MOLECULAR PLANT PATHOLOGY (2008) **9**(5), 609–631 DOI: 10.1111/J.1364-3703.2008.00486.X

Transcriptional analysis of the sweet orange interaction with the citrus canker pathogens *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas axonopodis* pv. *aurantifolii*

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SUMMARY

Xanthomonas axonopodis pv. *citri* (Xac) and *Xanthomonas axonopodis* pv. *aurantifolii* pathotype C (Xaa) are responsible for citrus canker disease; however, while Xac causes canker on all citrus varieties, Xaa is restricted to Mexican lime, and in sweet oranges it triggers a defence response. To gain insights into the differential pathogenicity exhibited by Xac and Xaa and to survey the early molecular events leading to canker development, a detailed transcriptional analysis of sweet orange plants infected with the pathogens was performed. Using differential display, suppressed subtractive hybridization and microarrays, we identified changes in transcript levels in approximately 2.0% of the ~32 000 citrus genes examined. Genes with altered expression in response to Xac/Xaa surveyed at 6 and 48 h post-infection (hpi) were associated with cell-wall modifications, cell division and expansion, vesicle trafficking, disease resistance, carbon and nitrogen metabolism, and responses to hormones auxin, gibberellin and ethylene. Most of the genes that were commonly modulated by Xac and Xaa were associated with basal defences triggered by pathogen-associated molecular patterns, including those involved in reactive oxygen species production and lignification. Significantly, we detected clear changes in the transcriptional profiles of defence, cell-wall, vesicle trafficking and cell growth-related genes in Xac-infected leaves between 6 and 48 hpi. This is consistent with the notion that Xac suppresses host defences early during infection and simultaneously changes the physiological status of the host cells, reprogramming them for division and growth. Notably, brefeldin A, an inhibitor of vesicle trafficking, retarded canker development. In contrast, Xaa triggered a mitogen-activated protein kinase signalling pathway involving WRKY and ethylene-responsive transcriptional factors known to activate downstream defence genes.

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INTRODUCTION

Xanthomonas axonopodis pv. *citri* (Xac) and *Xanthomonas axonopodis* pv. *aurantifolii* (Xaa) are bacterial pathogens responsible for citrus canker, a disease that seriously affects commercial citrus varieties worldwide (Shubert *et al*., 2003). Xac causes the most damaging 'Asiatic' or type A canker on a wide range of citrus species, including sweet oranges (*Citrus sinensis*), grapefruits (*Citrus paradisi*) and lemons (*Citrus limon*). Xaa, by contrast, has a narrower range of citrus hosts and is responsible for the B and C types of canker, which are restricted to some citrus-producing areas in South America (Shubert *et al*., 2003). The C strains of Xaa are exclusively found in Brazil and are limited to Mexican lime (*Citrus aurantifolia* Swingle) cultivars (Brunings and Gabriel, 2003; Coletta-Filho *et al*., 2006; Shubert *et al*., 2003). In addition, pathotype C strains induce a hypersensitive response (HR) in various citrus species including sweet oranges (Brunings and Gabriel, 2003).

The disease symptoms caused by Xac and Xaa on susceptible plants are in general very similar and include the formation of water-soaked eruptions and pustule-like lesions on the surface of leaves, stems and fruits (Shubert *et al*., 2003). Some of these symptoms are attributed to the phenomenon of cell enlargement (hypertrophy) and division (hyperplasia) that occurs in the host tissues upon infection. This eventually leads to a rupture of the epidermis, which significantly favours pathogen spreading and disease dissemination (Brunings and Gabriel, 2003; Wichmann and Bergelson, 2004).

The molecular mechanism by which Xac and Xaa induce lesions and rupture of the host epidermis is not entirely clear; nevertheless, it has been shown that members of the PthA/AvrBs3 family of effector proteins are required to elicit cankers on citrus (Swarup *et al*., 1992). Significantly, transient expression of *pthA* was sufficient to promote hypertrophy and hyperplasia in host cells (Duan *et al*., 1999).

PthA/AvrBs3 proteins are delivered by the type III secretion (TTS) system into the host cells, where they suppress host defences (Fujikawa *et al*., 2006) and are targeted to the nucleus to modulate transcription of host genes (Marois *et al*., 2002). In the interaction between *Xanthomonas campestris* pv. *vesicatoria*

(Xcv) and pepper plants, AvrBs3 directly activates transcription of a basic helix–loop–helix factor that controls cell enlargement (Kay *et al.*, 2007), a typical response induced by Xcv that resembles the citrus hyperplastic canker. Interestingly, AvrBs3 also mediated promoter activation of its cognate resistance gene in the incompatible interaction (Römer *et al.*, 2007). In citrus plants, however, the early transcriptional changes that occur in response to Xac or Xaa infection remain unknown. Thus, to gain insights into the molecular mechanisms involved in Xac/Xaa-induced hypertrophy and hyperplasia, and to understand the basis of the resistance reaction observed in sweet orange infected with Xaa, a systematic analysis of gene expression in response to these pathogens was conducted. By means of differential display (DD) and suppressed subtractive hybridization (SSH) we identified and categorized 100 genes from *C. sinensis* implicated in canker development and disease resistance. Furthermore, with the recent availability of commercial citrus arrays, a more complete transcriptional profiling of orange leaves infected with the canker pathogens was performed and a clearer picture of transcript alterations emerged. These analyses not only revealed significant transcriptional changes associated with resistance and canker symptom development, but also pointed to a great overlap with gene expression patterns associated with both innate immunity and gene-for-gene resistance mechanisms described for other plant–pathogen interactions (Navarro *et al.*, 2004; Truman *et al.*, 2006). To our knowledge, this is the first description of a detailed global gene expression analysis on citrus plants in response to bacterial pathogens.

The transcriptional change profiling described here may be exploited for future development of citrus plants resistant to Xac.

RESULTS AND DISCUSSION

Differential pathogenicity of Xac and Xaa on citrus cultivars

While Xac causes cankers on sweet orange Pêra and Mexican lime cultivars, Xaa pathotype C produces hyperplastic lesions only in Mexican lime whereas in Pêra leaves it induces a type of resistance response that visually appears as a slight yellowing at the site of bacterial infiltration, 5–10 days post-infection (Fig. 1A). The resistance response observed in Pêra leaves upon

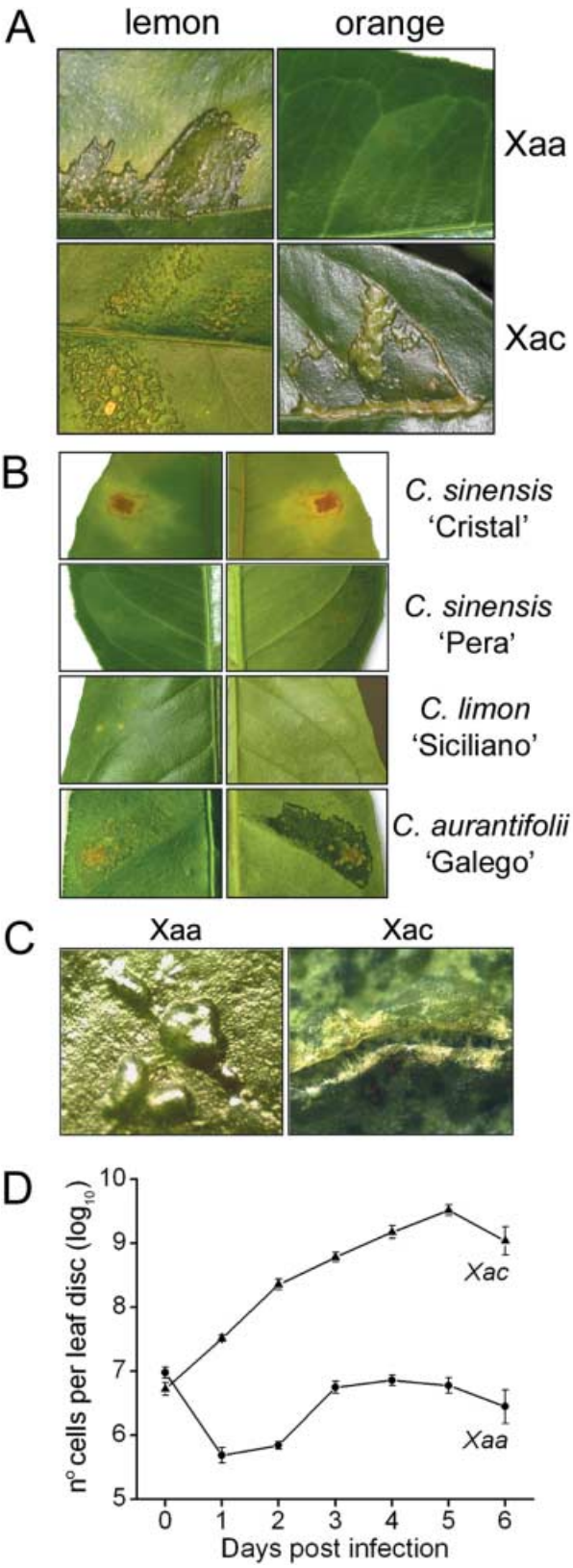

Fig. 1 Differential pathogenicity of Xac and Xaa on *Citrus* cultivars. (A) Leaves of sweet orange and Mexican lime infiltrated with suspensions of Xac or Xaa showing absence of water-soaked lesions or hyperplastic cankers in orange leaf infiltrated with Xaa. Pictures were taken 10 days after bacterial infiltration. (B) Typical HR induced by Xaa in the sweet orange 'Cristal' cultivar compared with the reaction observed in the Pêra or the *C. limon* 'Siciliano' cultivars. Canker symptoms induced by Xaa in Mexican lime 'Galego' are shown for comparison. Pictures were taken 8 days after bacterial infiltration. (C) Micropustules (100× magnification) induced by Xaa on sweet orange relative to the hyperplastic lesion with rupture of the epidermis caused by Xac. (D) Bacterial growth curves *in planta* showing absence of Xaa exponential growth on Pêra leaves.

Xaa infection does not appear to be a typical HR as we normally see with other sweet orange varieties (Fig. 1B), or to be what has been generally reported for an Xaa–sweet orange interaction (Brunings and Gabriel, 2003). For instance, tissue necrosis or cell death at the site of bacterial infiltration is not clearly observable. In contrast, close inspection of the Xaa-infiltrated leaf sectors remarkably revealed the presence of micropustules on one side of the epidermis, suggesting the initiation of hyperplastic canker (Fig. 1C). These micropustules appeared much later after Xaa infiltration and, as far as we could observe, they did not lead to rupture of the epidermis, as compared with the hyperplastic pustules induced by Xac, which typically develop within 5–10 days post-infection, under our experimental conditions (Fig. 1C).

To investigate this differential response further, we followed the growth of Xac and Xaa in Pêra leaves and we observed that while Xac grows vigorously after being infiltrated, Xaa does not grow exponentially; nevertheless, a stable number of cells remain alive for at least 6 days after bacterial infiltration (Fig. 1D). This pattern of *in planta* growth is more characteristic of a non-pathogenic invader. Thus, it appears that Xaa has either lost its ability to elicit full canker symptoms on Pêra plants or is evolving to become a pathogen to this cultivar. Hence, we found the Xaa–Pêra interaction an interesting pathosystem to study the citrus defence response at the molecular level, as the development of canker symptoms is aborted at some stage during infection, relative to the compatible Xac–Pêra interaction.

To gain insights into the molecular mechanisms involved in the differential pathogenicity of Xac and Xaa, we monitored the transcriptional changes in Pêra plants in response to these bacteria to identify the genes associated with the early events leading to canker and the resistance reaction, respectively. As Xac appears to induce cell division in citrus within 72 h of inoculation (Brunings and Gabriel, 2003), and the virulence effector PthA is capable of significantly suppressing a defence response as early as 12 h post-infection (hpi), as shown in tobacco (Fujikawa *et al.*, 2006), we analysed the transcriptional changes in sweet orange at 6 and 48 h after Xac/Xaa infiltration.

Identification and classification of orange genes modulated in response to Xac/Xaa infection

Early transcriptional changes that occur in orange leaves in response to Xac or Xaa infection were studied by three independent methods (DD, SSH and microarray) and the expression levels of a significant number of differentially expressed genes were verified by quantitative reverse-transcriptase PCR (qRT-PCR) and Northern blotting. Using DD and SSH, we identified 120 orange genes with altered expression between Xac- and Xaa-infiltrated leaves. The expression levels of 75% of these genes, measured by qRT-PCR at 48 hpi, confirmed their differential expression relative to water-infiltrated leaves (Supporting Information Table S1). BLAST searches revealed that most of these messages showed significant identities to known plant genes, which enabled us to categorize them by function (Table S1).

Using the *Citrus* genechip array from Affymetrix, the expression changes of approximately 32 000 citrus expressed sequence tags (ESTs) were analysed at 6 and 48 hpi (Supporting Information Tables S1–S9). Genes were considered as up- or down-regulated when their expression levels were equal or higher than a 3.0-fold increase/decrease relative to water-infiltrated leaves. However, in Xac versus Xaa comparisons, a $\geq$ 2.0-fold change cutoff was used to retain a substantial number of genes that were highly up- or down-regulated by the pathogens in respect to water but which showed fold changes lower than 3.0 in Xac versus Xaa analyses (compare fold changes between Xac and Xaa in Tables S8 and S9).

On average, the microarray analyses revealed significant changes in mRNA levels in approximately 2% of the probesets. The corresponding citrus genes were also functionally classified according to their similarity to genes of known function in plants and other organisms and good correlation was found between the gene categories identified by DD/SSH and microarray, as well as between the two microarray datasets of 6 and 48 hpi (Tables S1–S9). As expected, the microarray analyses identified a much greater number of differentially expressed genes but not as many additional functional classes, as compared with DD and SSH. Significantly, nearly half of the genes identified by DD/SSH found no matches to probesets represented in the citrus array, and those that perfectly matched probesets showed expression levels comparable with the levels determined by microarray analysis (Table S1). Thus, the three approaches were complementary and a high level of consistency was observed in the gene expression levels determined by microarray, qRT-PCR and Northern blotting.

Citrus genes with altered expression in response to Xac or Xaa infection were functionally classified into the following categories: cell-wall synthesis and remodelling, terpene and gibberellin (GA) synthesis, auxin signalling and mobilization, vesicle trafficking, disease resistance and defence, cell division and morphogenesis, ethylene synthesis and signalling, retroelement and transposition, carbohydrate and nitrogen metabolism, and putative transcriptional factors. A substantial number of genes were categorized as unknown/hypothetical or 'no hit' (Fig. 2 and Tables S1–S9).

Cell-wall remodelling and structural proteins

One of the major differences in the transcriptional profiling between Xac- and Xaa-infected leaves was observed in the cell-wall metabolism (Fig. 2). At 48 hpi, Xac strongly up-regulated several cell-wall-remodelling enzymes similar to endoglucanases, glycosylhydrolases, pectatelyases, pectinesterases and cell-wall proteins such as expansins, extensins and arabinogalactan proteins, known to be induced in tissues undergoing rapid expansion or during pericarp softening and fruit ripening (Li *et al.*, 2003;

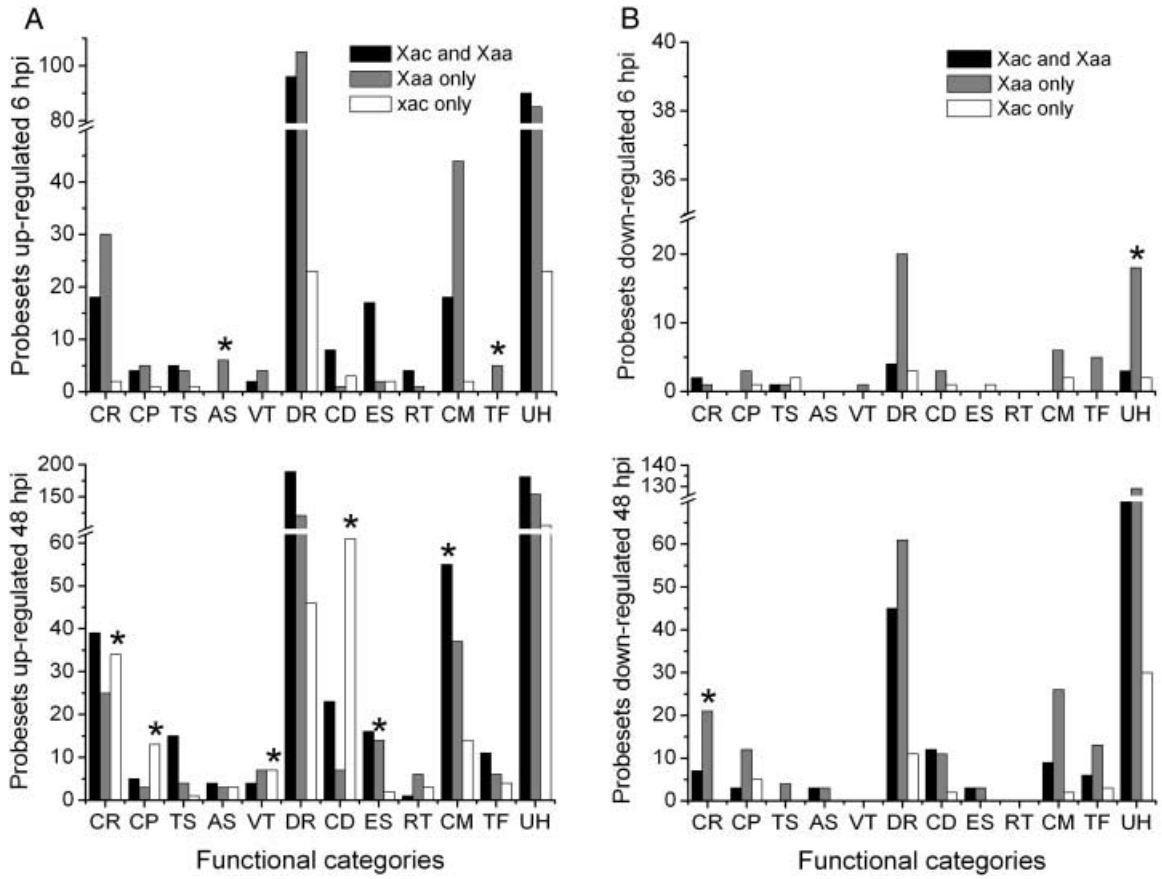

Fig. 2 Distribution and abundance of sweet orange genes modulated by Xac and Xaa in the different functional categories: cell-wall remodelling (CR), cell-wall proteins (CP), terpene and gibberellin synthesis (TS), auxin mobilization and signalling (AS), vesicle trafficking (VT), disease resistance and stress response (DR), cell division and morphogenesis (CD), ethylene synthesis and signalling (ES), retroelement and transposition (RT), carbohydrate and nitrogen metabolism (CM), transcriptional factors (TF) and unknown/hypothetical (UH). The graphics illustrate the number of probesets that were up- (A) or down- (B) regulated by the pathogens with a fold change equal or higher than 3, relative to water-infiltrated leaves. Major transcriptional changes between the 6- and 48-hpi treatments are indicated by asterisks.

Trainotti *et al.*, 1999). Xaa, by contrast, preferentially up-regulated a number of genes related to endoglucanase inhibitors and various *O*-methyltransferases, lacases, hydroxyproline-rich glycoproteins and enzymes of the phenylpropanoid biosynthetic pathway, most of which have been shown to play a central role in cell wall cross-linking and lignification (Ender *et al.*, 2002; Eudes *et al.*, 2006; Goujon *et al.*, 2003). Xaa also down-regulated various cell-wall-modifying and structural proteins whereas Xac down-regulated two proteins related to animal periaxin and cadherin, involved in cell adhesion (Straub *et al.*, 2003) (Table 1 and Supporting Information Tables S1–S4).

Some of these genes were also identified by DD and SSH and their expression levels measured by qRT-PCR are in agreement with the levels determined by microarray (Tables 1 and S1). In addition, Northern blot analysis confirmed the differential expression of some cell-wall-remodelling enzymes between Xac and Xaa treatments (Supporting Information Fig. S1).

The transcriptional changes associated with cell-wall metabolism were perceived earlier during infection, particularly after Xaa treatment (Fig. 2B). Increased expression of genes homologous to specific lignin biosynthesis enzymes including caffeic *O*-methyl transferases, cinnamate 4-hydroxilase and cinnamoyl CoA reductases (Eudes *et al.*, 2006; Goujon *et al.*, 2003; Kawasaki *et al.*, 2006) and cell-wall cross-linking proteins similar to pherophorins were detected at 6 h after Xaa infection (Table 1 and Supporting Information Tables S5–S7).

When we analysed the genes that were commonly regulated by the pathogens we noticed that Xac also induced some genes related to lignin biosynthesis, but at much lower levels than Xaa (Tables S8 and S9). In addition, among the cell-wall-remodelling genes regulated by both bacteria, we observed that Xac up-regulated an alpha-expansin (CK934531, CF837795) that was repressed by Xaa, whereas Xaa up-regulated the periaxin-like protein (CN183059) repressed by Xac (Tables 1 and S9).

Table 1 Major cell-wall proteins and remodelling enzymes modulated by Xac and/or Xaa at 6 and 48 hpi, relative to water-infiltrated leaves. The expression levels of genes identified by DD/SSH were determined by qRT-PCR. Negative numbers represent down-regulation. The complete list of cell-wall remodelling genes modulated by Xac/Xaa is found in Supporting Information Tables S1–S9. Fold change values of Xac versus Xaa treatments are shown for comparison.

			Change after treatments							
			Microarray						DD/SSH	
Target description	Citrus	Target	6 hpi			48 hpi			48 hpi	
Cell-wall synthesis and remodelling	EST	Gene ID	Xaa	Xac	Xac/Xaa	Xaa	Xac	Xac/Xaa	Xaa	Xac
Acidic cellulase [*C. sinensis*]	CF831790	AAB65155	—	—	—	—	36.8	34.8	1.4	8.0
Basic cellulase [*C. sinensis*]	CX663293	AAB65156	—	—	—	—	13.8	15.3	1.7	18.2
Endo-beta-1,4-glucanase [*Fragaria* × *ananassa*]	CV887291	CAC94006	—	—	—	—	13.8	21.6	1.3	35.8
Xyloglucan endo-1,4-beta-D-glucanase [*T. majus*]	CN182557	T10523	—	—	—	—	8.7	15.5	—	—
Beta-D-glucosidase [*G. hirsutum*]	CX667827	AAQ17461	—	—	—	—	8.4	10.1	—	—
Xyloglucan endotransglycosylase [*B. pendula*]	CX076871	ABB72441	—	4.0	—	—	7.9	2.3	—	—
Glycosyl transferase family 2 protein [*A. thaliana*]	CV709535	NP_197666	—	—	—	—	19.1	17.8	—	—
Alpha-D-xylosidase [*T. majus*]	CV710106	CAA10382	—	—	—	—	11.6	6.2	—	—
Pectate lyase family protein [*A. thaliana*]	CK933446	NP_563715	—	—	—	—	18.7	32.5	—	—
PS60 pectinesterase [*N. tabacum*]	CF653190	CAA65634	—	—	—	—	20.4	8.7	1.7	22.6
Cellulose synthase-like OsCslE1 [*O. sativa*]	CX071539	BAD46391	—	—	—	4.1	—	–2.2	—	—
Glucan endo-1,3-beta-D-glucosidase [*C. sinensis*]	CB291815	T10119	—	—	—	24.2	—	–8.9	6.3	2.1
O-methyltransferase [*P. balsamifera* × *P. deltóides*]	CX302017	AAF60951	12.9	—	–12.5	—	—	—	—	—
Caffeic acid O-methyltransferase [*R. chinensis*]	CK665535	BAC78828	4.5	—	—	—	—	—	—	—
Caffeic acid 3-O-methyltransferase [*C. annuum*]	AJ489039	Q9FQY8	8.7	—	–3.5	—	—	—	—	—
Lignin bispecific methyltransferase [*P. tremuloides*]	CX302051	CAA44006	3.5	—	—	—	—	—	—	—
Cinnamate 4-hydroxylase [*Citrus* × *paradisi*]	CK932830	AAK57011	3.9	—	–2.2	—	—	—	—	—
Cinnamoyl CoA reductase-like protein [*A. thaliana*]	CV706284	AAM62926	3.4	—	—	—	—	—	—	—
Cinnamyl alcohol dehydrogenase [*Prunus mume*]	CX667871	BAE48658	3.0	—	—	—	—	—	—	—
Phenylalanine ammonia-lyase [*C. limon*]	CD575471	Q42667	4.6	—	—	—	—	—	—	—
Shikimate kinase family protein [*A. thaliana*]	CN191392	NP_565393	3.2	—	—	—	—	—	—	—
Polygalacturonase-inhibiting protein [*C. jambhiri*]	CF828981	BAB78473	4.5	—	–2.0	—	—	—	—	—
Xyloglucanase-specific inhibitor [*L. esculentum*]	CF833518	AAN87262	6.2	—	–4.2	—	—	—	—	—
Xyloglucanase inhibitor [*S. tuberosum*]	CX302610	AAP84703	4.6	—	–2.6	5.0	—	–2.7	—	—
Glycoside hydrolase/polygalacturonase [*A. thaliana*]	CX305641	NP_567055	3.0	—	—	3.4	—	—	—	—
Beta-galactosidase [*C. sinensis*]	CX043592	AAK31801	8.0	—	–3.0	4.2	—	—	—	—
Cinnamate 4-hydroxylase CYP73 [*C. sinensis*]	AF255013	AAF66065	3.0	—	–2.0	4.5	—	—	—	—
Catechol O-methyltransferase [*N. tabacum*]	CX670983	CAA50561	—	—	–6.6	6.9	—	–6.7	—	—
Caffeic acid O-methyltransferase II [*N. tabacum*]	CX045519	AAL91506	—	—	–4.5	4.8	—	–3.0	—	—
Laccase/diphenol oxidase [*A. thaliana*]	CF836861	NP_187533	—	—	—	8.5	—	–5.0	—	—
Glycosyl hydrolase family 38 protein [*A. thaliana*]	CX671694	NP_201416	—	—	—	5.1	—	–2.0	—	—
Glucosyltransferase-1 [*V. angularis*]	CN187784	BAB86919	—	—	—	–4.1	—	—	—	—
UDP-glycosyltransfersase [*J. curcas*]	CX075207	AAL40272	—	—	—	–3.4	—	—	—	—
Xyloglucan endo-transglycosylase [*C. papaya*]	CX666073	AAK51119	—	—	—	–3.5	—	—	—	—
Polygalacturonase [*A. thaliana*]	CF417542	AAM91193	—	—	—	–3.4	—	—	—	—
Pectinacetylesterase [*A. thaliana*]	CX069783	AAO50621	—	—	—	–3.4	—	—	–5.0	–2.5
Cell-wall proteins										
Expansin [*C. sinensis*]	DN958759	ABG49444	—	—	—	—	16.0	8.1	2.6	91.2
Expansin [*P. communis*]	CX545018	BAC67193	—	—	—	—	19.6	10.8	—	—
Expansin [*P. cerasus*]	CV710432	AAK48848	—	—	—	—	15.9	17.3	—	—
Alpha-expansin 3 [*P. tremula* × *P. tremuloides*]	CK934531	AAR09170	—	—	—	–4.3	5.2	9.5	—	—
Arabinogalactan-protein; AGP [*P. communis*]	CD574246	AAB35283	—	—	—	—	49.4	41.0	—	—
Cim1 protein [*G. max*]	CX667721	S48032	—	—	—	—	38.4	47.0	—	—
Proline-rich protein [*N. glauca*]	CK701597	AAF28387	—	—	—	—	9.6	6.9	—	—
Cell-wall protein Exp4 precursor [*M. jalapa*]	CX640971	AAL87023	—	—	—	—	14.0	11.2	—	—
Fiber protein E6 [*G. barbadense*]	CK934786	S65063	—	—	—	—	9.2	10.2	—	—
Periaxin-like protein [*A. thaliana*]	CN183059	CAB89377	3.0	—	—	—	–6.2	–12.6	—	—
Proline-rich protein PRP1 [*C. sativa*]	CF829030	AAK25755	7.8	—	–2.9	—	—	—	—	—
Pherophorin-dz1 protein [*V. carteri*]	CX047057	CAD22154	7.8	—	–3.6	—	—	—	—	—
Prolyl 4-hydroxylase [*N. tabacum*]	CF831203	BAD07294	—	—	—	3.3	—	—	—	—
Beta-expansin 2 (AtEXPB2) [*A. thaliana*]	CX641267	Q9SHY6	—	—	—	–3.0	—	—	—	—
Fasciclin-like AGP 11 [*P. alba* × *P. tremula*]	CX051837	AAT37954	—	—	—	–4.3	—	—	—	—
Arabinogalactan protein [*G. hirsutum*]	CK701267	AAO92753	—	—	—	–3.4	—	–2.1	—	—

Table 2 Major transcriptional changes in terpene and GA synthesis, auxin mobilization and signalling genes induced by Xac and/or Xaa at 6 and 48 hpi, relative to water-infiltrated leaves. Genes identified by DD/SSH not represented in the citrus array are shown in bold and their expression levels were determined by qRT-PCR. Negative numbers represent down-regulation. The complete list of terpene and GA synthesis, auxin mobilization and signalling genes with altered expression upon Xac/Xaa infection is found in Supporting Information Tables S1–S9.

			Change after treatments					
			Microarray				DD/SSH	
Target description	Citrus	Target	6 hpi		48 hpi		48 hpi	
Terpene, GA and volatile synthesis	EST	Gene ID	Xaa	Xac	Xaa	Xac	Xaa	Xac
GGPP synthase [*D. carota*]	CX669501	BAA78047	7.5	—	28.8	40.6	—	—
Geranyl diphosphate synthase [*A. majus*]	CX290062	AAS82859	—	—	3.0	14.8	—	—
HGG transferase [*V. vinifera*]	CX306825	AAV74623	87.3	30.7	133.7	75.4	—	—
Transferase family protein [*A. thaliana*]	CF418090	NP_189233	31.3	6.7	17.4	3.3	—	—
Acetyltranferase-like protein [*A. thaliana*]	CN191360	BAB01067	35.6	9.8	23.0	5.5	—	—
Orcinol O-methyltransferase [*Rosa* hybrid]	CV714380	AAM23005	3.9	5.2	8.0	5.6	—	—
GHMP kinase family protein [*A. thaliana*]	CK935595	NP_566144	—	—	6.8	4.7	—	—
LYTB-like protein precursor [*A. palaestina*]	CF828804	AAG21984	5.3	—	3.0	—	—	—
Linalool synthase [*A. thaliana*]	CV885575	AAO85533	–8.0	–3.4	—	—	—	—
Cytochrome P450 / DDWF1 [*N. tabacum*]	CV885042	AAK62346	—	—	23.7	10.6	43.3	19.3
Methylcoclaurine 3′-hydroxylase [*E. californica*]	DN620712	**AF014802**	—	—	—	—	2.1	3.0
Terpene synthase [*A. thaliana*]	—	**AF497492**	—	—	—	—	3.7	6.9
UbiA prenyltransferase [*A. thaliana*]	—	**NM_112028**	—	—	—	—	1.0	3.2
α-Farnesene synthase [*Malus* × *domestica*]	CX044235	**AY182241**	—	—	—	—	–4.2	3.0
SAM-salicilate methyltransferase [*H. carnosa*]	CN182915	**AJ863118**	—	—	—	—	20.0	60.4
PDR-type ABC transporter 1 [*N. tabacum*]	CX641504	**AB109388**	—	—		—	4.1	2.5
Auxin signaling								
IAA-amino acid conjugate hydrolase [*A. thaliana*]	CF836363	AAL47552	3.6	—	—	—	—	—
gr1-protein/IAA-amino acid hydrolase [*A. thaliana*]	CN186513	CAA09330	5.1	—	—	—	—	—
Aux/IAA protein [*V. vinifera*]	CX306870	AAL92850	—	—	3.8	—	—	—
Nitrilase [*N. tabacum*]	CX289842	T03736	—	—	3.2	—	—	—
Auxin-induced BLHL factor [*G. hirsutum*]	CX544672	AAD48836	—	—	–3.1	–2.4	—	—
Aux/IAA protein [*P. tremula* × *P. tremuloides*]	CX301300	CAC84710	—	—	–3.8	–2.9	—	—
Rhodanese-like family protein [*A. thaliana*]	CK936971	AAM64600	–2.2	—	–3.2	–2.7	—	—
Auxin efflux carrier family protein [*A. thaliana*]	CN187762	NP_683316	4.0	—	15.7	6.2	—	—
GH3.1/IAA synthetase [*A. thaliana*]	CV714093	AAC61292	3.9	—	7.4	4.9	—	—
Nt-gh3 deduced protein [*N. tabacum*]	CF837443	AAD32141	3.6	—	5.3	4.3	—	—
Auxin-induced protein Aux22 [*V. radiata*]	DN799130	T10941	—	—	–4.6	–4.1	—	—
Nt-iaa4.5 deduced protein [*N. tabacum*]	CK934325	AAD32145	—	—	–3.9	–4.2	—	—
Putative microRNA167	CK936755	—	—	—	–6.7	–4.0	—	—
Multidrug resistance/P-glycoprotein [*A. thaliana*]	CX306117	NM_116080	—	—	3.6	3.3	3.0	2.0
NAC domain protein NAC6 [*G. max*]	DN619712	DQ028774	—	—	—	2.1	1.8	4.5
ER lumen-retaining receptor [*A. thaliana*]	CX676252	**AY086280**	—	—	—	—	2.0	2.2
ADP-ribosylation factor [*C. annuum*]	DR908238	AF108891	—	—	—	—	1.7	2.7

Terpene and GA synthesis

Various citrus genes encoding proteins similar to terpene synthase, geranylgeranyl diphosphate synthase, homogentisate geranylgeranyl transferase, cytochrome P450/DDWF1 and S-adenosyl methionine-salicilate methyltansferase, among others, were strongly up-regulated by both Xac and Xaa at 6 h and 48 h after bacterial infiltration (Table 2 and Supporting Information Tables S1–S9). These enzymes are known to be involved in the synthesis and transport of a variety of terpenes, gibberellins, brassinoesteroids, alkaloids and plant volatiles, which play diverse roles in plant development and defence (Bird *et al.*, 2003; Mercke *et al.*, 2004; Richman *et al.*, 1999).

Major differences in the transcriptional profiles of terpene and GA synthesis genes were found in the expression of an LYTB-like protein (CF828804) involved in isoprenoid biosynthesis, detected in Xaa treatments only (Tables 2, S4 and S7). Similarly, by DD/SSH we identified a putative UbiA prenyltransferase specifically

up-regulated by Xac, whereas a protein similar to an α-farnesene synthase (CX044235), implicated in defence volatile synthesis (Mercke *et al.*, 2004), was up-regulated by Xac but down-regulated by Xaa (Tables 2 and S1).

Auxin mobilization and signalling

Transcriptional changes in response to Xac and Xaa infection were also observed for a group of genes involved in auxin mobilization, transport and signal transduction. For instance, genes related to GH3-like proteins, nitrilase and IAA (indole-3-acetic acid)–amino acid conjugate hydrolases, involved in auxin synthesis and mobilization (Park *et al.*, 2003; Staswick *et al.*, 2005) were preferentially up-regulated by Xaa, particularly at 6 hpi (Fig. 2A, Table 2 and Supporting Information Tables S1–S9). This is in accordance with the induction of proteins similar to auxin efflux carrier, P-glycoprotein, ER lumen-retaining receptor and ADP-ribosylation factor implicated in the polar flux of auxin in the endoplasmic reticulum and Golgi (Multani *et al.*, 2003 Noh *et al.*, 2001; Zhuang *et al.*, 2005) (Table 2 and Supporting Information Tables S1–S9).

Interestingly, a number of related Aux/IAA proteins and a putative microRNA167 were similarly down-regulated by Xac and Xaa, relative to water-infiltrated leaves. Additionally, a NAM-like protein (DN619712) homologous to NAC transcriptional factors required for auxin and GA signal transduction (Robertson, 2004; Xie *et al.*, 2000) was up-regulated by the pathogens (Tables 2 and S2), suggesting changes in auxin signalling upon *X. axonopodis* infection. As repression of auxin signalling has recently been shown to play a role in antibacterial resistance (Navarro *et al.*, 2004), it is likely that the changes in expression of auxin mobilization and signalling genes described here are associated with canker development. Accordingly, Xcv also induces auxin-responsive genes including expansins, which may be involved in cell enlargement caused by this bacterium in pepper plants (Marois *et al.*, 2002).

Vesicle trafficking

In addition to the genes possibly involved in the polar flux of auxin, we identified several genes related to vesicle trafficking, docking and fusion that were induced by the pathogens mainly at 48 h after bacterial infiltration. For instance, genes encoding proteins homologous to SAR1, Sec1, Sec14, SNAREs, Rab-GTPases, epsin, coatomer and vacuolar-sorting receptor were similarly up-regulated by Xac and Xaa (Tables S1, S8 and S9).

Recent studies have highlighted the importance of polarized vesicle trafficking in plant–pathogen interactions (Hückelhoven, 2007). Transport and secretion of plant materials at the interface of interactions have been associated primarily with non-specific resistance, and syntaxins and ABC transporters play important roles in this process (Hückelhoven, 2007). Interestingly, citrus proteins related to syntaxins and PDR/ABC transporters implicated in secretion of antimicrobial terpenoids (van den Brûle and Smart, 2002) were specifically induced by Xaa. Notably, the citrus EST CX304749 is homologous to syntaxin SYP132, a novel component of effector-triggered immunity that contributes to plant resistance against bacteria and secretion of pathogenesis-related (PR) proteins (Kalde *et al.*, 2007). By contrast, some genes related to SNAREs, Rab-GTPases, SAR1, patellin and annexins were specifically up-regulated by Xac (Tables S2–S7). Particularly, the gene BQ624283 is similar to NPSN11, a cell plate-associated SNARE protein that is highly expressed in dividing cells and interacts with KNOLLE, a cytokinesis-specific syntaxin (Zheng *et al.*, 2002). Thus, although polar vesicle trafficking has been generally implicated in new cell-wall appositions for basal defence, the up-regulation of an NSP11-like protein in Xac-infected leaves suggests a link with the hyperplasia of canker lesions. This idea is supported by the fact that the number of vesicle trafficking, cell division and cell-wall remodelling genes up-regulated by Xac increased substantially from 6 to 48 hpi (Fig. 2A). Remarkably, brefeldin A, a well-known inhibitor of vesicle trafficking, significantly retarded initial pustule development induced by Xac (Fig. 3), thus indicating that vesicle trafficking in this case is mediating symptom development rather than defence.

Disease resistance, defence and stress responses

The major transcriptional changes after Xac/Xaa infection were observed for genes related to disease resistance, defence and stress responses (Fig. 2). In this functional category we incorporated many citrus ESTs homologous to disease resistance, signal perception, oxidative burst, protein turnover and HR-related genes, most of which have been implicated in the innate immune response or involved in the sensing of PAMPs (pathogen-associated molecular patterns). However, the expression of genes related to gene-for-gene resistance mechanisms was also altered by the pathogens.

Citrus genes that were induced by both pathogens and are likely to play roles in innate immunity are homologous to CYP450 monooxigenases, peroxidases and oxidases involved in the production of reactive oxygen species (ROS), as well as glutathione-S-transferase (GST), thioredoxins, leucoanthocyanidin dioxygenases, haemoglobins and flavonol synthases, known to counteract pathogen-induced oxidative stress in plants (Reddy *et al.*, 2007). These genes were strongly up-regulated at 6 and 48 hpi (Tables 3, S8 and S9). Notably, at 6 hpi, we detected an elevated expression of ESTs (CX077228, CX296261) homologous to BON1-associated protein BAP1, which has been recently shown to act as a general inhibitor of programmed cell death (Yang *et al.*, 2007). Interestingly, however, these and other citrus ESTs related to copines, BAP1 and BAP2 were found up-regulated by Xaa only at 48 hpi (Tables 3, S2 and S4), suggesting that Xac suppresses programmed cell death early during infection.

Table 3 Principal disease resistance, defence and stress response genes up-regulated by Xac and Xaa at 6 and 48 hpi, relative to water-infiltrated leaves. Genes identified by DD/SSH not represented in the citrus array are shown in bold and their expression levels were determined by qRT-PCR. The complete list of disease resistance, defence and stress response genes with altered expression upon Xac/Xaa infection is found in Supporting Information Tables S1, S8 and S9.

			Change after treatments					
			Microarray				DD/SSH	
Target description	Citrus	Target	6 hpi		48 hpi		48 hpi	
Disease resistance, defence and stress response	EST	Gene ID	Xaa	Xac	Xaa	Xac	Xaa	Xac
Disease resistance protein [*A. thaliana*]	CX301461	NP_199715	8.7	8.7	19.0	15.3	—	—
Leucine-rich repeat protein [*A. thaliana*]	CX642531	NP_172219	—	—	6.6	6.5	—	—
Resistance protein RPP8-like protein [*A. thaliana*]	CX669576	AAP82824	14.0	7.3	5.2	4.7	—	—
Receptor protein kinase [*A. thaliana*]	CV707423	AAO42089	13.4	9.5	7.6	7.3	—	—
EIX receptor 2 [*L. esculentum*]	CX043514	AAR28378	—	—	4.4	6.3	—	—
LRR receptor like kinase [*A. thaliana*]	—	**AK221860**	—	—	—	—	11.8	14.7
Disease resistance protein [*A. thaliana*]	—	**AC020579**	—	—	—	—	3.8	5.6
BON1-associated protein BAP1 [*A. thaliana*]	CX077288	NP_182100	11.6	19.7	6.1	—	—	—
WRKY-type DNA binding protein [*A. thaliana*]	CX050828	BAC42556	13.9	6.4	7.5	15.5	—	—
WRKY-type DNA binding protein [*A. thaliana*]	CF828414	BAC23031	—	—	24.7	7.5	—	—
Zinc finger (C3HC4-type RING finger) [*A. thaliana*]	BQ623333	NP_565376	3.1	6.1	—	—	—	—
RING-H2 zinc finger protein ATL3 [*A. thaliana*]	CX050623	AAD33581	4.8	4.7	7.7	4.0	—	—
ZPT2-12 [*Petunia* × *hybrida*]	CK939354	BAA21921	7.4	9.2	—	—	—	—
F-box family protein [*A. thaliana*]	CK935545	NP_566322	3.4	3.4	7.4	4.9	—	—
Calcium-binding EF hand protein [*A. thaliana*]	CK939355	NP_568568	3.7	10.0	—	—	—	—
NAC domain protein NAC2 [*A. thaliana*]	CK940145	AAM63330	11.3	4.5	39.0	7.8	—	—
HSR203J like protein [*C. chinense*]	CN190145	BAD11070	—	—	13.5	6.4	—	—
Bacterial-induced peroxidase [*G. hirsutum*]	CX078551	AAD43561	45.1	41.7	36.3	28.4	—	—
Peroxidase [*P. nigra*]	CF832245	BAA11853	14.6	7.0	9.5	7.5	—	—
Anionic peroxidase H [*Z. mays*]	CV718839	AAC79953	19.1	5.9	8.3	3.5	—	—
Apoplastic anionic gaiacol peroxidase [*G. hirsutum*]	CF835282	AAL92037	36.7	13.6	14.1	6.7	—	—
Peroxidase [*P. balsamifera*]	CV707821	CAA66037	12.0	4.6	9.4	3.8	—	—
Putative NADH-dehydrogenase [*P. sativum*]	CX293755	AAO27256	9.7	5.9	13.6	20.8	—	—
Ttropinone reductase [*A. thaliana*]	CX078323	NP_180480	38.3	21.6	—	—	—	—
Tropinone reductase I [*S. tuberosum*]	CN191155	CAC34420	11.9	12.9	26.7	10.3	—	—
Glutathione S-transferase [*C. maxima*]	CX297758	BAC21263	48.2	32.6	18.0	21.2	—	—
Respiratory burst oxidase [*N. benthamiana*]	CX301580	BAC56865	14.0	4.3	12.1	11.3	—	—
Alternative oxidase [*P. tremula* × *P. tremuloides*]	DN795132	CAB64356	4.6	3.5	17.3	3.2	—	—
Cytochrome P450 monooxygenase [*A. thaliana*]	CX636220	AAM61746	10.1	5.7	—	—	—	—
Cytochrome P450 [*C. sinensis*]	CX639356	AAL24049	7.3	8.8	43.7	5.7	—	—
Cytochrome P450 79A1 [*S. vulgare*]	CX296809	Q43135	23.9	13.3	13.6	6.9	—	—
Cytochrome P450 CYP79A2 [*A. thaliana*]	CB322199	NP_568153	21.6	11.6	17.3	8.3	—	—
Cytochrome P450 [*A. thaliana*]	CX301598	BAB09969	25.3	11.3	19.2	8.2	—	—
Cytochrome P450 like_TBP [*N. tabacum*]	CB322204	**D64052**	—	—	—	—	4.2	2.9
Lipase class 3 family protein [*A. thaliana*]	CN190043	NP_973975	30.4	5.0	79.3	15.8	—	—
Lipoxygenase LOX2 [*P. deltoids*]	CX671317	**DQ131179**	—	—	—	—	8.0	4.4
Phospholipase D gamma 1 [*A. thaliana*]	DN618160	NP_192922	—	—	8.0	5.1	18.9	9.2
Pathogenesis-related protein PR-1 [*C. annuum*]	CF653559	AAK30143	7.0	4.2	22.7	25.3	187	233
Pathogenesis-related protein 5-1 [*H. annuus*]	CF836158	AAM21199	39.7	6.2	11.7	10.1	—	—
Pathogenesis-related protein 4b [*C. chinense*]	CX643738	BAD11073	128.7	13.7	40.7	6.0	—	—
Pathogenesis-related protein 4A [*N. tabacum*]	CX637285	CAA41437	12.2	4.1	27.6	14.0	—	—
Phenylalanine-ammonia lyase [*Citrus* sp.]	CX643181	CAB42793	12.0	7.9	21.2	19.5	—	—
Lectin-like protein kinase [*A. thaliana*]	CX669884	BAB09808	11.0	3.8	39.7	6.3	—	—
Chitinase CHI1 [*C. sinensis*]	CX292066	AAC35981	3.5	3.2	13.4	24.1	—	—
Chitinase [*H. brasiliensis*]	CX671223	CAA09110	—	—	7.6	7.7	—	—
Chitinase [*G. hirsutum*]	CX637693	CAA92277	18.5	3.1	26.3	13.8	—	—
Leucoanthocyanidin dioxygenase [*A. thaliana*]	CX641508	BAB02603	9.7	6.3	37.2	12.4	—	—
Phytocyanin homolog [*P. taeda*]	CF836493	AAF75824	42.7	6.9	—	—	—	—
Dicyanin [*L. esculentum*]	CX297166	AAF66242	7.0	3.1	27.2	24.5	—	—
Patatin-like protein 1 [*N. tabacum*]	CX295316	AAF98368	11.0	4.1	—	—	—	—
Ionotropic glutamate receptor GLR4 [*O. sativa*]	CV710376	XP_478449	4.3	4.5	5.1	4.3	—	—
Wts2L [*C. lanatus*]	DN620336	BAA89230	4.9	5.2	5.1	3.3	—	—
CPRD2 [*V. unguiculata*]	CX291051	BAB33033	55.6	9.0	74.4	46.3	—	—
Nectarin 5 [*N. langsdorffii* × *N. sanderae*]	CX299253	AAP30841	84.65	18.65	123.7	106.7	—	—

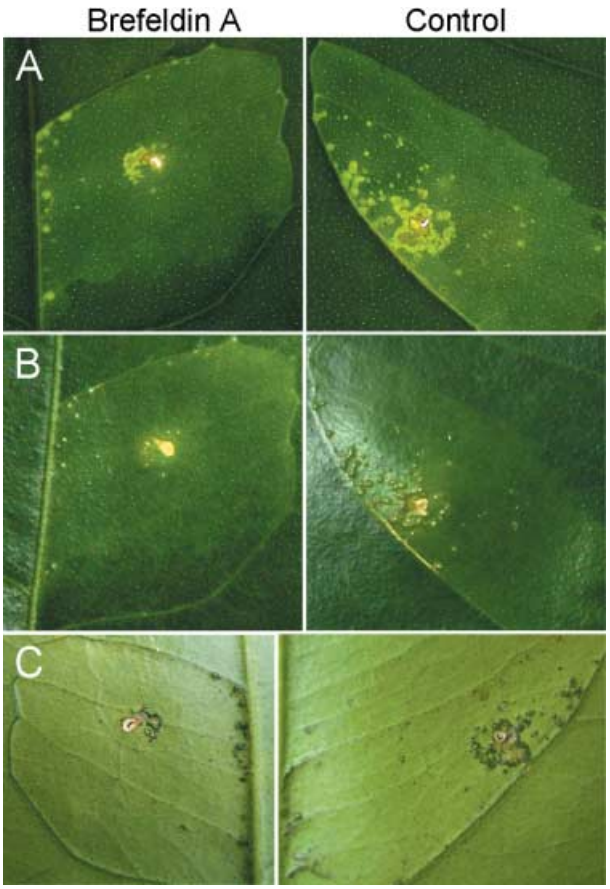

Fig. 3 Effect of brefeldin A on initial formation of canker pustules in Pêra leaves infiltrated with Xac. The number of pustules observed under transmitted light (A) or in the upper (B) and lower (C) surfaces of the leaf are significantly smaller in the leaf sector treated with brefeldin A, compared with control. Clearer zones in A and B correspond to the infiltrated sectors.

We also noticed high expression levels of phenylalanine ammonia lyases (PAL), tropinone reductases and related FAD-linked oxidases (CPRD2 and nectarin 5) similar to berberine bridge enzymes involved in phytoalexin and alkaloid production, as well as increased expression of proteins similar to lipases, jasmonic acid (JA) proteins, PR proteins, chitinases and a phospholipase D implicated in local defence and wound response (Laxalt and Munnick, 2002) (Tables 3, S8 and S9). In addition, the elevated expression of genes homologous to leucine-rich repeat (LRR) proteins, receptor protein kinases, WRKY transcription factors, C3HC4 Ring fingers, F-box and 26S proteosome proteins, NAC/NAM-like proteins, PDR ABC transporters and HSR203, known to play roles in defence (Dong *et al.*, 2006; Eulgem and Somssich, 2007; Oh *et al.*, 2005; van den Brûle and Smart, 2002) strongly suggests that an HR-like reaction is being mounted against both pathogens. Nonetheless, although most of these genes were induced at higher levels by Xaa (Tables 3, S2 and S5), this transcriptional profile would apparently be inconsistent with the compatible Xac–Pêra interaction. Thus, to find better clues to the differential pathogenicity exhibited by Xac and Xaa in the Pêra cultivar, we looked at the defence-related genes specifically modulated by each pathogen. Not surprisingly, we found that Xac altered the expression of a significantly smaller number of defence-related genes compared with Xaa in both infection treatments (Fig. 2 and Tables S2–S7), a clear indication of suppression of defence responses during canker development. This is in accordance with the fact that the effector protein PthA suppressed non-specific or basal defence responses in a heterologous plant system (Fujikawa *et al.*, 2006).

At 6 hpi, Xac specifically induced the expression of several genes that might be associated with an oxidative stress response, including a WRKY transcriptional factor, GST, S-like RNase and thioredoxin h (Table 4 and Supporting Information Tables S5–S7). Curiously, thioredoxin h was shown to reduce S-RNase in *Nicotiana alata*, whereas in Arabidopsis, a thioredoxin h induced by oxidative stress and *Pseudomonas syringae* infection was regulated by a WRKY factor (Juárez-Díaz *et al.*, 2006; Laloi *et al.*, 2004).

Interestingly, the transcriptional profile of Xac-regulated genes changed significantly from 6 to 48 hpi (Fig. 2A, Table 4 and Supporting Information Tables S2–S4), indicating a change in the physiological status of the host cell, as the infection progressed. At 48 hpi, Xac induced the expression of specific genes encoding putative disease-resistant proteins such as MLO, LysM-domain receptor kinase, cysteine protease (CP), and nodulins. Notably, the MLO, early nodulin and CP homologues were also identified by DD/SSH and their expression levels as measured by qRT-PCR confirmed their predominant responsiveness to Xac infection (Table 4 and Supporting Information Tables S1–S4).

The role of these proteins in disease resistance or canker development is not yet clear; however, it is interesting to note that MLO proteins function as negative modulators of antifungal defences and are associated with vesicle trafficking (Consonni *et al.*, 2006), whereas Lys-domain receptor kinases play crucial roles in the specific recognition of symbiotic bacteria during root nodule formation (Spaink, 2004). This suggests that overexpression of such proteins in citrus leaves might favour disease development rather than resistance. Consistent with this idea, the citrus CP (DN623815) is similar to nodule-specific cysteine proteinases (Naito *et al.*, 2000) and to Rcr3, required for *Clasdoporium fulvum* resistance but also suppression of autonecrosis in tomato (Krüger *et al.*, 2002). Inhibition of Rcr3 activity by *C. fulvum* Avr2 led to an HR reaction (Rooney *et al.*, 2005), suggesting that increased levels of citrus CP might as well be associated with disease susceptibility. This idea is supported by the fact that the citrus CP is overexpressed in Mexican lime in response to both Xac and Xaa infection (Supporting Information Fig. S2).

Xac up-regulated a number of cyclophilin-related peptidylprolyl isomerases and aquaporins, whereas it down-regulated some lipid-transfer proteins and a cysteine endopeptidase (Table 4 and

Table 4 Main disease resistance and defence-related genes specifically regulated by Xac, relative to water treatments. The expression levels of genes identified by DD/SSH were determined by qRT-PCR. Negative numbers represent down-regulation. The complete list of defence-related genes specifically modulated by Xac is found in Supporting Information Tables S3 and S6. Fold change values of Xac versus Xaa treatments are shown for comparison.

			Change after treatment					
			Microarray				DD/SSH	
Target description	Citrus	Target	6 hpi		48 hpi		48 hpi	
Disease resistance, defence and stress response	EST	Gene ID	Xac	Xac/Xaa	Xac	Xac/Xaa	Xaa	Xac
WRKY transcription factor 10 [*N. tabacum*]	DN799348	CAI38917	9.3	5.3	—	—	—	—
Hypersensitive-induced response protein [*O. sativa*]	DN798608	XP_450602	5.2	2.7	—	—	—	—
Avr9/Cf-9 rapidly elicited protein 65 [*N. tabacum*]	DR405210	AAG43557	6.5	2.4	—	2.8	—	—
Calcium-binding protein, putative [*A. thaliana*]	CX046527	NP_564143	4.2	2.2	—	2.3	—	—
S-like ribonuclease [*P. dulcis*]	CX044216	AAF82615	4.6	2.6	—	—	—	—
Thioredoxin h [*H. brasiliensis*]	BQ623844	AAD33596	3.0	3.8	—	—	—	—
Glutathione S-transferase [*A. thaliana*]	CX293561	AAG30140	4.7	2.2	—	—	—	—
Glutathione S-transferase [*A. thaliana*]	CX293561	AAG30140	4.7	2.2	—	—	—	—
Lipase/hydrolase, putative [*A. thaliana*]	CX044184	AAM64368	4.9	2.4	—	—	—	—
Disease resistance protein/LRR protein [*A. thaliana*]	CN181971	NP_197731	—	—	6.6	7.4	—	—
Leucine-rich repeat protein-related [*A. th*aliana]	CX303178	NP_564237	—	—	5.2	6.3	—	—
Receptor protein kinase family [*A. thaliana*]	CF510003	NP_200249	—	—	7.1	3.4	—	—
Putative protein kinase APK1A [*Oryza sativa*]	CD573725	NP_913119	—	—	5.2	2.9	—	—
Seven transmembrane MLO family protein [*A. thaliana*]	CK933849	NP_192169	—	—	3.0	3.0	1.0	4.0
LysM-domain GPI-anchored protein 1 [*A. thaliana*]	CV707862	Q93ZH0	—	—	4.0	2.8	—	—
Peroxidase ATP26a homolog [*A. thaliana*]	CX048985	BAB10896	—	—	10.9	7.8	—	—
Cysteine protease-like protein [*A. thaliana*]	DN623815	CAB66413	—	—	4.9	—	1.6	20.2
Putative early nodule-specific protein [*A. thaliana*]	CV887212	BAC43003	—	—	5.7	6.2	5.2	44.1
Nodulin-like protein [*P. trifoliata*]	CX671683	AAN62343	—	—	6.1	7.5	—	—
Lipase-related [*A. thaliana*]	CD574243	NP_564291	—	—	3.8	4.8	—	—
Multi-copper oxidase [*A. thaliana*]	CX671357	AAN38699	—	2.6	8.5	7.9	—	—
Syringolide-induced protein 19-1-5 [*G. max*]	CK939135	BAB86890	—	—	3.6	—	—	—
Aquaporin TIP3 [*V. berlandieri* × *V. rupestris*]	CX673014	AAF78757	—	2.3	5.0	5.3	—	—
Zinc finger (C3HC4-type RING finger) protein [*A. thaliana*]	BQ624718	NP_193956	—	—	4.9	5.8	—	—
FAD-linked oxidoreductase [*A. thaliana*]	CX675564	AAO50720	—	—	19.0	11.1	—	—
Protein disulphide isomerase [*E. guineensis*]	CN182165	AAO26314	—	—	3.4	—	—	—
70 kDa heat shock protein [*P. vulgaris*]	CV704678	CAA47345	—	—	3.6	—	—	—
HSP70 [*A. thaliana*]	CV720046	NP_567510	—	—	3.6	—	—	—
10 kDa chaperonin (CPN10) [*A. thaliana*]	CV713701	NP_563961	—	—	4.5	—	—	—
Mitochondrial chaperonin-60 [*O. sativa*]	CX671431	NP_921872	—	—	4.3	—	—	—
Glutaredoxin [*A. thaliana*]	CX289567	CAB69043	—	—	3.0	—	—	—
FKBP-type peptidyl-prolyl cis-trans isomerase [*A. thaliana*]	CK934189	NP_567717	—	—	3.1	—	—	—
Peptidylprolyl isomerase [*O. sativa*]	CX053295	BAD46607	—	—	3.3	—	—	—
Peroxidase ATP23a [*A. thaliana*]	CX666965	CAA70035	—	—	–3.2	—	—	—
Glycosylhydrolase/beta-glucosidase [*A. thaliana*]	CK935993	NP_181976	—	—	–3.1	—	—	—
Cysteine endopeptidase [*R. communis*]	CO91268	AAC62396	—	—	–3.2	—	—	—
Tropinone reductase [*Calystegia sepium*]	CN181671	CAD62568	—	2.1	–3.8	—	—	—
Cold-regulated LTCOR12—snakin 2 [*L. thuringiaca*]	CF837470	AAC15460	—	—	–3.0	—	—	—
Lipid transfer protein [*A. thaliana*]	CX302649	AAM60950	—	—	–3.4	—	—	—
Non-specific lipid transfer-like protein [*P. vulgaris*]	CX069746	AAC49370	—	—	–3.1	—	—	—
Protease inhibitor/lipid transfer protein (LTP) [*A. thaliana*]	CK935516	NP_190966	—	2.0	–3.1	—	—	—
Germin [*A. thaliana*]	CX663291	AAM61433	—	—	–3.0	—	—	—

Supporting Information Tables S1–S4). The role of such proteins in defence is not clear, although elevated expression of lipid-transfer proteins and cysteine endopeptidase resulted in increased resistance to pathogens (Jayaraj and Punja, 2007) and programmed cell death (Greenwood *et al.*, 2005), respectively. Increased expression of aquaporins, on the other hand, might be associated with diffusion of ROS across membranes during oxidative stress (Bienert *et al.*, 2006) or with the water soaking

phenomenon that occurs prior to canker development (Brunings and Gabriel, 2003). Interestingly, cyclophilin has been recently shown to activate a bacterial effector protein (Coaker *et al.*, 2005), suggesting that it may be required to properly fold effector proteins after they are delivered into the plant cell. Significantly, we identified a citrus cyclophilin that interacted with PthA in a two-hybrid assay (unpublished results).

As opposed to the expression profiles of defence-related genes in Xac-infiltrated leaves, there was a remarkable overlap between the transcriptional profiles of Xaa-regulated genes of the 6- and 48-h infection treatments (Table 5 and Supporting Information Tables S2–S7). Notably, most of the defence-related genes altered by Xaa have been shown in other plants to be rapidly activated during incompatible interactions or to act in the establishment of HR. For instance, some Xaa-induced genes are similar to MYB transcription factors and HIN1 involved in resistance against bacterial and virus pathogens (Daniel *et al.*, 1999; Fujikawa *et al.*, 2006; Yang and Klessig, 1996), F-box and Ring fingers known to play crucial roles in protein turnover during pathogen attack (Azevedo *et al.*, 2002; Kawasaki *et al.*, 2005), and NAC/NAM transcriptional factors associated with incompatible interactions and stress responses (Hu *et al.*, 2006; Oh *et al.*, 2005) (Table 5 and Supporting Information Tables S2–S7). Xaa also induced the expression of several genes related to protein kinases implicated in defence, including a homologue of the rice XA21 (Table 5), a receptor-like kinase that confers gene-for-gene resistance to races of *Xanthomonas oryzae* pv. *oryzae* (Wang *et al.*, 2006).

The citrus ESTs CX046706, CX044130 and CX643787 are homologous to WRKY factors induced by *Xanthomonas* pathogens, JA and ethylene, and that when overexpressed enhanced resistance against *Xanthomonas* and other pathogens (Liu *et al.*, 2007a; Oh *et al.*, 2006; Xu *et al.*, 2006). Members of the WRKY family have been implicated in the regulation of the transcriptional reprogramming associated with plant immune responses and they can act as positive and negative regulators of disease resistance (Eulgem and Somssich, 2007). This is consistent with the fact that both Xac and Xaa induced the expression of WRKY genes and that Xaa altered the expression of a greater number of such genes (Tables 4 and 5, and Supporting Information Tables S1–S7).

The increased expression of WRKY genes specifically induced by Xaa might also be associated with other Xaa-modulated genes. For instance, the EST AJ489090 (Table 5) is similar to the Arabidopsis receptor-like kinase RLK4, a downstream target of a WRKY induced by bacterial pathogens (Du and Chen, 2000). Similarly, CX640171 and CX292287 are related to members of the S gene family of receptor kinases SFR2 and ARK3, which are rapidly induced by bacterial pathogens and their expression requires W-box motifs in their promoters (Pastuglia *et al.*, 2002; Rocher *et al.*, 2005). Interestingly, W-box motifs have also been found in the promoters of defence-related GST and cytochrome P450 genes (Narusaka *et al.*, 2004; Shimono *et al.*, 2007) homologous to many citrus genes preferentially induced by Xaa (Tables 3 and 5).

Most significantly, Xaa induced the expression of other protein kinases involved in HR-like cell death that are functionally connected to WRKY proteins. In particular, the citrus EST CX073386 is related to the tobacco mitogen-activated protein kinases (MAPK) SIPK, WIPK and Ntf4, known to play pivotal roles in plant innate immunity in the regulation of cell death triggered by different pathogens (Ren *et al.*, 2006). These protein kinases are rapidly activated via a MAPK cascade after pathogen recognition (Ren *et al.*, 2006). Surprisingly, the citrus EST CB610685 (Table 5) is similar to MAPK kinases (MAPKK or MEK) that interact and activate SIPK/WIPK/Ntf4 (Liu *et al.*, 2007b; Takahashi *et al.*, 2007). Activation of SIPK and WIPK by MEK2 in tobacco led to the induction of several defence-related genes including PR proteins and HIN1, which are also found up-regulated in Xaa-infected leaves. Remarkably, WRKYs have been recently shown to be substrates of MAPK and MEK (Andreasson *et al.*, 2005; Miao *et al.*, 2007), supporting the idea that the MEK2–SIPK/WIPK cascade activates transcription of WRKY factors which eventually activates downstream defence genes (Kim and Zhang, 2004).

Accordingly to the overall induction of HR-related genes, Xaa also induced the expression of several enzymes involved in oxidative burst responses (Table 5 and Supporting Information Tables S5–S7). In particular, we highlight the expression of two proline oxidase/dehydrogenases (CX544362, CK934047), which have been shown to induce ROS-dependent cell death in yeast and animal cells through depletion of protective proline levels (Chen *et al.*, 2006; Maxwell and Rivera, 2003). In addition, several cytochrome P450s and an FAD-linked oxidase (CX302002) implicated in hydrogen peroxide production (Carter and Thornburg, 2004) were specifically and strongly up-regulated by Xaa (Tables 5, S2, S4, S5 and S7).

Cell division and morphogenesis

A substantial number of citrus ESTs related to cell cycle, growth and differentiation showed altered expression preferentially in response to Xac, which strongly correlates with the development of hypertrophy and hyperplasia of the canker lesions (Fig. 1). However, genes that were commonly induced by both pathogens included ribosomal proteins and fibrillarins, indicating increased ribosomal RNA processing and ribosome assembly during bacterial infection. In addition, Xac and Xaa up-regulated genes homologous to prohibitins and immuno-reactant natriuretic peptide, known to control cell volume and expansion (Chen *et al.*, 2005) (Tables S8 and S9).

By contrast, genes homologous to TRIPTYCHON (TRY), suspensor-specific protein and rapid alkalinization factor (RALF), were specifically up-regulated by Xac at 6 hpi (Table S5). TRY controls trichome and root hair cell patterning in Arabidopsis (Schellmann *et al.*, 2002), whereas RALF and suspensor-specific protein have

Table 5 Main disease resistance and defence-related genes specifically regulated by Xaa, relative to water treatments. The expression levels of genes identified by DD/SSH were determined by qRT-PCR. Negative numbers represent down-regulation. The complete list of defence-related genes specifically modulated by Xaa is found in Supporting Information Tables S6 and S7. Fold change values of Xac versus Xaa treatments are shown for comparison.

			Change after treatment					
			Microarray				DD/SSH	
Target description	Citrus	Target	6 hpi		48 hpi		48 hpi	
Disease resistance, defence and stress response	EST	Gene ID	Xaa	Xac/Xaa	Xaa	Xac/Xaa	Xaa	Xac
WRKY-type DNA binding protein [*A. thaliana*]	CX046706	AAP21232	3.3	−2.2	4.0	—	—	—
WRKY transcription factor 30 [*V. aestivalis*]	CX044130	AAR92477	3.6	—	—	—	—	—
WRKY transcription factor [*C. annuum*]	CX643787	AAW66459	—	—	9.4	−3.5	—	—
SPF1 protein [*I. batatas*]	CX294239	BAA06278	3.3	—	4.8	—	—	—
MYB family transcription factor (MYB63) [*A. thaliana*]	CN188989	NP_178039	3.6	−2.2	4.0	−2.0	—	—
No apical meristem (NAM) family protein [*A. thaliana*]	CN191283	NP_187057	3.4	−2.4	6.5	−2.2	—	—
Ubiquitin [*L. esculentum*]	CX296271	CAA51679	3.1	—	3.3	—	—	—
Ubiquitin-conjugating enzyme E2 [*O. sativa*]	CX298761	XP_466921	—	—	5.2	−3.2	—	—
F-box family protein [*A. thaliana*]	DN623568	AAN31083	—	—	5.0	—	—	—
F-box family protein [*A. thaliana*]	CF509222	AAN18072	—	—	3.6	−2.2	—	—
Zinc finger (C3HC4-type RING finger) (BRH1) [*A. thaliana*]	CN190502	NP_191705	—	—	3.5	—	—	—
Zinc finger (C3HC4-type RING finger) [*A. thaliana*]	CX287180	NP_567222	—	—	3.4	−2.0	—	—
RING/C3HC4/PHD zinc finger-like protein [*C. melo*]	CN186687	AAO45753	—	—	4.9	−2.0	—	—
Hin1 protein—common tobacco [*N. tabacum*]	CB293794	T03265	—	—	3.7	—	—	—
Disease resistance protein (CC-NBS-LRR) [*A. thaliana*]	CX544709	NP_195056	—	—	3.5	—	—	—
Putative protein kinase Xa21 [*O. sativa*]	CN190501	NP_918567	4.0	—	—	—	—	—
MAP kinase [*A. thaliana*]	CX073386	BAA04866	3.1	—	—	—	—	—
Putative receptor-like protein kinase 4 RLK4 [*A. thaliana*]	AJ489090	BAC42412	4.2	−2.6	—	—	—	—
Serine/threonine protein kinase isolog [*A. thaliana*]	CV717931	AAM20520	3.8	—	6.3	−2.6	—	—
Serine/threonine kinase [*O. sativa*]	CX640171	BAD53292	—	—	3.0	—	—	—
Receptor kinase 1 [*A. thaliana*]	CX292287	BAB02668	—	—	3.3	−2.4	—	—
Protein kinase [*A. thaliana*]	CX302153	NP_177852	4.8	−2.4	5.6	−2.7	—	—
Mitogen-activated protein kinase kinase MKK9 [*A. thaliana*]	CB610685	NP_177492	—	—	3.3	—	—	—
Receptor-like protein kinase [*N. tabacum*]	CX297031	BAC07504	—	—	4.0	—	—	—
Protein kinase-like protein [*A. thaliana*]	CX297218	CAB62310	—	—	10.6	−4.2	—	—
Respiratory burst oxidase 2 [*M. truncatula*]	CX309380	AAW78864	—	—	7.4	—	—	—
FAD-linked oxidase protein [*A. thaliana*]	CX302002	AAQ56827	26.4	−14.2	20.8	−14.0	—	—
Glycolate oxidase [*M. crystallinum*]	CK935794	AAB40396	3.3	−2.0	6.7	−2.3	—	—
CER1-like protein [*A. thaliana*]	CV704769	AAC24373	4.0	—	5.2	—	—	—
Peroxidase [*N. tabacum*]	CV884382	AAK52084	—	—	6.1	−2.3	—	—
Glutathione S-transferase [*E. esula*]	CX669862	AAF72197	—	—	4.2	—	—	—
Hemoglobin [*C. glauca*]	CX636043	CAA37898	—	—	9.9	−4.2	—	—
Thioredoxin family protein [*A. thaliana*]	CN186629	NP_200952	4.7	−2.2	—	—	—	—
Proline oxidase/dehydrogenase 1 [*N. tabacum*]	CX544362	AAT57674	6.0	−2.5	—	—	—	—
Proline dehydrogenase [*G. max*]	CK934047	AAR86686	9.2	−3.8	10.5	−3.8	—	—
Aldehyde dehydrogenase 1 precursor [*L. corniculatus*]	CX297720	AAO72532	4.1	−2.3	3.6	—	—	—
NADH glutamate dehydrogenase [*V. vinifera*]	CX306249	CAC18730	4.3	−2.8	11.7	−4.3	—	—
Leucoanthocyanidin dioxygenase-like protein [*A. thaliana*]	CX674852	AAM91495	8.5	−5.2	4.5	—	—	—
Syringolide-induced protein 1-3-1A [*G. max*]	CN189156	BAB86892	3.7	—	3.8	—	—	—
Ferulate 5-hydroxylase [*C. acuminata*]	CF417783	AAT39511	4.7	—	3.1	—	—	—
N-hydroxylating cytochrome P450 [*M. esculenta*]	CN191734	AAF27289	7.4	−2.4	5.3	—	—	—
Cytochrome P450 [*H. tuberosus*]	CF504933	CAA04117	—	—	3.7	—	—	—
Cytochrome P450 [*P. communis*]	CX644731	AAL66194	—	—	3.5	—	—	—
Cytochrome P450 [*A. majus*]	CX308646	AAS90126	—	—	5.4	−2.2		—
Cytochrome P450 protein [*A. thaliana*]	CF509035	AAM14385	—	—	4.6	−2.0	—	—
Cytochrome P450 [*A. officinalis*]	CX668706	BAB40324	—	−3.9	11.5	−10.2	—	—
Germin-like protein [*A. thaliana*]	CN186507	P92997	—	—	26.9	−19.7	—	—
Nodulin26-like intrinsic protein [*P. sativum*]	DN958192	CAB45652	4.9	−2.0	—	—	—	—
MTD1 [*M. truncatula*]	CX669382	AAF86687	6.5	−3.8	—	—	—	—
Putative lipase [*A. thaliana*]	DN621566	NP_568327	3.6	—	4.0	—	—	—
12-oxophytodienoate reductase OPR2 [*A. thaliana*]	CX043279	BAC42387	6.0	−5.3	10.9	−6.0	—	—
Putative phosphatase [*L. esculentum*]	CX544543	CAD30864	4.7	−2.1	7.8	−2.9	—	—
Putative phosphatase [*L. esculentum*]	CX070476	CAD30863	5.5	−2.2	8.0	−3.5	19.8	3.0
Cysteine endopeptidase, papain-type (XCP2) [*A. thaliana*]	CK935239	NP_564126	−3.3	—	−3.3	—	—	—
Chalcone synthase 2 [*C. sinensis*]	CF833966	Q9XJ57	−3.2	—	−3.6	—	—	—
Aquaporin [*R. communis*]	BQ623782	CAE53882	−4.4	—	—	—	—	—

Table 6 Principal citrus genes related to cell division and morphogenesis up-regulated by Xac at 48 hpi. The complete list of genes related to cell division and morphogenesis modulated by Xac and Xaa is found in Supporting Information Tables S1–S9.

Target description	Citrus	Target	Fold change	
Cell division and morphogenesis	EST	Gene ID	Xac × H_2O	Xac × Xaa
Minichromosome maintenance 7 [*P. sativum*]	CX297398	AAQ72567	4.1	4.4
Minichromosomal maintenance factor [*T. aestivum*]	CV717182	AAS68103	3.4	3.6
Xylogen protein 1 [*Z. elegans*]	CK932766	NP_568984	5.3	—
Nucleosome assembly protein (NAP) [*A. thaliana*]	CX668339	NP_564063	4.5	—
Nucleolin homolog [*P. sativum*]	CV884300	AAA74208	3.5	—
Glycine-rich RNA-binding protein AtGRP2-like [*A. thaliana*]	CX640162	AAM62842	3.1	—
Putative RNA-binding protein [*A. thaliana*]	DN622183	AAM61313	3.4	—
RNA binding protein-like [*A. thaliana*]	CX075891	AAM61280	7.1	—
Putative GAR1 protein [*A. thaliana*]	CF833093	AAM14173	3.4	—
Histone H2A [*C. arietinum*]	CK935733	CAA07234	—	2.4
Histone H2A [*E. esula*]	CX667228	AAF65769	—	2.7
Putative histone H3 [*O. sativa*]	CF836726	XP_475315	—	2.3
Histone H3 [*O. sativa*]	CX643907	NP_910496	—	2.6
Lateral organ boundaries (LOB) domain protein [*A. thaliana*]	BQ623314	NP_172268	26.9	14.7
Gibberellin-regulated protein GASA5 [*A. thaliana*]	CX637545	S71371	24.2	24.9
GASA5-like protein [*P. mariana*]	CB250380	T51963	16.5	15.5
Putative cyclin-dependent kinase CDC2C [*O. sativa*]	CF503956	BAC79804	3.7	3.8
Cyclin d2 [*G. max*]	CV720002	AAS13370	—	2.6
Replication protein A1 [*O. sativa*]	CN190507	AAB71836	3.2	2.4
Calcium-binding protein [*L. corniculatus*]	CK932634	CAB63264	4.3	4.5
Putative WD-repeat protein [*A. thaliana*]	CB417354	AAL87290	3.6	—
Subtilase family protein [*A. thaliana*]	CK936343	NP_565330	7.8	3.5
Putative subtilisin serine protease [*A. thaliana*]	CX045698	AAN13182	7.2	4.6
Prohibitin 1-like protein [*B. napus*]	CV713016	AAK07610	3.1	
Actin [*V. radiata*]	CN184021	AAF31643	3.0	2.1
Beta-tubulin [*G. hirsutum*]	CX672740	AAL92118	3.2	—
Beta-tubulin 2 [*L. albus*]	CX076714	AAB03267	—	3.7
Alpha-tubulin [*P. dulcis*]	CX078454	CAA47635	—	2.3
BY-2 kinesin-like protein 10 [*N. tabacum*]	CF828325	BAB40710	—	2.4
Putative dynein light subunit lc6 [*A. thaliana*]	CX644949	AAM20087	—	3.6
FH protein NFH2 [*N. tabacum*]	CV712442	AAF24497	—	3.6
Myosin [*D. discoideum*]	DT214435	EAL62703	—	3.3
Putative myosin heavy chain-like protein [*S. demissum*]	CV708208	AAT39303	—	2.5
Microtubule-associated protein MAP65-1c [*N. tabacum*]	CX673127	CAC17796	—	2.3
Microtubule-associated protein MAP65/ASE1 [*A. thaliana*]	CF832572	NP_200334	—	3.1

been implicated in cell growth and embryo development, respectively (Germain *et al.*, 2005; Weterings *et al.*, 2001).

The majority of differentially expressed genes implicated in cytokinesis were nevertheless detected 48 h after Xac infection (Fig. 2A and Tables 6, S2 and S3). Such a change in the transcriptional profile from 6 to 48 hpi indicates an alteration in the physiological status of the host cells, which would be reprogrammed for division and growth. For instance, the various histones and ribosomal proteins up-regulated by Xac may reflect the requirements of dividing cells but also an increase in general transcriptional activity. It is known that the state of chromatin has a major effect on the levels of gene expression, and histone modifications such as acetylation play an important role in regulation of transcription in eukaryotes (Sterner and Berger, 2000). It is therefore interesting to note that Xac specifically up-regulated a GCN5 histone acetyltransferase (CF417594) known to regulate histone acetylation required for gene expression (Benhamed *et al.*, 2006), plant growth and development (Vlachonasios *et al.*, 2003) in Arabidopsis (Tables 6, S5 and S6).

Consistent with an increased transcriptional activity of ribosome biosynthesis genes, Xac induced the expression of several RNA recognition motif (RRM) proteins such as nucleolins (CV884300, CF833651) and GAR1 (CF833093) required for rDNA chromatin organization and ribosome synthesis (Girard *et al.*, 1992; Pontvianne *et al.*, 2007) (Tables 6, S5 and S6). Significantly, expression of nucleolin correlated with cell proliferation in alfafa

(Bogre *et al.*, 1996) and mutations in the Arabidopsis nucleolin PARL1 affected leaf development (Petricka and Nelson, 2007).

Xac also up-regulated genes implicated in cell cycle control, including nucleosome assembly protein (NAP1), minichromosome mantainance (MCM) factors, fringe, response regulators and cyclin-dependent kinases (Tables 6, S2 and S3). NAP1 facilitates nucleosome assembly by acting as a histone chaperone, and in tobacco it binds to histone 2A, cyclin and tubulin (Dong *et al.*, 2005), proteins that were abundantly detected in Xac-infected leaves (Tables 6, S2 and S3). Similar to NAP1, which controls cell proliferation and expansion during Arabidopsis leaf development (Galichet and Gruissem, 2006), MCM proteins play an important role in cell cycle control and are essential for the initiation of DNA replication (Braun and Breeden, 2007; Stevens *et al.*, 2002). Notably, fringe genes regulate dorsal–ventral boundary determination in animal cells through modulation of the Notch pathway, which is connected to the MCM complex during cell cycle progression (Jinek *et al.*, 2006; Noseda and Karsan, 2006).

In addition to tubulins, Xac induced various cytoskeleton proteins including actin, formin, myosin, kinesin, dynein and microtubule-associated proteins, known to be required for the progression of cytokinesis (Favery *et al.*, 2004 Shima *et al.*, 2006; Tanaka *et al.*, 2004). For instance, the citrus EST CV712442 is homologous to AtFH6, an Arabidopsis formin that is up-regulated in hypertrophied giant cells induced by nematodes (Favery *et al.*, 2004) (Tables 6, S2 and S3). Thus, the up-regulation of cytoskeleton proteins in Xac-infected leaves is consistent with the idea that actin cables and microtubules are not only required for cytokinesis but also for the vesicle trafficking associated with cell-wall biogenesis discussed above.

Xac also induced the expression of genes homologous to LATERAL ORGAN BOUNDARIES (LOB), GASA and subtilases related to ABNORMAL LEAF SHAPE (ALE1) (Tables 6, S2 and S3). These proteins have been implicated in lateral organ fate, GA responses and epidermal surface formation, respectively (Ha *et al.*, 2007; Roxrud *et al.*, 2007; Tanaka *et al.*, 2001). In particular, both LOB (BQ623314) and GASA (CX637545 and CB250380) homologues were strongly induced by Xac at 48 hpi (Table 6), and this might be associated with the up-regulation of GA biosynthesis genes (Table 2).

Ethylene synthesis and signalling

Ethylene regulates a variety of developmental processes and stress responses in plants, including seed germination, cell elongation, senescence, fruit ripening and defence. Nonetheless, it has long been known that ethylene can either promote disease resistance or susceptibility depending on the host–pathogen interaction (Broekaert *et al.*, 2006). Here, we observed considerable changes in the transcriptional profiles of genes related to ethylene synthesis, signalling and perception after Xac and Xaa infection, suggesting that these bacteria stimulate ethylene production in citrus plants (Fig. 2). However, although both bacteria were capable of modulating the expression of key genes of ethylene biosynthesis including 1-aminocyclopropene-1-carboxylic acid (ACC) synthase and ACC oxidases, ethylene receptors and various transcriptional factors carrying an AP2/EREBP-like domain, most of these genes were induced at higher levels by Xaa at 6 and 48 hpi (Fig. 2A, Table 7 and Supporting Information Tables S2–S9).

ACC synthase and ACC oxidase are known to play pivotal roles in ethylene biosynthesis and in many cases their expression levels are affected by pathogen attack (Broekaert *et al.*, 2006). Interestingly, Xaa specifically up-regulated an ACC synthase (AJ012696) at 48 hpi (Table 7), indicating that ethylene synthesis is maintained during Xaa relative to Xac infection. This idea is supported by the fact that genes related to ACC oxidases (CX292966, CX302270), ethylene receptor (CX674473), ethylene-induced esterases (CB290370, CF835645) and ethylene response factors (ERF) (CN185598, CX043799) were detected at higher levels in Xaa-infiltrated leaves and additional ERF and AP2/EREBP genes were specifically modulated by Xaa at 48 hpi (Table 7). By contrast, Xac up-regulated a smaller number of ethylene synthesis and signalling genes, including AP2/EREBP, and specifically down-regulated a beta-cyanoalanine that catalyses the detoxification of cyanide formed during ethylene biosynthesis (Yip and Yang, 1988), suggesting that ethylene production in Xac-infiltrated leaves was lowered by 48 hpi (Fig. 2, Table 7).

These observations suggest that, at least in the Xaa–Pêra interaction, ethylene synthesis and signalling might be associated with disease resistance rather than susceptibility. The increased number of ERF- and AP2/EREBP-related genes modulated by Xaa supports this notion. These factors control the expression of many PR proteins and effectors of the defence response (Broekaert *et al.*, 2006) and their increased expression in Xaa-infiltrated leaves correlates with the higher PR gene expression induced by Xaa (Table 3). Accordingly, as ethylene and JA can act synergistically to induce certain PR proteins in defence reactions it is interesting to note that Xaa also up-regulated a number of genes related to JA biosynthesis including lipoxygenases (Table S1) and 12-oxophytodienoate reductase (Table 5). Significantly, consistent with the cross-talk that occurs between defence response pathways controlled by JA, ethylene and salicylic acid (SA), the EST CB290370, which is preferentially up-regulated by Xaa, encodes an ethylene-induced esterase that is similar to SABP2, an SA-binding protein from tobacco required for plant immune response (Kumar and Klessig, 2003).

Taken together, the transcriptional profiles of ethylene synthesis and signalling genes of Xac- and Xaa-infiltrated leaves strongly suggest that ethylene plays a key role in the defence response against Xaa.

Table 7 Principal ethylene synthesis and signalling genes modulated by Xac and Xaa at 6 and 48 hpi, relative to water-infiltrated leaves. The complete list of ethylene synthesis and signalling genes with altered expression upon Xac/Xaa infection is found in Supporting Information Tables S2–S9.

Target description	Citrus	Target	Change after treatments			
			6 hpi		48 hpi	
Ethylene synthesis and signalling	EST	Gene ID	Xaa	Xac	Xaa	Xac
Xac- and Xaa-regulated genes						
ACC synthase [*C. sinensis*]	CX643923	CAB60722	3.05	4.0	—	—
ACC oxidase [*C. sinensis*]	CB322167	AAG49361	4.21	3.9	4.8	3.8
Ethylene-forming-enzyme [*A. thaliana*]	CX292966	BAB10730	16.48	12.5	18.8	5.9
Ethylene-forming-enzyme [*A. thaliana*]	CX302270	AAM65315	26.52	21.3	26.9	9.3
Ethylene-induced esterase [*C. sinensis*]	CB290370	AAK58599	8.54	4.8	27.7	5.3
Ethylene-induced esterase [*C. sinensis*]	CF835645	AAK58599	10.40	4.8	105.9	18.4
AP2 domain transcription factor [*A. thaliana*]	CX299615	BAB08875	17.22	4.5	12.9	50.0
AP2 domain transcription factor [*A. thaliana*]	CF509751	NP_196837	—	—	10.1	6.4
ATERF5 [*A. thaliana*]	CK936491	BAA97157	5.81	5.7	—	—
AtERF1 [*A. thaliana*]	CK939541	O80337	5.64	5.0	—	—
ERF [*L. esculentum*]	CN185598	AAN77067	—	—	15.7	4.5
Ethylene receptor [*Fragaria* × *ananassa*]	CX674473	CAC48386	—	—	32.3	13.0
Putative ethylene receptor [*C. sinensis*]	CX667638	AAC99435	—	—	5.7	4.0
DNA binding protein S25-XP1 [*N. tabacum*]	CX043799	T03927	—	—	16.3	6.8
AP2/EREBP transcription factor [*A. thaliana*]	CX546122	AAT44918	—	—	–4.1	–4.2
AP2 domain transcription factor [*A. thaliana*]	CK938211	NP_201520	—	—	–4.9	–3.5
Xac-regulated genes						
EREBP [*N. tabacum*]	DN617716	BAA07323	—	3.0	—	—
AP2 domain-containing protein [*A. thaliana*]	CX298932	NP_172723	—	3.3	—	—
DREBP 5 [*G. max*]	CX665765	ABQ53928	—	–3.2	—	5.9
Beta-cyanoalanine synthase [*B. pendula*]	DN958104	AAN86822	—	—	—	–5.6
Xaa-regulated genes						
ACC synthase [*C. sinensis*]	AJ012696	CAB60831	—	—	5.0	—
AP2/EREBP [*G. hirsutum*]	CB292635	AAV51937	—	—	5.6	—
ERF like protein [*C. melo*]	CK936741	BAD01556	—	—	4.0	—
Transcriptional activator PTI5 [*L. esculentum*]	CN187333	O04681	—	—	3.6	—
DNA binding protein S25-XP1 [*N. tabacum*]	DN795374	T03927	—	—	9.4	—
AP2 domain-containing protein [*A. thaliana*]	CB304607	NP_176620	—	—	–4.2	—
AP2 domain-containing protein [*A. thaliana*]	CX288967	CAB96654	—	—	–3.9	—

Retroelement and transposition

A number of differentially expressed genes identified by microarray (CX072598, CN182725, CN182543, CN183624) and DD/SSH (AF369930, CX052914) are homologous to copia-like retrotransposons and polyproteins. These genes were up-regulated by both Xac and Xaa at 6 and 48 hpi; however, their expression levels were relatively higher in Xaa-infected leaves (Tables S1–S9).

In plant genomes, retrotransposons constitute the major mobile genetic elements and are important for the evolution of the genome structure and function (Grandbastien, 1992). Interestingly, most of the citrus genes identified here are homologous to retrotransposons that have been mapped in close proximity or within clusters of resistance genes (Parniske and Jones, 1999; Wei *et al*., 2002), and in such genome organizations they are thought to provide resistance gene duplications, insertions or deletions generating new recognition specificities (Paal *et al*., 2004). This is reminiscent of the systemic DNA rearrangement induced by a viral pathogen in tobacco, which suggested the existence of a systemic recombination signal (Kovalchuk *et al*., 2003). Accordingly, the expression of the tomato TCL1.1 retrotransposon is induced by several plant hormones, but most notably by ethylene (Tapia, *et al*., 2005). Thus, it is tempting to speculate that the up-regulation of citrus mobile elements by Xac and Xaa might be the result of the transcriptional changes in the ethylene synthesis and signalling genes described above. Also in agreement with the up-regulation of retrotransposons in citrus leaves, Xac and Xaa induced the expression of various citrus ESTs (CK933117, CN186009, CF509054, CF832466) homologous to tobacco MYB2, a transcriptional factor that recognizes conserved DNA motifs in the Tto1 retrotransposon promoter and activates its transcription (Sugimoto, *et al*., 2000).

Carbon and nitrogen metabolism

Both Xac and Xaa altered the expression of a significant number of genes related to carbon and nitrogen metabolism (Fig. 2). Among the commonly modulated genes, we highlight the up-regulation of invertases, asparagine, glutamine and tryptophan synthases, phosphoenolpyruvate-carboxylase kinases, succinyl-CoA ligases, prolyl-carboxypeptidase, 3-deoxy-D-arabino-heptulose 7-phosphate synthases and a number of sugar, amino acid and nitrate transporters, most of which were strongly induced at 6 and 48 hpi. By contrast, genes commonly down-regulated by Xac and Xaa include the small subunit of Rubisco, Rubisco activase, fructose-1,6-bisphosphatase, fructose-bisphosphate aldolases and serine hydroxylmethyltransferase (Tables S1, S8 and S9).

These transcriptional changes are thus in agreement with the fact that plants infected with biotrophic pathogens normally show lower photosynthesis rates, increased protein degradation and higher nutrient mobilization towards infection sites. For instance, reduction in photosynthesis has been attributed to an early expression of invertases and the accumulation of hexoses (Swarbrick *et al.*, 2006; Walters and McRoberts, 2006). Interestingly, among the various sugar transporters up-regulated at 6 and 48 hpi is a hexose transporter (CF509967) (Tables S8 and S9). In addition, the elevated expression of asparagine synthase, glutamine synthase and nitrate transporters suggest an increased mobilization of nitrogen in the infection site, whereas the strong induction of a prolyl-carboxypeptidase indicates aumented protein catabolism.

We found, however, that Xaa altered the expression of a significantly greater number of carbon and nitrogen metabolism genes relative to Xac at 6 and 48 hpi (Fig. 2). In particular, Xaa induced various amino-transferases, beta-amilases and additional sugar and amino acid transporters (Tables S1, S2, S4, S5 and S7). This suggests that nutrient mobilization might be associated with the energy supply required for defence reactions or, as pointed out recently, it could act as a signal for defence (Halford and Paul, 2003; Swarbrick *et al.*, 2006). Accordingly, Xaa induced the expression of specific trehalose-6-phosphate synthase (CX299159) and trehalose-6-phosphate phosphatases (CX636014, CF831824), both involved in the synthesis of trehalose (Tables S2, S4, S5 and S7). These sugar metabolites have gained the status of signalling molecules that regulate plant metabolism, development and stress responses (Grennan, 2007; Wilson *et al.*, 2007). Moreover, two Xaa-induced ESTs (CX672184, CX639947) are homologous to sucrose nonfermenting-1 (SNF1), a protein kinase that plays a central role in trehalose metabolism, sugar signalling and homeostasis (Polge and Thomas, 2007). Most significantly though, an Arabidopsis SNF1-related protein kinase (SnRK1) was shown to interact with proteins implicated in pathogen resistance (Gissot *et al.*, 2006). Hence, it appears that the changes in trehalose biosynthesis enzymes and SNF1-related kinase observed in Xaa-infected leaves might indeed be associated with a defence response.

Xac induced the expression of a trehalose-6-phosphate phosphatase (CD575394) but not trehalose-6-phosphate synthase or SNF1 (Table S3). It is interesting to note that Arabidopsis plants overexpressing trehalose-6-phosphate phosphatase exibited larger leaves than wild-type plants and the accumulation of trehalose-6-phosphate caused inhibition of seedling growth (Schluepmann *et al.*, 2003; 2004), indicating that up-regulation of trehalose-6-phosphate phosphatase alone promotes cell growth.

Putative transcription factors

Several genes homologous to transcription factors showed altered expression after Xac and Xaa infection, particularly at 48 hpi (Fig. 2 and Tables S2–S9). Some of the genes that were up-regulated by Xac and Xaa (CV884345, CX295885, CK739629 and CN187819) are similar to tobacco BZI-1, a bZIP factor that binds the GH3 gene promoter and modulates auxin-induced transcription (Heinekamp *et al.*, 2004). This correlates with the up-regulation of GH3 genes by both pathogens at 48 hpi (Table 2). In addition, we highlight the up-regulation of a gene (CX669831) homologous to ORCA3, a JA-induced transcription factor involved in the activation of terpenoid biosynthetic genes (Vom Endt *et al.*, 2007).

Interestingly, Xac specifically up-regulated a bZIP transcriptional activator (CV719678) similar to RSG (repression of shoot growth), which regulates tobacco cell elongation by controlling the levels of gibberellins (Fukazawa *et al.*, 2000) (Table S3). In contrast, Xaa specifically repressed the expression of a basic helix–loop–helix factor (CX045057) implicated in cell proliferation (Heim *et al.*, 2003), whereas it up-regulated a gene (CF509564) homologous to CCR4-associated factor CaCAF1, required for defence response. Notably, silencing of CaCAF1 in pepper plants increased susceptibility to Xcv infection (Sarowar *et al.*, 2007). In addition, Xaa up-regulated two ESTs (CX543536, CF832559) related to GT-2 factors implicated in auxin signalling (O'Grady *et al.*, 2001), which might be associated with the increased up-regulation of auxin-responsive genes induced by Xaa at 6 hpi (Fig. 2 and Table S7).

Unknown genes

On average, approximately 35% of all citrus genes identified by microarray analyses as differentially expressed in response to Xac or Xaa infection show no similarities to plant genes or are homologous to genes of unknown function (Fig. 2). In addition, nearly 40% of the genes identified by DD/SSH showed no similarities to known genes or to citrus ESTs (Table S1). This reflects our current limited knowledge of gene functions related to disease resistance and symptom development.

GENERAL DISCUSSION

In this study, we focused on the early transcriptional modifications that occur in sweet orange leaves after Xac and Xaa infection with the aim of understanding the differential pathogenicity exhibited by these bacteria on Pêra plants and identifying the genes involved in canker development. We provide strong evidence for a coordinated expression of cell-wall remodelling, vesicle trafficking and cell division genes that are likely to play a role in symptom development. This is supported by the observation that the formation of canker lesions is inhibited by brefeldin A, a clear indication of the functional connection between these categories of genes. On the other hand, our data point to a much stronger defence response triggered by Xaa. Interestingly, however, the expression profiles of the Xac/Xaa commonly regulated genes are remarkably similar to transcriptional changes triggered by PAMPs in other plant–pathogen interactions, which is in accordance with the idea that transcriptional differences in response to bacterial challenges are predominantly determined by PAMPs (Navarro *et al.*, 2004; Truman *et al.*, 2006). Moreover, the major differences observed in the transcriptional profiles between Xac- and Xaa-infected leaves are in agreement with the proposed zigzag model of plant immunity (Jones and Dangl, 2006), in which Xac would suppress PAMP-triggered immunity (PTI) via translocation of effector proteins including PthAs (Fujikawa *et al.*, 2006). This idea is supported by the fact that a significantly smaller number of defence-related genes are altered by Xac at 6 and 48 hpi relative to Xaa, and although Xac induced a great number of plant defence genes, the amplitude of this response is not sufficient to halt Xac's growth or to establish an effective HR, as envisaged in the zigzag model.

Although it is not yet clear how Pêra plants stop Xaa exponential growth, the results shown here indicate that Xaa induces a MAP kinase cascade that would culminate with the activation of a variety of defence-related genes involved in ROS production and programmed cell death. The inability of Xaa to suppress defences on sweet orange suggests that it cannot successfully translocate effector proteins to block PTI, or one of its effectors might be recognized by the host triggering defence. Significantly, we cloned two *pthA* homologues from Xaa that are more similar to *pthB* and *pthC* genes recently characterized in another representative C strain of Xaa (Al-Saadi *et al.*, 2007) than to the four *pthA*s from Xac (da Silva *et al.* 2002). This raises the possibility that one of them may act as an avirulence factor in sweet orange, and our current knowledge on the mechanism of action of this class of bacterial effectors suggests that they could transactivate a citrus resistance gene (Römer *et al.*, 2007). Although *pthC* from Xaa strain C340 does not appear to act as an *avr* gene (Al-Saadi *et al.*, 2007), a recent study has shown that the *pthA* homologue *hssB3.0* is responsible for host-specific suppression of virulence in *Citrus grandis* (Shiotani *et al.*, 2007). This is the first evidence indicating that PthA proteins can behave as avirulence factors on citrus. Interestingly, *hssB3.0* reduced the ability of Xac to multiply in plant tissues, enhanced PAL expression and partially interrupted canker development elicited by another PthA homologue (Shiotani *et al.*, 2007), a phenomenon that in many aspects resembles the Pêra–Xaa interaction, where micropustules developed in spite of a resistance response. As citrus canker is dependent on very similar PthA-like proteins (Al-Saadi *et al.*, 2007) that dimerize in the host cell prior to nuclear import (Gürlebeck *et al.*, 2005), it is possible that depending on the repertoire of PthA proteins delivered, different heterodimers may form and their interaction with host targets may determine the outcome of the host response, whether disease resistance or canker development.

EXPERIMENTAL PROCEDURES

Plant material and bacterial infiltration

Six-month-old plants of sweet orange (*C. sinensis*) 'Pêra' and 'Cristal' cultivars as well as Mexican lime 'Galego' (*Citrus aurantifolia*) and 'Siciliano' lemon (*C. limon*) were obtained from certified nurseries and kept in a growth room at 25–28 °C under a 14-h/day fluorescent light. Plant leaves were infiltrated with suspensions of *Xac* (strain 306, da Silva *et al.*, 2002) or *Xaa* pathotype C (strain ICMP 8435). Bacterial cells grown in LB medium without NaCl (LBON) for 48 h at 28 °C with shaking at 200 r.p.m. were recovered by centrifugation and resuspended in sterile water (OD_{600nm} = 0.6). Leaf sectors were infiltrated with approximately 0.3 mL of the bacterial suspensions or water as control.

For brefeldin A experiments, Pêra leaves were pre-infiltrated with solutions of 0.2 mM brefeldin A (Sigma) in 0.5% DMSO or 0.5% DMSO only as control. After 24 h, the same leaf sectors were infiltrated with a water suspension of Xac (OD_{600nm} = 0.5) and the appearance of canker pustules was monitored daily. Brefeldin A did not affect Xac growth on culture media.

Bacterial growth curves

Bacterial cells grown in LBON for 24 h were pelleted and resuspended in sterile water to a cell density of 10^7 colony forming units (cfp) per mL. Cells were infiltrated on *C. sinensis* leaves and after different time intervals leaf discs were collected for bacterial recovery and counting of the number of viable cells.

Plant mRNA purification

Total RNA was extracted from sweet orange leaves at different time intervals after water and bacterial infiltration treatments using Trizol (Invitrogen), followed by mRNA purification with FastTrack 2.0 (Invitrogen). The quality and quantity of the mRNA

samples were verified by agarose gel and UV spectroscopy (Sambrook and Russel, 2001).

Differential display and suppressed subtractive hybridization

Differential display was performed according to standard procedures (Sambrook and Russel, 2001), except that first-trand cDNA was synthesized using ordinary oligo-dT, instead of anchoring primers, and the cDNAs were amplified by PCR using a set of decamer oligonucleotides (Operon). A total of 40 different combinations of decamer pairs were used in PCR reactions in the presence of α^{33}P-dATP. PCR products were separated in denaturing sequencing gels and the differentially expressed bands, detected by autoradiography, were re-amplified, cloned in pGemT (Promega) and sequenced.

Suppressed subtractive hybridization was carried out according to Diatchenko *et al*., (1999) with the exception that an *Nla*III site was inserted at the 3′-end of the adaptors in order to favour adaptor ligation to *Nla*III-cut cDNAs. Double-strand cDNAs were prepared from mRNAs isolated from sweet orange leaves infiltrated with Xac or Xaa for 48 h and from leaves infiltrated with Xac or water for 10 days, when canker lesions were visible. Subtractions were made between cDNAs from Xac- (tester) versus Xaa-infiltrated leaves (driver) and from Xac- (tester) versus water-infiltrated leaves (driver). After subtraction, approximately 1000 clones were isolated and screened for differentially expressed cDNAs by dot blot, as described (Diatchenko *et al*., 1999).

Quantitative PCR and Northern blot analysis

Primers for qRT-PCR were designed using the Primer Express 3.0 software (Applied Biosystems). cDNAs from water- or pathogen-infiltrated leaves were reverse transcribed using oligo-dT (Sambrook and Russel, 2001) and used at different concentrations to test the efficiency of the amplifications between targets and internal controls. Two internal controls were used (CK937155-hypothetical protein and AAR89627-elongation factor). The expression levels of both internal controls in Xac-, Xaa- or water-infiltrated leaves were verified by Northern blotting and shown to have similar banding patterns (not shown). Three qRT-PCR reactions were performed on each mRNA sample using the SYBR-green mix and the universal conditions of amplification provided by the 7500 System (Applied Biosystems). Dissociation curves were analysed in every amplification mixture and when more then one peak was observed, the qRT-PCR conditions were changed so as to obtain a single dissociation product. The results were analysed by the 7500 System software (Applied Biosystems) using the relative quantification mode.

Northern blotting was performed using formaldehyde gels (Sambrook and Russel, 2001). Approximately 10 μg of total RNA extracted from water- or pathogen-infiltrated orange leaves was separated in denaturing agarose gels and transferred to nylon membranes. After hybridization to specific ^{32}P-labelled DNA probes, membranes were washed and images were captured from the exposed phosphoimager screens.

Microarray analysis

Two independent mRNA samples (0.6 μg) extracted from citrus leaves infiltrated with water, Xac or Xaa at 6 and 48 hpi, were hybridized to Affymetrix GeneChip citrus genome arrays (Affymetrix, Santa Clara, CA) according to Affymetrix's instructions. Stained arrays were scanned on the Gene Chip Scanner 3000–7G using the Gene Chip Operating Software (GCOS) version 1.4. Filter on calls, variance stabilization, log transformation, background subtraction, average computing and significance analysis ($P \leq 0.05$) were obtained from two GCOS CEL files of each treatment using the MAS5 algorithm from the ArrayAssist software package (ArrayAssist x.5, Stratagene, USA). The microarray data have been deposited in GEO database as GSE10798 series.

ACKNOWLEDGMENTS

We thank Júlio Rodrigues Neto for providing the Xaa pathotype C strain and Patrick Cooke and Adriana C. Alves for technical help with the Affymetrix microarray system. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grants 98/14138-2; 00/10266-8; 03/08316-5). R.A.C. and C.E.B. received fellowships from FAPESP and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), respectively.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Differential expression of cell-wall-remodelling genes between Xac- and Xaa-infected leaves analysed by Northern blot. Total RNA extracted from sweet orange leaves 48 h after Xac or Xaa infiltration was hybridized to DNA fragments corresponding to endo-beta glucanase (CV886058), acidic cellulase (AAB56555), pectinesterase (CN181845) and expansin (DN621554).

Fig. S2 Expression levels of a putative cysteine protease (DN623815) in sweet orange (Pêra) and Mexican lime plants infected with Xac or Xaa at 48 hpi, determined by qRT-PCR.

Table S1 *C. sinensis* genes with altered expression in response to Xac or Xaa infection identified by DD and/or SSH and validated by qRT-PCR. Fold change in gene expression relative to water-infiltrated leaves (= 1.0) is the average of three qRT-PCR measurements. Positive and negative values represent up- and down-regulation, respectively- and 'nd' means quantification not determined. *Citrus* ESTs that showed a perfect match with probesets of the Affymetrix citrus array are shown in bold, whereas bold-underlined indicates ESTs that were represented in the citrus array but were not detected in the microarray experiments. Fold change values of Xac versus Xaa at 48 hpi, determined by microarray, are shown for comparison with the qRT-PCR measurements.

Table S2 Sweet orange genes regulated by Xac relative to Xaa at 48 hpi with fold change = 2.0.

Table S3 Sweet orange genes specifically regulated by Xac at 48 hpi with fold change = 3.0.

Table S4 Sweet orange genes specifically regulated by Xaa at 48 hpi with fold change = 3.0.

Table S5 Sweet orange genes regulated by Xac relative to Xaa at 6 hpi with fold change = 2.0.

Table S6 Sweet orange genes specifically regulated by Xac at 6 hpi with fold change = 3.0.

Table S7 Sweet orange genes specifically regulated by Xaa at 6 hpi with fold change = 3.0.

Table S8 Sweet orange genes commonly regulated by Xac and Xaa at 6 hpi, relative to water-infiltrated leaves, with fold change = 3.0.

Table S9 Sweet orange genes commonly regulated by Xac and Xaa at 48 hpi, relative to water-infiltrated leaves, with fold change = 3.0.

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