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Short communication

Optimization of pathogenicity assays to study the *Arabidopsis thaliana–Xanthomonas campestris* pv. *campestris* pathosystem

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SUMMARY

The cruciferous weed *Arabidopsis thaliana* and the causal agent of black rot disease of Crucifers *Xanthomonas campestris* pv. *campestris* (*Xcc*) are both model organisms in plant pathology. Their interaction has been studied successfully in the past, but these investigations suffered from high variability. In the present study, we describe an improved Arabidopsis–*Xcc* pathosystem that is based on a wound inoculation procedure. We show that after wound inoculation, *Xcc* colonizes the vascular system of Arabidopsis leaves and causes typical black rot symptoms in a compatible interaction, while in an incompatible interaction bacterial multiplication is inhibited. The highly synchronous and reproducible symptom expression allowed the development of a disease scoring scheme that enabled us to analyse the effects of mutations in individual genes on plant resistance or on bacterial virulence in a simple and precise manner. This optimized Arabidopsis–*Xcc* pathosystem will be a robust tool for further genetic and post-genomic investigation of fundamental questions in plant pathology.

Arabidopsis thaliana has become the model plant in the past 15 years, and the number of tools available for it imposes its use for the study of fundamental questions in plant biology (Somerville and Meyerowitz, 2004). Numerous studies of plant–pathogen interactions have used this model crucifer and have provided considerable information. Although pathosystems of Arabidopsis with a large number of viral, bacterial and fungal plant pathogenic micro-organisms have been developed, the Arabidopsis–*Pseudomonas syringae* (*P. syringae*) system occupies an outstanding position, both because of the large number of groups using it and because of the quantity of exciting insights into fundamental aspects of plant–pathogen interaction it has provided (Katagiri *et al.*, 2002; Quirino and Bent, 2003). By contrast, our knowledge about the interaction between Arabidopsis and *Xanthomonas campestris* pv. *campestris* (*Xcc*), which was described even before the Arabidopsis–*P. syringae* system (Simpson and Johnson, 1990), is still rudimentary (Buell, 2002). Many Xanthomonads including *Xcc* have, however, been intensively studied and *Xcc* is a natural pathogen of cruciferous plants and provokes considerable economical loss by causing black rot diseases (Alvarez, 2000; Onsando, 1992; Williams, 1980). Moreover, the genome sequence of three different *Xanthomonas* species/strains and two closely related *Xylella fastidiosa* strains are publicly available, which makes *Xanthomonas* an interesting plant pathogen for comparative genomics (da Silva *et al.*, 2002; Simpson *et al.*, 2000; Vorholter *et al.*, 2003). In order to gain a better understanding of plant–pathogen interactions and to undertake post-genomic approaches, it is desirable to work with model species and therefore we made an effort to improve existing, and to develop new, Arabidopsis–*Xanthomonas* pathosystems.

Our group has been working on the Arabidopsis–*Xcc* pathosystem for several years. We isolated plant mutants impaired in resistance to *Xcc* and identified genes involved in the establishment of hypersensitive response (HR) and defence (Godard *et al.*, 2000; Lacomme and Roby, 1999; Lummerzheim *et al.*, 1993). Nevertheless, our previous work with the Arabidopsis–*Xcc* pathosystem suffered from the fact that only qualitative changes—i.e. from resistance to susceptibility or from virulence to avirulence—could be detected. Inoculation was mainly performed by syringe infiltration of a restricted zone of fully expanded leaves (one-third of the half leaf) of 4-week-old plants with a bacterial suspension of 10^8 cfu/mL (Lummerzheim *et al.*, 1993). Plants were grown for this purpose on Jiffy pots in a growth chamber at 22 °C, with a 9-h light period and a light intensity of 192 μmol/m^2/s. After inoculation, plants were covered and kept at nearly 100% relative humidity for 2 days and were subsequently transferred to 70% relative humidity. Plants were scored as resistant if an HR of the infiltrated zone limited bacterial development, or susceptible if chlorosis spreading out of the infiltration zone invaded the entire

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leaf, 3–5 days post-inoculation (dpi). Problems associated with this method (hereafter method 1) are as follows.

1 The outcome of the interaction depends on the physiological state of the plants at the moment of inoculation, and on the environmental conditions after the inoculation: disease will only be observed if conditions, mainly hygrometry, but also temperature, are favourable for the pathogen. Resistance, by contrast, will only be observed if the conditions are not too favourable for the pathogen.

2 We can only score qualitatively (resistance or susceptibility) because of the phenotype observed and because of the variability observed within a given experiment.

Work on the interaction of *Xcc* with its natural hosts (e.g. *Brassica oleracea* and *Brassica napus*) has shown that it is a vascular pathogen (Alvarez, 2000). *Xcc* enters the leaf mainly via hydathodes at the leaf margin and subsequently colonizes the xylem vessels. In the mesophyll it is found only at the latest stages of the infection process (Bretschneider *et al.*, 1989). Accordingly, stomatal infiltration is not considered to be the natural route of infection, and when introduced into leaf mesophyll, *Xcc* induced nonspecific symptoms on *Brassica* species instead of eliciting typical black rot symptoms (Shaw and Kado, 1988). We therefore speculated that the problems with method 1—in which bacteria are infiltrated into the leaf mesophyll—are due to the infiltration technique and that tissue-specific virulence functions of the bacteria and tissue-specific defence responses of the plant are important features in the Arabidopsis–*Xcc* pathosystem.

In order to test this hypothesis and to develop an inoculation procedure that gives highly reproducible results and allows their quantitative analysis, we compared two different techniques on plants grown as described above: leaf infiltration (method 2), in which one half of a fully expanded leaf was infiltrated with a low bacterial inoculum (5×10^5 cfu/mL); and wound inoculation (method 3), in which the central vein was pierced three times with a needle that had been dipped in a bacterial suspension (10^7–10^9 cfu/mL). After inoculation, plants were kept in both cases for 1 day at 100% relative humidity and were then transferred to 65% relative humidity. Method 2 is commonly used in the study of Arabidopsis–*P. syringae* interaction (Katagiri *et al.*, 2002), whereas method 3 is largely employed for the study of the interaction of *Xcc* with *Brassica* species (Shaw and Kado, 1988; Vicente *et al.*, 2001). The tests were done with two different *Xcc* strains, which largely gave the same results: strain 147 (*Xcc*147), which is commonly used in our group, and strain LMG568/ATCC33913 (*Xcc*568), the genome of which has been sequenced (da Silva *et al.*, 2002).

When using the leaf infiltration test (method 2) with *Xcc*147 and *Xcc*568, the inoculated leaf zone became chlorotic 3 dpi and necrotic 5 dpi on diverse ecotypes of Arabidopsis (Fig. 1A,B for Col-0 and Sf-2, other ecotype results not shown). Leaves infiltrated with a mutant strain of *Xcc*568, carrying an insertion in the *hrpG* regulatory gene (XCC1166), remained symptomless (Fig. 1B, details of the generation of the mutant can be obtained from the authors upon request). Although we found slight differences in the timing of symptom development between the different ecotypes, no qualitative differences could be observed. Notably, Col-0 and Sf-2, behaved identically. This is different from the results we obtained with the previous infiltration technique, method 1, in which Col-0 was resistant and Sf-2 was susceptible (Lummerzheim *et al.*, 1993), and which allows the observation of

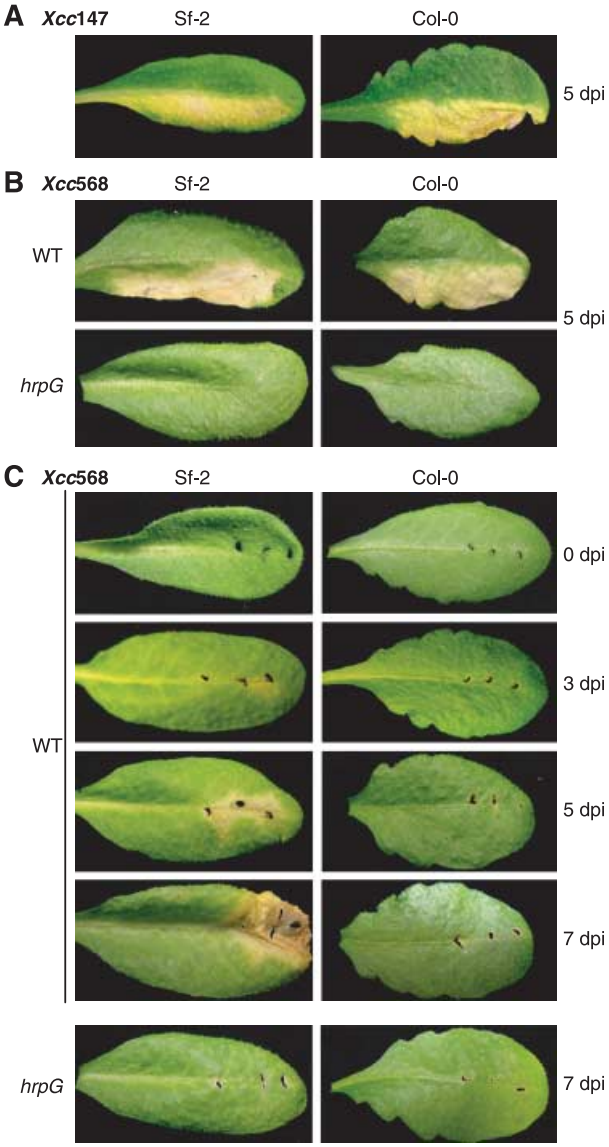

Fig. 1 Symptoms on *Arabidopsis thaliana* ecotypes Sf-2 and Col-0 after leaf infiltration (method 2) and wound inoculation (method 3) with *Xcc* strains. Leaf infiltration with (A) *Xcc*147 and with (B) *Xcc*568 wild-type (wt) or *hrpG* mutant bacteria (*hrpG*) was done by using a bacterial suspension adjusted to 5×10^5 cfu/mL. (C) Wound inoculation was performed with *Xcc*568 wild-type (wt) or *hrpG* mutant bacteria (*hrpG*) adjusted to 1×10^7 cfu/mL.

pathogen spreading from the inoculation zone in the compatible interaction (not observed in the incompatible interaction).

In wound inoculation tests (method 3) with *Xcc*568 (Fig. 1C) and *Xcc*147 (data not shown), we observed spreading chlorosis on ecotype Sf-2 that started 3 dpi with weak chlorosis around the wound site, developed 5 dpi into V-shaped chlorotic sectors of varying size that occasionally invaded the entire leaf, and became necrotic 7 dpi. These symptoms are reminiscent of *Xcc* disease symptoms observed on cabbage and other *Xcc* hosts belonging to the Brassicaceae (Shaw and Kado, 1988; Vicente *et al.*, 2001) and are in accordance with the previous classification of Sf-2 as susceptible (Lummerzheim *et al.*, 1993). The ecotype Col-0, which we previously classified as resistant, showed no symptoms or, in rare cases, very weak symptoms. Both ecotypes showed no symptom development when inoculated with the *Xcc*568 *hrpG* mutant strain.

In order to correlate symptom development with bacterial multiplication in the plant, we determined *in planta* bacterial growth. After leaf infiltration with method 2, we found strong bacterial growth of *Xcc* strains 147 and 568 on both Col-0 and Sf-2 plants (Fig. 2A,B). This is in accordance with the symptoms we observed but it is again contrary to the results we obtained previously, where we found restriction of *Xcc*147 growth on Col-0 (Lummerzheim *et al.*, 1993). The *Xcc*568 *hrpG* mutant strain did not show any *in planta* growth. After wound inoculation (method 3), we only observed bacterial growth in the interactions of the wild-type strains with Sf-2 and not with Col-0 or the interactions with the *hrpG* mutant strain (Fig. 2C for *Xcc*568, data not shown for *Xcc*147). Multiplication in Sf-2 depended to a large extent on the initial inoculum. When a bacterial density of 10^9 cfu/mL was used, an overall multiplication of 20–50-fold was observed; when a suspension of 10^7 cfu/mL was used, we found pathogen multiplication of more than 1000-fold. These results demonstrate that disease symptom development is truly correlated with bacterial multiplication and not due to a difference in tolerance between Sf-2 and Col-0. Moreover, it confirms that Col-0 is resistant against *Xcc* strains 147 and 568.

Our tests showed that the outcome of the Arabidopsis–*Xcc* interaction greatly depends on the inoculation procedure. This supported our hypothesis that different plant tissues are invaded after the different inoculation procedures and that the outcome of the rendezvous between plant and bacteria is determined by the plant tissue-specific responses and by the equipment of *Xcc* for tissue colonization. When *Xcc* is infiltrated in the mesophyll, defence mechanisms that restrict the colonization of the vascular system and that are relevant in a natural context may be less operational or inefficient in restricting bacterial colonization. This is perhaps why we found strong bacterial growth of *Xcc* after leaf infiltration with method 2, whichever ecotype of Arabidopsis was used.

In order to analyse the spatial and tissue-specific aspects of the Arabidopsis–*Xcc* interaction and to verify whether there is indeed vascular colonization after wound inoculation (method 3) and mesophyll colonization after leaf infiltration (method 2), we generated by transposon tagging an *Xcc*568 reporter strain, carrying the LUX operon of *Photorhabdus luminescens* (Winson *et al.*, 1998) and emitting constitutively high luminescence. This strain showed the same growth characteristics and the same

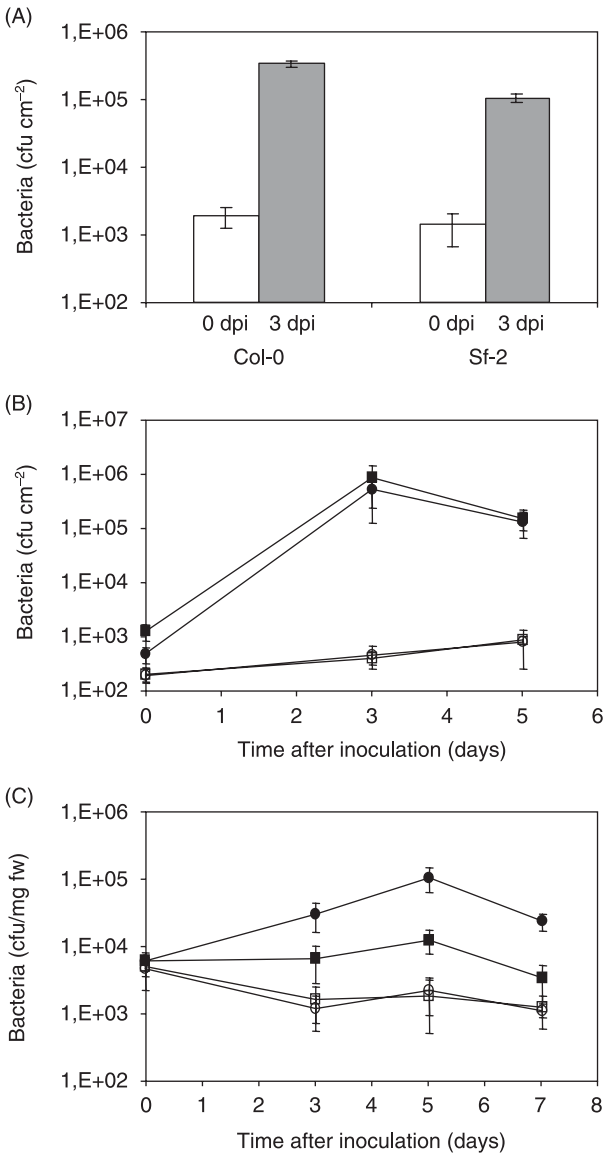

Fig. 2 *In planta* bacterial growth of *Xcc* strains after infiltration (method 2) and wound inoculation (method 3). (A) Infiltration of Sf-2 and Col-0 leaves with *Xcc*147 was done at 5×10^5 cfu/mL. (B) Infiltration of Sf-2 (circles) and Col-0 leaves (squares) with *Xcc*568 wild-type (solid) or *Xcc*568 *hrpG* mutant bacteria (open) was done at 5×10^5 cfu/mL. Bacterial numbers were determined as described by Katagiri *et al.* (2002). (C) Wound inoculation with *Xcc*568 was done at 10^9 cfu/mL. At the indicated time points, three entire inoculated leaves per plant were harvested, weighted, ground and taken up in water. Bacterial populations were determined by dilution plating. Each data point represents the mean and the standard deviation calculated from four replicates. The experiments were repeated at least twice with equivalent results.

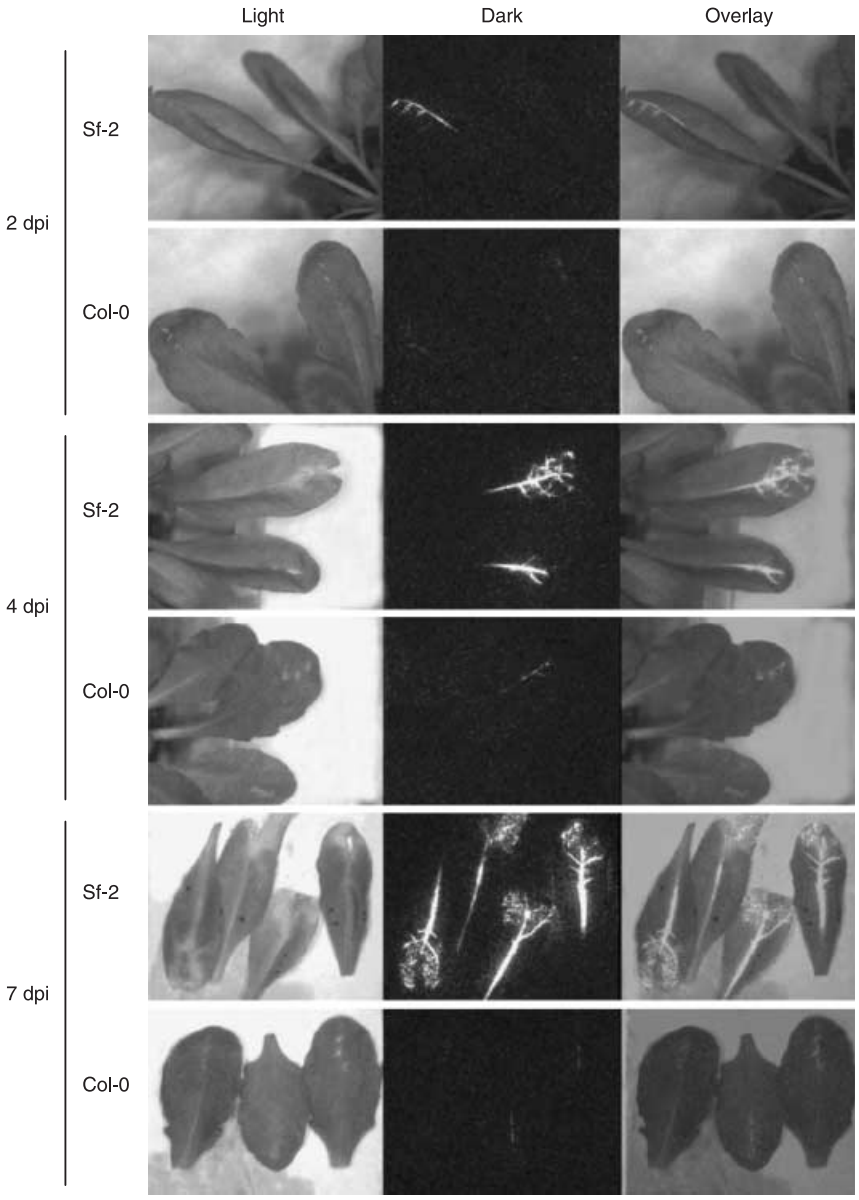

Fig. 3 Luminescence imaging illustrates spatial aspects of the Arabidopsis–*Xcc* interaction. Arabidopsis leaves were inoculated by wound inoculation with an *Xcc*568 reporter strain (10^7 cfu/mL) that carries the *Photorhabdus luminescens* lux operon and emits constitutively high luminescence. Pictures were taken with a CCD camera under dark conditions (exposure time 10 s) and under light conditions at the indicated times after inoculation and an overlay of the images was generated.

virulence as the wild-type strain. By using this reporter strain and a CCD-video camera (Photonic Science, Robertsbridge, UK), we could follow the infection process in individual leaves in a noninvasive and very sensitive manner (Fig. 3). Until 36 h after wound inoculation, leaves showed no luminescence. At 2 dpi, luminescence along the midvein and secondary veins could be observed in Sf-2 leaves. Thereafter, the luminescence became increasingly intensive and progressively invaded the leaf vascular system. Luminescence was present approximately 2 days before symptom development, and when chlorosis became apparent, strong luminescence was also found outside the chlorotic region. Within the chlorotic region, luminescence became rapidly more diffuse and invaded nonvascular tissue. Uninfected leaves never showed luminescence, indicating that we do not get systemic infection of the plant, which is in accordance with the observation that noninoculated leaves never show *Xcc* disease symptoms. In Col-0 plants, we detected no or only very weak luminescence, which remained restricted to the vascular system close to the wound site. After leaf infiltration, we also observed strong luminescence, which was proceeding and accompanying leaf chlorosis in Sf-2 and Col-0, but in this case the luminescence was equally distributed over the entire leaf blade and did not give any indication of tissue specificity of *Xcc* multiplication or differences in susceptibility/resistance between Col-0 and Sf-2 (data not shown).

Together, these results suggest that Col-0 is resistant against *Xcc* strains 147 and 568 when bacteria are directly introduced into the vascular system by wound inoculation (method 3), whereas Sf-2 is susceptible. The same differential response was found

previously when we used localized high-titre inoculum infiltration (method1) but could not be reproduced when bacteria were infiltrated with low-titre inoculum into the mesophyll (method 2). Wound inoculation proved, in addition, to be much more robust and clear cut than our former inoculation procedure (method 1). Our conclusion is that leaf infiltration, commonly used for *P. syringae*, is not well suited to study Arabidopsis–*Xcc* interactions because it does not take into account the tissue specificity of *Xcc* infection. It does not allow us to study the vascular tissue-specific resistance responses on the plant side and virulence mechanisms allowing xylem colonization on the bacterial side. Similar conclusions on *Xcc* pathosystems can be found in the literature, where the tissue specificity of *Xcc* in natural infections has been well documented (Alvarez, 2000) and the importance of appropriate inoculation procedures for distinguishing between compatible and incompatible interactions has been highlighted (Shaw and Kado, 1988; Simpson and Johnson, 1990). The fact that our wound inoculation procedure closely reflects aspects of a natural infection process is further reinforced by the following observation: in a spray-inoculation procedure, Col-0 was found to be resistant—leaves remained symptomless—whereas Sf-2 showed the development of typical V-shaped chlorosis and vein darkening (Lummerzheim *et al.*, 1993). This was found to be associated with extensive multiplication of *Xcc* in the vascular system of Sf-2 (data not shown). Thus, our system allows us to study ecotype-specific responses that seem to operate in the vascular system of the plant and virulence functions of the bacteria, contributing to their capability to colonize leaf xylem.

The highly reproducible and synchronous symptom development after wound inoculation (method 3) prompted us to develop a symptom annotation procedure, which is based on an individual score for each inoculated leaf. It allows us to analyse accurately the effects of mutations in individual genes in the plant or the bacteria on resistance or virulence, because we can easily and precisely quantify decreases or increases in resistance and virulence; this is illustrated in Fig. 4 with two examples. A mutation in the Arabidopsis *HXC2* gene leads to partial loss of resistance against *Xcc* strains 147 (Godard *et al.*, 2000) and 568. In accordance with this, the disease score that we find for *hxc2* is much higher than that of the wild-type Col-0, which is almost 0, but significantly lower than that of the fully susceptible ecotype Sf-2 (Fig. 4A). A mutation in the *Xcc rpfC* gene (XCC1856) leads to a loss of production of exopolysaccharides, reduced secretion of hydrolytic enzymes and reduced *Xcc* virulence (Slater *et al.*, 2000; Tang *et al.*, 1991). As expected, an *rpfC* mutant shows a significantly reduced disease score on Sf-2 plants as compared with the wild-type strain. Nevertheless, we do not find complete loss of pathogenicity as with an *hrpG* mutant strain, for which the disease score is 0.

In addition to the analysis of the genetic bases of resistance of Col-0 to *Xcc*147 and *Xcc*568 and the identification of the corresponding plant resistance and bacterial avirulence genes, this

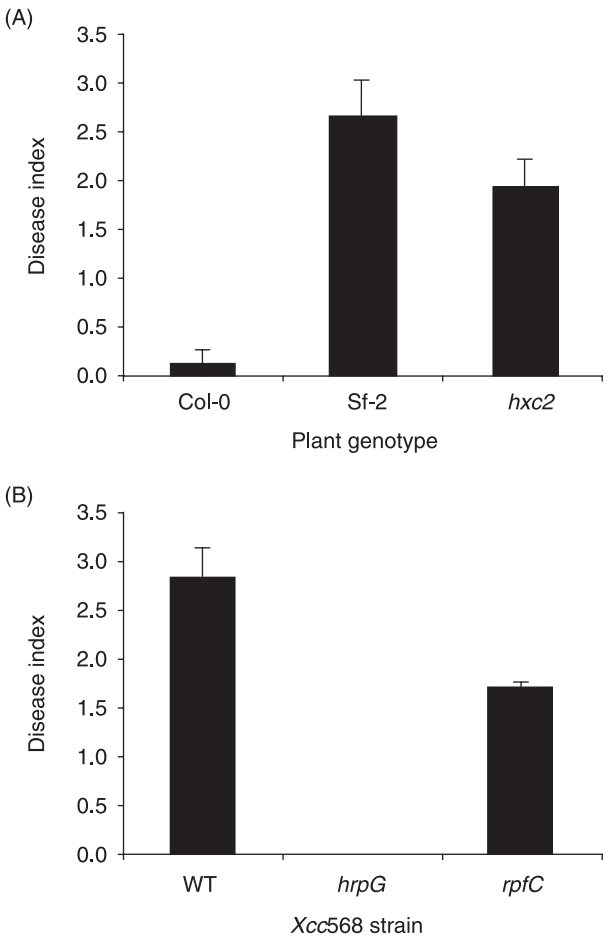

Fig. 4 Quantitative analysis of the interaction phenotype of plant and bacterial mutants by disease scoring. Disease symptoms were scored 7 days after wound inoculation of (A) Col-0, Sf-2 and *hxc2* mutant plants with *Xcc*568 wild-type bacteria and of (B) Sf-2 plants with *Xcc*568 wild-type, *rpfC* mutant and *hrpG* mutant bacteria. Each inoculated leaf was individually scored as: no symptoms = 0; weak chlorosis surrounding the wound sites = 1; strong V-shaped chlorosis = 2; developing necrosis = 3; leaf death = 4. The represented average disease scores and the standard deviations were calculated from the values of four plants with four inoculated leaves per plant.

new inoculation procedure (method 3) opens the way to address many exciting questions: is the Arabidopsis resistance response to *Xcc* restricted to the vascular system, what is the specificity of this resistance response and which aspects are shared in common with the resistance response to other pathogens, vascular or non-vascular? As a complement to this, it will be interesting to analyse precisely to what extent signal transduction elements, known to be implicated in other Arabidopsis resistance responses, are involved in *Xcc* resistance. Last but not least, further characterization of the *hxc* mutants that show attenuated resistance to *Xcc* (Godard *et al.*, 2000; Lummerzheim *et al.*, 2004) will benefit from the improvements of this pathosystem. It is particularly interesting that within the species *Xanthomonas*

campestris, the different pathovars *raphani* (*Xcr*), *armoraciae* (*Xca*) and *campestris* have all been described to infect Arabidopsis but with different tissue specificities (Hugouvieux *et al.*, 1998). Whereas *Xcc* is a vascular pathogen, *Xcr* and *Xca* colonize the mesophyll and in Arabidopsis, as in their natural hosts, cause leaf spot diseases. A comparative study of those interactions will be particularly interesting and promises to provide considerable information on the tissue specificity of bacterial virulence functions and plant resistance responses.

Finally, the availability of the genome sequences of different *Xcc* strains and of three strains from other *Xanthomonas* species or closely related *Xylella* species opens the way to the systematic analysis of virulence functions in the *Xcc* genome. In this context, the Arabidopsis–*Xcc* pathosystem will be particularly valuable because it permits us to search relatively easily for plant targets of bacterial virulence factors and to analyse them by molecular, genetic and cell biological approaches. This can only be done in a model plant and not on the natural hosts of *Xcc* or other *Xanthomonas* species.

To conclude, we believe that the optimized Arabidopsis–*Xcc* pathosystem will be a powerful tool for postgenomic approaches intended to investigate fundamental aspects of plant–pathogen interactions.

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