliable method, as it provides the ultimate confirmation of the identification of the pathovar (Roberts and Koenraadt, 2006). However, all of these methods are time consuming and inadequate for high-throughput screening.

Several molecular methods have been used for the identification and characterization of the molecular diversity of *Xcc* and related pathovars. Rademaker *et al*. (2005) used PCR primers that amplified repetitive sequences dispersed across bacterial genomes to generate a method to distinguish DNA 'fingerprinting' of isolates. Several studies have demonstrated that rep-PCR (using REP, ERIC and BOX primers) can differentiate isolates at the species, pathovar and intrapathovar level of *X. campestris* (Rademaker *et al*., 2005; Vicente *et al*., 2006). Nevertheless, the comparison of gel profiles and the standardization of the method between laboratories are still difficult to achieve (Parkinson *et al*., 2007). A DNA probe was developed for the detection of *Xcc*, but, although the method worked for infected leaves, it was generally not sufficiently sensitive to detect the pathogen in seeds (Shih *et al*., 2000).

The *hrp* (hypersensitive response and pathogenicity) genes encode type III secretion systems (T3SSs) (see section on Secretion systems). This gene cluster is involved in plant–pathogen interactions, the growth and development of symptoms in plants and is largely conserved; therefore, these genes are good candidates for molecular diagnostics of different species or pathovars. Berg *et al*. (2006) and Zaccardelli *et al*. (2007) developed PCR methods using primers that amplify part of the *hrpF* gene and the *hrcC* secretin-like gene, respectively. These methods allowed the identification of a range of *Xcc* isolates, but were also positive for isolates of the closely related pathovars *aberrans*, *armoraciae*, *raphani*, *barbarea* and *incanae*.

In the near future, the comparison of whole genome sequences might constitute the basis for the classification and identification of *X. campestris*, and PCR methods with primers related to pathogenicity genes might become part of the routine protocol for the identification of *Xcc*. Presently, for the molecular identification to the genus or species level, MLSA has the advantage of being a cheaper, more flexible technique to compare bacteria, and is a practical, easier alternative to hybridization studies and full sequencing. It is possible that different sets of housekeeping genes need to be selected to target variation between and within different species (Young *et al*., 2008), but, as the number of genes increases, the analysis of results becomes more complicated. At the pathovar level, primers based on *hrp* or other genes linked to pathogenicity might partially substitute the need to test susceptible plants to confirm the identification.

PATHOGEN RACES

A race structure for *Xcc* was first proposed by Kamoun *et al*. (1992). The authors described five races (numbered 0–4) based on the reaction of different brassica species (Table 1). Vicente *et al*. (1998) and Ignatov *et al*. (1998b) have subsequently shown that race 1 can be subdivided into two or three races on the basis of their reaction on several accessions of *B. oleracea* and *B. carinata*. A revised race classification was proposed by Vicente *et al*. (2001) based on a much larger collection of isolates (Tables 2 and 3). Three races (1, 2 and 4) were retained from Kamoun *et al*. (1992); however, no isolate was found that matched race 3, and so this race was dropped from the new race classification. Three variant classes were identified amongst the previous race 1 isolates based on the reactions of two *B. oleracea* accessions and an accession of *B. carinata*: a new race 1 that refers to the most commonly found variant, a new race 3 to accommodate a rare variant represented by the type strain of *Xcc* (ATCC33913; NCPPB 528) and an additional race 5 for three non-UK isolates, including an isolate previously included in *X. campestris* pv. *aberrans* (Vicente *et al*., 2001). It was proposed that race 0 should be reassigned to a new race 6 to avoid the implication that these isolates lacked avirulence genes; although these isolates are pathogenic in all the differentials currently used, partial resistance to this race has been observed in brassica accessions (J. D. Taylor *et al*., unpublished data; Horticulture Research International, Warwick, UK). Race 2 is only represented by a single isolate (HRI 3849A), which was used in the earliest molecular investigations of black rot resistance in *A. thaliana* (Buell and Somerville, 1997; Kamoun and Kado, 1990; Tsuji *et al*., 1991) (Table 3). More recently, race 7 has been added by Jensen *et al*. (2007, 2010) and Fargier and Manceau (2007). In addition, Fargier and Manceau (2007) included races 8 and 9 for the classification of isolates that have a narrow host range in the differential cultivars (Tables 2 and 3).

Interestingly, the type strain (ATCC33913; NCPPB528) can cause atypical blight symptoms in brassicas (Alvarez *et al*., 1994;

Table 1 Differentiation of *Xanthomonas campestris* pv. *campestris* races according to Kamoun *et al*. (1992).

	Race				
Differential cultivar	0	1	2	3	4
Early Jersey Wakefield (*Brassica oleracea*)	+	+	+	+	+
Just Right Turnip, Tokyo Turnip Hybrid (*Brassica rapa*)	+	+	+	–	–
Seven Top Turnip (*B. rapa*)	+	+	–	+	–
Florida Broad Leaf India Mustard (*Brassica juncea*)	+	–	+	–	–

+, compatible interaction (susceptibility); –, incompatible interaction (resistance).

Table 2 Postulated gene-for-gene model to explain the relationship between *Brassica* lines and races of *Xanthomonas campestris* pv. *campestris* (adapted from Vicente *et al*., 2001, Fargier and Manceau, 2007 and Jensen *et al*., 2010).

					Race/avirulence gene (A)								
					1	2	3	4	5	6	7	8	9
					A1		A1	A1?				A1	A1
						A2						A2	
						A3	A3		A3			A3	A3
								A4					A4
Differential cultivar or accession	Resistance gene (R)										A5		
Wirosa F_1 (*Brassica oleracea*)					+	(+)/+	+	+	+	+	+	+	+
Just Right Hybrid Turnip (*Brassica rapa*)				R4	+	+	+	–	+	+	+	+	–
COB60 (*Brassica napus*)		R2?		R4	+	(+)	+	–	+	+	+	nt	nt
Seven Top Turnip (*B. rapa*)		R2		R4	+	–	+,v	–,v	+,v	+	+	–	–
PIC1 (*Brassica carinata*)	R1			R4?	-	(+)	–	–	+	+	+	–	–
FBLM2 (*Brassica juncea*)	R1			R4?	R5 -	+	–	–	(+)	+	–	–	–
Miracle F_1 (*B. oleracea*)		R2?	R3		+	–/(+)	–/(+)	+	–	+	+	–	–
SxD1 (*B. oleracea*)			R3		+	–/(+)	–	+	–	(+)/+	+	nt	nt

+, compatible interaction (susceptibility); –, incompatible interaction (resistance); (+), weakly pathogenic; nt, not tested; v, variable; ? indicates that the gene might be present, although it might not be necessary to explain the interaction.

Table 3 Race type strains of *Xanthomonas campestris* pv. *campestris* (adapted from Vicente *et al*., 2001 and Fargier and Manceau, 2007).

HRI/UW isolate number	Other collections (number)	Host	Country (year)	Race
3811	P. Williams (PHW1205)	*Brassica oleracea*	USA	1
3849A	C.I. Kado (2D520)	*B. oleracea* var. *botrytis*	USA	2
5212	NCPPB (528), CFBP (524)1, LMG (568), ATCC (33913)	*B. oleracea* var. *gemmifera*	UK (1957)	3
1279A	–	*B. oleracea* var. *capitata*	UK (1984)	4
3880	NCPPB (2986)	*B. oleracea* var. *capitata*	Australia (1975)	5
6181	–	*Brassica rapa*	Portugal (1996)	6
–	CFBP (4953)	*B. oleracea* var. *botrytis*	Belgium (1999)	7
8450A	B. D. Jensen (N47)	*B. oleracea* var. *capitata*	Nepal (2001)	7
–	CFBP (1124), LMG (8032)	*B. oleracea* var. *botrytis*	France (1967)	8
3961	NCPPB (1145), LMG (8004), CFBP (6650)	*B. oleracea* var. *botrytis*	UK (1958)	9

CFBP, Collection Française de Bactéries Phytopathogènes, INRA, France; HRI/UW, The University of Warwick (ex-Warwick HRI), Wellesbourne, UK; LMG, Laboratorium voor Microbiology, Gent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Sand Hutton, UK.

Chen *et al*., 1994) characterized by dark necrotic lesions with limited chlorosis, and, in this respect, is not typical of the majority of *Xcc* isolates. These symptoms have also been observed in *B. oleracea* accessions inoculated with isolates of races 5 and 6. The ability to elicit blight symptoms may be under genetic control (Chen *et al*., 1994), but may also be influenced by environmental conditions, in particular temperature.

Homozygous brassica lines (i.e. inbred or doubled haploid lines) provide the best means for reproducible, routine identification of *Xcc* races, and seed stocks can be readily regenerated by researchers or public stock centres. However, the current host differential set includes, for example, the cultivar Seven Top Turnip, which exhibits variable reactions (possibly as a result of a genetic mixture from open pollination in the commercial seed production). F_1 hybrids are also included, which are impossible for researchers to regenerate, and may have limited availability in future years depending on the commercial success of each of these cultivars. To overcome part of these constraints, doubled haploids from several accessions of *B. oleracea*, *B. napus*, *B. carinata* and *B. juncea* were produced at the University of Warwick, Warwick HRI (now part of the School of Life Sciences), to replace the previous differential lines described by Vicente *et al*. (2001). These include doubled haploid lines that replace Cobra, PI199947, Florida Broad Leaf Mustard and Miracle F_1.

Gene-for-gene interactions can be used to explain the relationship between bacterial isolates and differential lines. The proposed gene-for-gene model presented in Table 2 is based on the interaction of at least five matching gene pairs. The genes that confer

Table 4 Postulated gene-for-gene model to explain the relationship between *Brassica* spp., radish and pepper lines and isolates of *Xanthomonas campestris* pv. *campestris* (adapted from He *et al*., 2007).

					Avirulence gene (A)			
					xop AH (avrXccC)			
					.	*avrRc2**		
					.		*xopE (avrXccE1)1*	
					.			*avrBs1*
Differential cultivar or line	Resistance gene (R)							
Guangtou (*Brassica juncea* var. *megarrhiza*)	*Rc1*				–	+	+	
Jingfeng-1 (*Brassica oleracea)* and Huaye (*Raphanus sativus* var. *longipinnatus*)		*Rc2*			+	–	+	
Zhongbai-83 (*Brassica rapa* var. *pekinensis*)			*Rc3*		+	+	–	
ECW10R (*Capsicum annuum* v. *latum*)				*Bs1*	+	+	+	HR

*This gene has not yet been identified.

+, compatible interaction (susceptibility); –, incompatible interaction (resistance); HR, hypersensitive response.

resistance to the most important races (1 and 4) are designated *R1* and *R4*. The model allows for the possible inclusion of additional gene pairs if new races and differentials are identified. In general, the model was constructed in a manner that reflects the origin of the allotetraploid brassica species (Nagaharu, 1935): *R1* originates from the B genome, *R3* from the C genome and *R4* from the A genome. The proposed model needs to be supported by genetic and molecular data from both the host and the pathogen to be fully validated. In the case of the host, results of crosses made to establish the inheritance of resistance to some of the races indicate that *R1*, *R3* and *R4* are single dominant genes (Vicente *et al*., 2002).

A simpler gene-for-gene model has been proposed by He *et al*. (2007) based on the interactions between *Xcc* isolates and cultivars of *Brassica* (*B. juncea*, *B. oleracea*, *B. rapa*), radish (*Raphanus sativus*) and pepper (*Capsicum annuum*) (Table 4). It is possible that some of the resistance/avirulence genes proposed by the authors correspond to genes included in Table 2: the gene Rc1 might correspond to R1, Rc2 to R3 and Rc3 to R4.

Worldwide, races 1 and 4 are predominant, but their relative frequencies in *B. oleracea* crops appear to vary with geographical region. For example, race 1 appears to be more common than race 4 in the UK, whereas race 4 has been shown to be the predominant race in Portugal (Vicente, 2004), northwestern Spain (Lema *et al*., 2012) and some East African countries, such as Tanzania and Uganda (Mulema *et al*., 2012). Other races are generally rare, but may be more common in other host species that are less frequently surveyed. Races 2 and 6 were absent in a collection of isolates from Japan and Russia (Ignatov *et al*., 1998a). Nepal and northwest Spain seem to have diverse populations of *Xcc*, with five different races identified in *B. oleracea* crop plants (Jensen *et al*., 2010; Lema *et al*., 2012). The low frequency of race 3 worldwide may be a result of the extensive use of cultivars that are resistant to this race. However, this may not have been the case 50 years ago when the type strain of *Xcc* (ATCC33913, NCPPB528) was collected in 1957. Race 5 is also rare, but was recently found in Nepal (Jensen *et al*., 2010) and in a field in northwest Spain (Lema *et al*., 2012). Race 6 was not found in the UK, but this may have been a result of the preponderance of isolates obtained from *B. oleracea*. In Portugal and northwest Spain, race 6 was common in turnip (Lema *et al*., 2012; Vicente, 2004).

The gene-for-gene model and the availability of defined 'race type strains' should assist in the selection and evaluation of plant material for breeding programmes and may be the basis for molecular studies. Disease resistance screening should be performed with isolates that represent the pathogenic variation of *Xcc*, and therefore should at least include the major races 1 and 4. In addition, isolates of race 6 should be useful to detect potential race-nonspecific resistance. The monitoring of the frequency and distribution of races worldwide is essential to the development of effective strategies for the breeding of black rot-resistant cultivars. Future brassica crops will benefit from the combination of major genes that confer strong resistance to the most common races of the pathogen (*R1* and *R4*) and, if possible, race-nonspecific genes that could confer quantitative resistance to all known races.

THE PATHOGEN GENOME

Three isolates of *Xcc* have been fully sequenced, including the type strain ATCC33913 (NCPPB528; LMG568; ICMP13; DSM3586) (da Silva *et al*., 2002), isolate 8004 (a spontaneous rifampicin-resistant mutant from NCPPB1145) (Qian *et al*., 2005) and the industrial high-xanthan-producing isolate B100 (Vorhöelter *et al*., 2008). The type strain has been identified as race 3 (Vicente *et al*., 2001), isolate 8004 has been assigned to race 9 (Fargier and Manceau, 2007) and B100 belongs to race 1 (J. G. Vicente,

Table 5 *Xanthomonas campestris* pv. *campestris* strains that have been completely sequenced and features of the genome (adapted from Vorhöelter *et al*., 2008).

Isolate	ATCC 33913	8004	B100
Other designations	NCPPB (528), HRI5212, LGM 568, CFBP 5241	Rifampicin-resistant laboratory strain derived from NCPPB 1145, CFBP 6650	
Host of origin	Brussels sprouts (*Brassica oleracea* var. *gemmifera*)	Cauliflower (*B. oleracea* var. *botrytis*)	
Country of origin	UK	UK	
Year of isolation	1957	1958	
Race	3	9	1
Size (bp)	5 076 187	5 148 708	5 079 002
G + C content (%)	65.0	64.9	65.0
CDS—predicted number	4181	4273	4471
CDS—function assigned	2708	2671	2878
Ribosomal RNA operons	2	2	2
Transfer RNAs	54	54	54
Insertion sequence elements	109	115	59
Reference	da Silva *et al*. (2002)	Qian *et al*. (2005)	Vorhöelter *et al*. (2008)

CDS, coding DNA sequence; CFBP, Collection Française de Bactéries Phytopathogènes, INRA, France; HRI, School of Life Sciences (ex-Warwick HRI), University of Warwick, Wellesbourne, UK; LMG, Laboratorium voor Microbiology, Gent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Sand Hutton, UK.

unpublished results). The isolate 8004 has featured in several studies of phytopathological properties, such as the secretion of extracellular enzymes and exopolysaccharides (Tang *et al*., 1991), cell–cell signalling and biofilm formation (Torres *et al*., 2007). Further information about the *Xcc* genomes is summarized in Table 5. An isolate of *X. campestris* pv. *raphani* (756C), a closely related pathogen that causes a nonvascular, leaf spot pathogen on Brassicaceae and other hosts such as tomato, has also been sequenced recently (Bogdanove *et al*., 2011). These *X. campestris* genomes comprise circular chromosomes of approximately 5 000 000 base pairs (bp), have a high G + C content and do not carry plasmids.

The comparison of the sequences of *Xcc* type strain ATCC33913 and 8004 indicated that significant genomic-scale rearrangement across the replication axis between two IS1478 elements and the loss and acquisition of blocks of genes, rather than point mutations, constitute the main genetic variation between the strains (Qian *et al*., 2005). The sequence of B100 is extensively collinear to the strain 8004, but differs from the type strain ATCC33913 by the inversion of a large chromosomal fragment (Vorhöelter *et al*., 2008). This may indicate that the strains 8004 and B100 have originated via recent recombination events (Qian *et al*., 2005).

He *et al*. (2007) have constructed a whole genome microarray of the *Xcc* strain 8004 and have used this resource to study the genetic diversity and host specificity of this pathogen by array-based comparative hybridization with genomes of 18 pathogenic isolates collected from different host plants and various geographical regions of China. A core set of 3405 conserved coding sequences was identified and 730 coding sequences that were absent or highly divergent amongst the isolates. Of the 304 proven or postulated pathogenicity genes currently known in *Xanthomonas*, 258 were conserved and 46 were highly divergent amongst the isolates.

These current reference genomes of *Xcc* are useful for genome-wide comparisons with publicly available genomes from other *Xanthomonas* species, including strain 306 of *X. axonopodis* pv. *citri* (da Silva *et al*., 2002), which represents the citrus canker pathogen, strain 85-10 of *X. axonopodis* pv. *vesicatoria* (Thieme *et al*., 2005), which represents the bacterial spot pathogen which specifically attacks pepper (and not tomato), strains of the rice bacterial blight pathogen *X. oryzae* pv. *oryzae*, KACC10331 (Lee *et al*., 2005), MAFF 311018 (Ochiai *et al*., 2005) and PXO99^{A} (Salzberg *et al*., 2008), strain BLS256 of *X. oryzae* pv. *oryzicola* (Bogdanove *et al*., 2011), strain GPE PC73 of *X. albilineans* (Pieretti *et al*., 2009) and strains from *X. vasicola* pv. *vasculorum* and *musacearum*, the cause of banana wilt (Studholme *et al*., 2010). For example, Lu *et al*. (2008) compared six gene clusters associated with pathogenicity across genomes of eight *Xanthomonas* isolates representing vascular and nonvascular diseases of rice, brassicas, pepper and citrus. Interestingly, plasmid DNA was only observed in the reference isolates of *X. axonopodis* pv. *citri* and pv. *vesicatoria*.

SECRETION SYSTEMS

Reference genomes of *Xcc* have begun to reveal an extensive inventory of genes that may be required for plant associations on the basis of homology with known genes from other *Xanthomonas* or *Pseudomonas* species. Most importantly, genome-wide comparisons have revealed three secretion systems (types II, III and IV) in *Xcc* that have been associated with pathogenic bacteria of plants or animals.

The type II secretion system (T2SS) mediates the transport of proteins into the extracellular space. This system can secrete plant cell wall-degrading enzymes, including cellulose, polygalacturonase, xylanase and protease. Two classes of T2SS have been identified in *Xanthomonas*: *xps* and *xcs*. The *xps* cluster is present in all *Xanthomonas* genomes currently sequenced and in other genera (e.g. *Xylella*), whereas the *xcs* genes are only present in some of

the *Xanthomonas* genomes, including *Xcc* and *X. campestris* pv. *raphani* (Lu *et al*., 2008). Qian *et al*. (2005) obtained six pathogenicity-deficient mutants (*xpsD*, *xpsE*, *xpsF*, *xpsK*, *xpsL* and *xpsM*) related to the *xps* system, whereas no pathogenicity-deficient mutants were found on the 12 annotated genes related to the *xcs* system; therefore, the *xps* genes are related to pathogenicity, whereas the *xcs* genes may have other roles not essential for pathogenicity or may be nonfunctional.

The mutation of DsbB, a protein involved in disulphide bond formation in the periplasm of *Xcc*, reduced virulence, cell mobility and bacterial growth *in planta* and resulted in ineffective T2SS and T3SS (Jiang *et al*., 2008b).

The type IV secretion system (T4SS) is important for the release of macromolecules, and this system is used by Gram-negative bacteria to translocate protein and DNA substrates across the cell envelope into target cells (Souza *et al*., 2011). However, the role of T4SS in the pathogenicity of *Xanthomonas* is uncertain. The T4SS carried in the race 9 isolate 8004 is encoded mainly by genes in the *virD4* and the *virB* cluster that consists of nine open reading frames (ORFs). Qian *et al*. (2005) identified a mutated gene encoding the channel-forming protein VirB8, and concluded that the T4SS contributes to the virulence of the pathogen. However, He *et al*. (2007) have shown that deletion of *virD4* and the *virB* cluster of strain 8004 does not affect the virulence of the pathogen.

The T3SS is generally thought to be essential for pathogenicity and for the initiation of disease in susceptible host plants, and can be involved in the elicitation of cell death and other defence responses in resistant plants. The T3SS is encoded by the *hrp* cluster of genes and translocates effector proteins directly into the plant cells; it is also possible that effectors can exit the bacterial phytopathogens via the Hrp pathways (Lindgren, 1997). Individual genes have been named *hrp*, *hrp*-conserved (*hrc*) or *hrp*-associated (*hpa*). Most *hrp* gene sequences are only found in *Xanthomonas* and related genera, whereas *hrc* genes are conserved in many plant and animal pathogens. In *Xcc*, the *hrp* cluster consists of 26 genes extending from *hpa2* to *hrpF* (da Silva *et al*., 2002). The *Xcc* genomes do not contain *hpa3* and the N-terminal region of *xopF1*, although these genes are present in other *Xanthomonas* species, including *X. campestris* pv. *raphani* (Lu *et al*., 2008). Four mutants of genes encoding the T3SS machinery (*hrcJ*, *hrcU*, *hrcV*, *hrpE*) and one encoding a regulatory protein (*hrpG*) were nonpathogenic (Qian *et al*., 2005). Mutations in the regulator *hrpG* of strain 8004 can make all the *hrp* genes express constitutively and enhance the intensity of the HR reaction in pepper (Jiang *et al*., 2006).

Wei *et al*. (2007) showed that *hpaR*, a putative *marR* family transcriptional regulator, is essential for the pathogenicity of strain 8004 on cabbage, is required for the HR response on the nonhost pepper and is under the positive control of the two key *hrp* gene regulators HrpG and HrpX. The GntR family is a frequent group of helix–turn–helix transcriptional regulators in bacteria. One of the six GntR regulators, HpaR1, positively and negatively affects the expression of HR and pathogenicity *hrp* genes that encode the type III secretion via hrpG (An *et al*., 2011).

Several genes that are regulated in an HrpX-dependent manner possess a consensus nucleotide sequence TTCGB–N_{15}–TTCGB (B can be base C, G or T), which has been termed the plant-inducible-promoter box, or PIP box. The detection of PIP boxes provides a refined tool for the identification of candidate genes in the hrpX regulon, as well as effector protein genes of the type III pathway. For example, 17 putative PIP box sequences have been described in the promoter regions of *Xcc* ATCC33913 (da Silva *et al*., 2002) and 56 genes have been predicted to have PIP boxes in strain 8004 (Jiang *et al*., 2009).

TYPE III EFFECTOR GENES

There has been considerable research effort to identify and understand type III effector function in *Xanthomonas* species. White *et al*. (2009) reviewed in detail the type III effectors from *Xanthomonas* and classified them into 39 different groups (called Xop) reflecting sequence similarity. From these, 21 appear to be present in the *Xcc* strain ATCC33913. Nine effectors, considered to be core effectors, are present in almost all *Xanthomonas* isolates, with the exception of *X. albilineans* and, in some cases, *X. campestris* pv. *raphani*; other effectors are found in a more limited number of species/isolates (Bogdanove *et al*., 2011; Ryan *et al*., 2011). From the extensive list of genes encoding putative T3SS proteins in *Xcc* and *X. campestris* pv. *raphani*, summarized in Table 6, effort has begun to identify potential effector proteins (which are important for pathogenicity in a susceptible host) and avirulence determinants (which elicit defence in a resistant host). Effector proteins are generally thought to be involved in the suppression of the host defence system or in the alteration of host metabolism to favour nutrient uptake of the pathogen. However, experimental verification of effector function can be difficult. For example, deletion of individual effector genes can often result in little or no reduction in pathogenicity because of the presence of alternative functional (redundant) effector proteins (Cunnac *et al*., 2009). Some T3SS effectors may activate defences (e.g. rapid host cell death) when infiltrated into plant tissue, and thus indicate a potential role as avirulence (Avr) proteins in a resistant host (Flor, 1971; Keen, 1990).

Xu *et al*. (2006) showed that the mutagenesis of eight candidate genes from strain 8004 had no affect on pathogenicity in Chinese radish and cabbage. However, Xu *et al*. (2006) and He *et al*. (2007) showed that mutants of *avrBs1* lost the ability to elicit an HR in the pepper line ECW10R that contains the corresponding R gene *Bs1* (Table 4). The AvrBs1 effector is responsible for the elicitation of HR on pepper dependent on T3SS (Xu *et al*., 2006). However Castañeda *et al*. (2005) did not detect an HR variation between the *avrBs1* mutants and the wild-type 528T. He *et al*.

Table 6 Occurrence, features and function of putative and proven effector/avirulence genes of *Xanthomonas campestris* pv. *campestris*.

Effector class	Synonym	Gene ID in ATCC33913	Gene ID in 8004	Gene ID in B100	Gene ID in *Xcr* 756C	PIP box	Features and function on pathogenicity
Core effectors*							
AvrBs2		XCC0052	XC 0052	xcc-b100 0057	No	Yes	Glycerophosphoryl diester phosphodiesterase Avirulence in *Brassica juncea*, *B. carinata* and *B. oleracea* and HR on pepper (Ignatov *et al*., 2002) or no effect (Castañeda *et al*., 2005)
XopK		XCC2899	XC 1210	xcc-b100 1254	No		Unknown
XopL	XopLR	XCC4186	XC 4273	xcc-b100 4400	No	Yes	Leucine-rich protein Reduced virulence on radish (Jiang *et al*., 2009)
XopN		XCC0231	XC 0241	xcc-b100 0253	No		ARM/HEAT repeat Reduced virulence on radish (Jiang *et al*., 2008a)
XopP		XCC1247	XC 2994	xcc-b100 3057	XCA_1500	Yes	Unknown Reduced virulence on radish (Jiang *et al*., 2009)
XopQ		XCC1072	XC 3177	xcc-b100 3274	No	Yes	Putative inosine–uridine nucleotide N-ribohydrolase Reduced virulence on radish (Jiang *et al*., 2009)
XopR		XCC0258	XC 0268	xcc-b100 0280	XCA_4254		Unknown
XopX		XCC0529 XCC0530	XC 0541 XC 0542	xcc-b100 0558 and 0559	No		Methionine-rich protein
XopZ		XCC1975	XC 2210	xcc-b100 2274	No		Unknown
Other effectors†							
AvrBs1		XCC2100	XC 2081	xcc-b100 2396	No	No	HR on pepper (He *et al*., 2007)
XopD		XCC2896	XC 1213	xcc-b100 1256	No		SUMO cysteine protease (C48 family), EAR motif, DNA binding, nuclear localization(Canonne *et al*., 2012)
XopE	AvrXccE1	XCC1629	XC 2602	No	No	Yes	Putative transglutaminase Avirulence on *B. rapa* (He *et al*., 2007)
XopF		XCC1218	XC 3024	xcc-b100 3087	XCA_1470		Unknown
XopG		XCC3258	XC 0967	xcc-b100 2655	No		M27 family peptidase (*Clostridium* toxin)
XopH	AvrBs1.1	XCC2099	XC 2082	xcc-b100 2395	No	No	Putative tyrosine phosphatase
XopJ	AvrXccB	XCC3731	XC 3802	No	No	Yes	Putative cysteine protease (C55 family) or serine/threonine acetyltransferase. Ubiquitin-like protease No effect on pathogenicity (Jiang *et al*., 2009)
XopAC	AvrAC	XCC2565	XC 1553	xcc-b100 1596	XCA_2914	Yes	Leucine-rich protein Avirulence recognized in vascular tissues of Arabidopsis (Xu *et al*., 2008)
XopAD		No	No	No	XCA_1464		SKWP repeat protein
XopAG		XCC3600	XC 0563	xcc-b100 0580	No		Unknown
XopAH	AvrXccC	XCC2109	XC 2004	xcc-b100 2071	No	Yes	Dual function. Avirulence in *B. juncea* and *B. rapa*; virulence in *B. oleracea* (Castañeda *et al*., 2005; He *et al*., 2007; Wang *et al*., 2007)
XopAL1	AvrPhpE	XCC1246	XC 2995	xcc-b100 3058	XCA_1499	Yes	Reduced virulence on radish (Jiang *et al*., 2009)
XopAL2		Downstream XCC3574	Downstream XC_3916	xcc-b100 0616	No		Unknown
XopAM	XopR1	XCC1089	XC 3160	xcc-b100 3256	No	Yes	Reduced virulence on radish (Jiang *et al*., 2009)
XopAT		No	No	No	XCA_1464a	Yes	Unknown. No similarity with other known proteins (Bogdanove *et al*., 2011)
XopA		XCC1240	XC 3002	xcc-b100 3065	XCA_1492		Harpin, may not be a t3e
HpaA		XCC1224	XC 3018	xcc-b100 3081	XCA_1476		T3 secretion control protein, may not be a t3e
HrpW		XCC1219	XC 3023	xcc-b100 3086	XCA_1471		Pectate lyase, may not be a t3e
AvrXccA1	AvrXca	XCC4229	XC 4318	xcc-b100 4450	XCA_4581	No	May not be a t3e. Virulence of *Xcr* in Arabidopsis (Parker *et al*., 1993)
AvrXccA2		XCC2396	XC 1716	xcc-b100 1770	XCA_2696	No	Unknown, may not be a t3e

Adapted from da Silva *et al*. (2002), White *et al*. (2009), Jiang *et al*. (2009) and Ryan *et al*. (2011).
*Nine core effectors are found in most *Xanthomonas* spp., with the exception of *X. albilineans* and, in some cases, *X. campestris* pv. *raphani* (*Xcr*).
†Other effectors are found in a more limited number of *Xanthomonas* spp. isolates.
t3e, Type III effector.

(2007) suggested that it is possible that the function of *avrBs1* is redundantly encoded in the *Xcc* type strain and that the expression is different in the two strains.

Ignatov *et al*. (2002) reported that an *avrBs2* homologue, designated *avrRxc1/3*, determined the avirulence of *Xcc* 512/2 (a natural mutant strain of a race 3 isolate) on brassica plants with the B genome, including *B. juncea* cv. Florida Mustard and *B. carinata* line PI199947, and on some *B. oleracea* cultivars (including the lines SR1/3, Badger Inbred-16/2 and cv. Miracle F_1). This gene was also responsible for a mesophyllic HR on leaves of Florida Mustard and nonhost pepper plants carrying *Bs2*. Xu *et al*. (2006) showed that *avrBs2* is required for full virulence of strain 8004 on Chinese radish and cabbage. In contrast, Castañeda *et al*. (2005) showed that the deletion of the entire putative *avrBs2* homologue in the type strain had no effect on the interaction with Florida Mustard and pepper. The strains employed by Ignatov *et al*. (2002) are probably derived from the type strain used by da Silva *et al*. (2002) and Castañeda *et al*. (2005); therefore, it is difficult to reconcile these results and the role of the *avrBs2* homologue on pathogenicity/race specificity still needs to be clarified.

Genes from the AvrBs3 family have been found in a number of *Xanthomonas* species and pathovars, and in *Ralstonia solanacearum* (White *et al*., 2009). This large family of closely related T3 effectors constitutes the TAL effector family. All members of the family have a common structure with tandem repeats of a sequence of amino acids, typically of 34 amino acids in the central part of the proteins, nuclear localization signals (NLSs) and an acidic activation domain (AD); the avirulence and virulence specificities of an AvrBs3 member are dependent on the number and order of the repeats and the NLSs and AD (Boch and Bonas, 2010; Gurlebeck *et al*., 2006).

Interestingly, the three *Xcc* strains sequenced do not contain genes from the *avrBs3/pthA* family, whereas three members of this family have been identified in an isolate named as *X. campestris* pv. *armoraciae*. These three genes were designated *hax2*, *hax3* and *hax4* (homologue of *avrBs3* in *Xanthomonas*) (Kay *et al*., 2005). The three Hax proteins are translocated via the T3SS. The Hax3 and Hax4 proteins have the typical structure of the AvrBs3-like effectors, with 34 amino acid repeats in the central part, but Hax2 has 35 amino acid repeats that contain an additional proline residue. The three *hax* genes have an additive effect on disease symptoms in radish, with hax2 having the strongest influence, and two *hax* genes (*hax3* and *hax4*) have a *Bs4*-dependent avirulence activity in tomato (Kay *et al*., 2005).

Transconjugants of Chinese isolates containing *avrXccE1* (*xopE*) became avirulent on Chinese cabbage (*B. rapa*) cv. Zhongbai-83 (He *et al*., 2007) (Table 4). In contrast, Castañeda *et al*. (2005) did not notice any effect on pathogenicity on the plants tested, but it is possible that the lines tested by these authors did not possess the *R* gene responsive to *avrXccE1*.

Jiang *et al*. (2008a) showed that XopN from strain 8004 (designated XopXccN) is required for full virulence. A mutant of the strain 8004 with an insertion in *xopXccN* was significantly weaker than strain 8004 when inoculated on Chinese radish (*Raphanus sativus* var. *radiculus*) cv. Manshenhong. The expression of *xopXccN* is regulated by the key *hrp* regulators HrpG and HrpX.

The protein sequence of XopD has been identified in strain B100 (Canonne *et al*., 2012). This type III effector targets the Arabidopsis transcription factor MYB30, possibly as part of a virulence strategy that allows *Xcc* to suppress the plant cell responses to infection (Canonne *et al*., 2011).

Mutagenesis of the eight candidate effector genes from type strain ATCC33913 (syn. 528^T) had no effect on pathogenicity when the mutants were used to inoculate six crucifer species, and none of the mutated genes singly or in any combination affected the nonhost HR elicited by the type strain in pepper. However, insertion or deletion mutants in a locus of a gene designated *avrXccFM* (XopAH) became virulent on Florida Mustard, and therefore changed the race specificity of the isolate (Castañeda *et al*., 2005). The T3SS effector AvrXccC of strain 8004, which belongs to the AvrB effector family of *Xanthomonas*, has been shown to have a dual effect: this effector is required for full bacterial virulence in a susceptible *B. oleracea* cv. Jingfeng 1 and for avirulence in a *B. napiformis* L.H. Bailey [syn. *B. juncea* (L.) Czern. var. *napiformis* (Pailleux & Bois) Kitam.] accession (not specified) (Wang *et al*., 2007). The *avrXccC* gene was expressed in the race 2 strain HRI3849A (which causes symptoms on the mustard accession used by these authors) and the resulting strain was avirulent. He *et al*. (2007) also showed that an *avrXccC* mutant of 8004 became pathogenic on mustard (*B. juncea* var. *megarrhiza* Tsen et Lee) cv. Guangtou and Chinese cabbage (*B. rapa* ssp. *pekinensis*) cv. Zhongbai-83. The *avrXccC* gene of strain 8004, which confers avirulence on some mustard cultivars, is identical to *avrXccFM* of strain ATCC33913 (syn. 528T), which confers avirulence on Florida Mustard (He *et al*., 2007). Wang *et al*. (2007) showed that AvrXccC is anchored to the plant plasma membrane and its avirulence function for host recognition depends on its location. The expression of *avrXccC* is *hrpG/hrpX* dependent.

Xcc also contains genes encoding leucine-rich repeat (LRR) proteins. These motifs are commonly involved in protein–protein interactions and are found in several classes of plant disease resistance genes. Xu *et al*. (2008) showed that one of these genes, *xopAC*, named $avrAC_{Xcc8004}$, encodes a protein containing LRRs; this gene is a type III effector that appears to be restricted to strains of *X. campestris*. The *A. thaliana* accession Col-0 was resistant to the wild-type 8004 strain and to an $avrAC_{Xcc8004}$ mutant when the leaf mesophyll was infiltrated with bacterial suspensions, but the Col-0 ecotype became susceptible to the mutant when the bacterium was introduced into the vascular system by piercing the central vein of the leaves, indicating that

the product of *avrAC*$_{Xcc8004}$ is recognized in vascular tissues and this gene might be related to the ability to colonize the xylem of a host.

Some *avr*/effector genes have also been identified in closely related pathovars, including isolates identified as *X. campestris* pv. *armoraciae* and *raphani* (possibly synonymous). Parker *et al*. (1993) cloned an avirulence gene of an isolate identified as *X. campestris* pv. *raphani* (isolate 1067). This isolate was avirulent in all the *A. thaliana* accessions tested by the authors. When the *avrXca* gene was transferred to *Xcc* strain 8004, it strongly reduced symptom development and bacterial growth in Columbia plants, but did not affect virulence to *Brassica* plants. The *avrXca* gene encodes a protein of 67 kDa that has no homology with known sequences and confers avirulence in a number of *A. thaliana* accessions, except one ('Kas-1'). The avirulence phenotype is not *Hrp* dependent and the interaction with *A. thaliana* did not lead to a characteristic HR, indicating that this may not be a type III effector. More recently, Corbett *et al*. (2005) have characterized a virulence factor, designated Svx, of *E. carotovora* ssp. *atroseptica* which shows homology to AvrXca. This protein is secreted by the type II secretion apparatus and the transcription of the *svx* gene is regulated by quorum sensing; the function of this protein is unknown, but the authors consider that it may play a role in the pathogenicity of *E. carotovora* pv. *atroseptica*.

So far, there is some evidence that 12 of the putative effector genes from *Xcc* are phenotypically functional in a very restricted number of isolates. The avirulence genes presented in the model in Table 2 have not been proven and linked to the effectors of *Xcc* (Table 6), although it is likely that A1 might correspond to XopAH and A4 to XopE. Some putative effectors could be pseudogenes or poorly expressed genes in the isolates studied, or the genes might have small effects on pathogenicity or fitness that have not been detected in the assays (Castañeda *et al*., 2005). Further research into the putative effectors using other isolates and hosts might still show that they have a function in pathogenicity and/or race specificity. It is also possible that some effectors were once pathogenicity genes that determined the host range of the pathogen; the function of some of these effectors could be gratuitous or even detrimental, but their structure might allow rapid adaptive selection for pathogenic function (Gabriel, 1999).

OTHER VIRULENCE AND PATHOGENICITY FACTORS

The surface structure and appendages of phytopathogenic bacteria are important for the attachment, colonization and infection of the host. Type IV pili may contribute to bacterial pathogenesis by affecting surface motility, microcolony and biofilm formation, adhesion, immune evasion and cell signalling (Craig *et al*., 2004). The comparison of genomes from isolates ATCC33913 and 8004 showed that at least 26 genes related to the pili assembly are highly conserved, and mutations in two of the assembly genes (*pilB* and *pilC*) have reduced virulence (Qian *et al*., 2005).

Many phytopathogenic bacteria produce a large number of factors that might be essential or contribute to cause disease. The bacteria from the genus *Xanthomonas* typically produce yellow, membrane-bound pigments, called xanthomonadins. These pigments have a role in the maintenance of the ecological fitness of the bacteria, protecting the cells against photooxidative stress. Xanthomonadin biosynthesis is encoded by the *pig* cluster of genes. Poplawsky and Chun (1998) have shown that the mutation of *pigB* results in a reduction in epiphytic survival and decrease in infection via the hydathodes. The biosynthesis of xanthomonadin is regulated by 3-hydroxybenzoic acid, a diffusible factor (DF) that is associated with a range of biological functions; DF-deficient mutants are nonpigmented, impaired in survival ability and less virulent (He *et al*., 2011).

Xcc produces a range of extracellular enzymes (including proteases, pectinases and endoglucanase). The extracellular enzymes are capable of degrading the plant cell components and may be required to overcome plant defence responses, to allow bacteria to move into uncolonized plant tissues and to mobilize plant polymers for nutritional purposes (Torres *et al*., 2007).

In *Xcc*, the synthesis of extracellular degrading enzymes and exopolysaccharides is regulated by products of the *rpf* genes (rpfABFCHGDIE) (da Silva *et al*., 2002). The *rpf* gene cluster of *Xcc* regulates the genes involved in the synthesis of extracellular hydrolytic enzymes and extracellular polysaccharides (EPSs) (including xanthan) and plays a role in motility, toxin production, oxidative stress resistance, aerobic respiration and biofilm formation (Dow *et al*., 2003; Wilson *et al*., 1998). A diffusible signal factor (DSF) responsible for cell–cell signalling is involved in the transcriptional control of these genes (Barber *et al*., 1997). Some genes under the control of the rpf/DSF system are required for the first stages of endophytic colonization (Gudesblat *et al*., 2009). Genes that encode proteins involved in the synthesis or degradation of cyclic-di-guanosine-3′,5′-monophosphate (cyclic-di-GMP), a second messenger involved in the regulation of cellular functions, have been shown to play significant roles in virulence to plants. Complex regulatory networks possibly allow the bacteria to adapt to different environments and to modulate virulence factors (Ryan *et al*., 2007). The expression levels of proteases and endoglucanases are reduced when the *rpfI* gene is inactivated in *Xcc*, suggesting that *rpfI* may have a function in the extensive tissue degeneration that is characteristic of black rot (Dow *et al*., 2000). RpfF is involved in the synthesis of DSF, and a two-component system comprising RpfG and the complex sensor kinase RpfC is involved in sensing and responding to DSF (Ryan *et al*., 2010). Mutants deficient in RpfG, a phosphodiesterase that degrades cyclic-di-GMP, or the elevation of cyclic-di-GMP via the overexpression of a GGDEF domain protein WspR, caused the

aggregation of cells, reduction in mobility and decrease in production of virulence factors, extracellular enzymes and exopolysaccharides (Hsiao *et al*., 2011). Mutations of *rpfF* or *rpfC* also lead to a reduction in the synthesis of virulence factors. The cell–cell interactions are highly regulated and dynamic; the interactions involving HD-GYP and GGDEF domain proteins are dependent on DSF signalling, influence the localized expression of cyclic-di-GMP and mediate virulence (Ryan *et al*., 2007).

The Mips (macrophage infectivity potentiators) genes encode proteins reported as virulence factors in human pathogens. Zang *et al*. (2007) showed that a *mip*-like gene of *Xcc* is involved in pathogenicity through an effect on the production of exopolysaccharides and on the activity of extracellular proteases.

EPSs play an important role in the pathogenicity and virulence of many bacteria, both in terms of direct interactions with host cells and in conferring protection against hostile environments (Coplin and Cook, 1990). The most important EPS secreted by *Xcc* is xanthan gum, an EPS that has many industrial applications. Xanthan is a complex EPS with a cellulosic backbone and trisaccharide side chains of two mannose and one glucuronate residues that are attached to every second glucose in the backbone (Becker *et al*., 1998; Jansson *et al*., 1975; Vorhöelter *et al*., 2008; Yun *et al*., 2006). Yun *et al*. (2006) have shown that a xanthan minus mutant strain and a mutant strain that produces truncated xanthan fail to cause disease in *A. thaliana* and *Nicotiana benthamiana*, and induce the deposition of callose in these plants; pretreatment of the plant with xanthan restores the pathogenicity of both strains. The results indicate that xanthan induces susceptibility to *Xcc* by suppressing callose deposition. The bacterial cyclic β-(1,2)-glucan also has an effect on plant defence suppression and callose deposition, but, unlike xanthan, which acts locally, cyclic glucan acts systemically as part of a counter-defensive strategy that might facilitate the spread of the pathogen in the plants (Rigano *et al*., 2007).

Xanthan has a broad range of applications in food and nonfood products from the oil, pharmaceutical, cosmetic, paper, paint and textile industries, and is mainly used as a thickening, stabilizing, gelling and emulsifying agent (Becker *et al*., 1998). The biosynthesis of xanthan production and the genes that encode for the enzymes involved have been studied (Ielpi *et al*., 1993; Becker *et al*., 1990). Xanthan synthesis is encoded by twelve *gum* genes (*gumBCDEFGHIJKLM*), which are located in a single gene cluster of 12 kb that is mainly expressed as an operon from a promoter upstream of the first gene, *gumB* (Vojnov *et al*., 2001). The genes required for the synthesis of xanthan and its nucleotide sugar precursors are highly conserved among the three sequenced strains (ATCC33913, 8004 and B100), but are significantly different when compared with the other sequenced *Xanthomonas*; a model for the biosynthesis of xanthan based on the genome annotation has been proposed (Vorhöelter *et al*., 2008). The disruption of the *rmlA*, *xanA* and *gumK* genes, which are responsible for the biosynthesis of intermediates for xanthan production, can lead to a loss of pathogenicity (Qian *et al*., 2005).

The lipopolysaccharide (LPS) is an essential component of the outer membrane of Gram-negative bacteria, and is an important virulence factor in *Xanthomonas* (Dow *et al*., 1995; He *et al*., 2007; Patil *et al*., 2007). Over 20 genes for LPS synthesis have been characterized in *Xcc*, including *xanAB*, *rmlABCB*, *rfaXY*, *lpsIJ* and 15 genes that constitute the *wxc* cluster (Vorholter *et al*., 2001). The *wxc* gene cluster is involved in the synthesis of the LPS O-antigen, the most variable part of LPS. The *wxc* genes are highly divergent between *Xcc* strains, indicating that different isolates might produce varied LPSs (He *et al*., 2007). The LPS gene cluster of *Xcc* B100 is significantly different from those of 8004 and ATCC33913, but is almost identical to the LPS cluster of *X. campestris* pv. *raphani* 756C. This could indicate that the LPS cluster of B100 was acquired recently via horizontal gene transfer from *X. campestris* pv. *raphani* 756C or, more likely, that the *X. campestris* pv. *raphani* strain diverged recently from an *Xcc* with a B100-type LPS locus (Patil *et al*., 2007). Qian *et al*. (2005) showed that the disruption of a number of *wxc* genes resulted in a significant reduction in virulence.

FUTURE PERSPECTIVES

Research into *Xcc* and closely related pathovars has now reached the genomic age, although it still lags behind the progress made from the investigation of *Pseudomonas* pathogens, such as *P. syringae* pv. *tomato* and *maculicola*. Our understanding of *Xcc* is increasing rapidly through functional and comparative genomic studies, and we are starting to understand the role of some of the key genes involved in pathogenicity. Nevertheless, there are still many areas that require further work, including the study of the mode of entry of the pathogen, such as comparisons between the vascular pathogen *Xcc*, which generally penetrates the host via the hydathodes, and the nonvascular pathogen *X. campestris* pv. *raphani*, which generally penetrates the host through stomata. The effect of the environment and genetic factors in determining the preferred mode of entry of these pathogens is still under-studied. The importance of epiphytic survival and factors that contribute to it are also not well understood.

Recent studies have shown the role of certain effector proteins, but most have still not been characterized and their molecular targets and function are still unknown. Most of the effector/avirulence genes in the postulated gene-for-gene model have not yet been identified molecularly.

With the advances in sequencing, additional sequences of different *Xcc* isolates, including multiple isolates from each race and additional sequences of isolates of closely related pathovars, should soon be available. The comparison of multiple sequences should then provide more clues on the important genes that determine pathogenicity, including effectors; the presence and

absence of particular genes and the variability between isolates will provide clues on the role and evolution of these genes. Functional analysis of the genes can then confirm their role in the plant–pathogen interactions.

On the host side, a number of disease resistance genes in brassicas and *A. thaliana* have been postulated, some have been mapped, but none have been cloned. The effort to identify more disease resistance genes should be continued, and some of the most important crops could be improved with the incorporation of disease resistance genes from closely related species via interspecific crosses and embryo rescue, or via transformation. It is also possible that the engineering of the promoters of known R genes to recognize multiple effectors could contribute to the development of broad-spectrum disease-resistant crops (Ryan *et al*., 2011). The control of the disease could be improved by pyramiding disease resistance genes and/or by using multiple lines with different gene combinations that could be mixed and balanced to match the avirulence genes present in the bacterial population (Pink and Puddephat, 1999).

The application of functional genomics and proteomics to bacteria *in planta* to identify virulence factors, and the application of functional genomics and proteomics to both resistant and susceptible host plants inoculated with *Xcc*, will provide key information on the interaction between the bacteria and the hosts. Research on the diversity of *Xcc*, pathogenicity factors and evolution, together with host–pathogen interaction studies, should lead to improvements in the prevention and control of the black rot of crucifers.

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