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Responsiveness of different citrus genotypes to the *Xanthomonas citri* ssp. *citri*-derived pathogen-associated molecular pattern (PAMP) flg22 correlates with resistance to citrus canker

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SUMMARY

The bacterial agent of citrus canker disease (*Xanthomonas citri* ssp. *citri*, *Xcc*) has caused tremendous economic losses to the citrus industry around the world. Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) is important to plant immunity. In this study, we compared the defence responses of citrus canker-resistant and citrus canker-susceptible genotypes to the *Xcc*-derived PAMP flg22 (Xflg22) by analysing the expression of 20 citrus defence-associated genes. We showed that, in the most resistant genotype, 'Nagami' kumquat, there was significant induction of several defence genes (*EDS1*, *NDR1*, *PBS1*, *RAR1*, *SGT1*, *PAL1*, *NPR2* and *NPR3*) as early as 6 h and up to 72 h after Xflg22 treatment. At the other end of the spectrum, highly susceptible 'Duncan' grapefruit showed no induction of the same defence genes, even 120 h after treatment. Citrus genotypes with partial levels of resistance showed intermediate levels of transcriptional reprogramming that correlated with their resistance level. Xflg22 also triggered a rapid oxidative burst in all genotypes which was higher and accompanied by the induction of PTI marker genes (*WRKY22* and *GST1*) only in the more resistant genotypes. Pretreatment with Xflg22 prior to *Xcc* inoculation inhibited bacterial growth in kumquat, but not in grapefruit. A flagellin-deficient *Xcc* strain (*XccΔfliC*) showed greater growth increase relative to wild-type *Xcc* in kumquat than in grapefruit. Taken together, our results indicate that Xflg22 initiates strong PTI in canker-resistant genotypes, but not in susceptible ones, and that a robust induction of PTI is an important component of citrus resistance to canker.

Keywords: canker, citrus, flg22, PAMP, PTI, resistance, *Xanthomonas citri* ssp. *citri*.

INTRODUCTION

Citrus is one of the most economically important crops for both the fresh and processed fruit markets. The bacterial disease citrus canker, caused by *Xanthomonas citri* ssp. *citri* (*Xcc*), has been damaging to the industry and has become endemic in many parts of the world. In certain areas (e.g. Florida), this has caused losses as a result of the eradication of millions of trees in an attempt to control this disease. In addition, restrictions have been imposed in the interstate and international transportation of citrus products because of quarantines and other regulatory measures (Bronson and Gaskalla, 2007), and has increased the costs of management of the groves. Most commercially grown citrus types, including sweet orange (*C. sinensis* Osb.), grapefruit (*C. paradisi* Macf.) and lemon [*C. limon* (L.) Burm. f.], are susceptible to canker (Goto, 1992; Gottwald *et al*., 1993, 2002), and disease control methods are limited in effectiveness (Dewdney and Graham, 2011; Graham and Leite, 2004; Granado *et al*., 1995; Leite and Mohan, 1990). Genetic resistance has the potential to become a solution for the management of this disease. Genotypes with disease-resistant traits have been identified and serve as valuable resources for breeding. Kumquat [*Fortunella margarita* (L.) Swing.], a citrus relative that is highly resistant to canker, is one such example (Goto, 1992; Reddy, 1997). Moreover, the recent characterization of the kumquat–*Xcc* interaction offers insights into the canker resistance response at the cellular and molecular levels (Khalaf *et al*., 2007).

Plants have two levels of immunity to defend themselves against microbial invasions. At one level, plants respond to pathogen-associated molecular patterns (PAMPs). Plant recognition of PAMPs is mediated by cell membrane-localized pattern recognition receptors (PRRs), which trigger complex signalling events and a defence response resulting in the limitation of the microbe's multiplication and disease development (PAMP-triggered immunity, PTI) (Asai *et al*., 2002; Jones and Dangl, 2006; Zipfel and Felix, 2005; Zipfel *et al*., 2004). At another level, adapted pathogens are able to secrete effector proteins into host cells and modulate plant defences. In turn, plants have evolved resistance (R) proteins to recognize such effectors and initiate a strong defence response that often leads to pathogen resistance (effector-triggered immunity, ETI) (Dangl and Jones, 2001; Jones and Dangl, 2006; Qutob *et al*., 2006).

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Table 1 List of primer and probe sequences used to analyse the expression of defence-associated genes in citrus. The assay names are based on homology to genes characterized in *Arabidopsis*. Loci of the respective genes are from the *Citrus sinensis* genomic database available at the National Center for Biotechnology Information (NCBI). Forward primer (f), reverse primer (r) and probe (p) sequences were generated using Primer Express Software 3.0 (Applied Biosystems, Foster City, CA, USA)**.**

Assay	Primer/probe	*Citrus sinensis* locus	Assay	Primer/probe	*Citrus sinensis* locus
5.8S rRNA	f: CGACTCTCGGCAACGGATA r: CGCATTTCGCTACGTTCTTCA p: CTCGGCTCTCGCATC	JQ990165	*NPR1*	f: CGTGGCATATTGTGATGCAAA r: GTTGACATCAGCAAGTCCAAGATC p: ACCACAACTGAGCTTC	LOC102617188
AZI1	f: CCATCAAAGCGAACATTTTGG r: CGTTCAAAAGAAGGCTGAGTGA p: ATCAACCTTAATATCCC	LOC102617861	*NPR2*	f: ACCTTAGACGAAGCCAATGCA r: CAGACAACACCTTGGGATCACA p: TCCATTATGCTGCAGCGTA	LOC102621158
CHI	f: GCCGGCTTCCGGGATAC r: CTTGGCCACATTCAATTCCA p: CTAACCACAAATATAATCAACG	Z70032	*NPR3*	f: TTTTATACTGGCCTTTCAGCATCA r: CGTCTCAACTGTTTTAAGCAAAGC p: TGCAAGCCAAGAGAC	LOC102624339
COI1	f: GGGAATGGAGGATGAAGAAGGT r: GCCCTGAGCCAAAGCAATTA p: TTGTCTCGCAAAGAGGA	LOC102620384	*PAL1*	f: CTCGGCCCTCAGATCGAA r: CCGAGTTGATCTCCCGTTCA p: TGATTCGGTTTGCAACCA	LOC102620173 LOC102620464
EDR1	f: TCCAGGAGTGCTTTGAGTGGTA r: GCCCATTTAACTGACTTGTGCTAGA p: TGGCCCATCATTGG	LOC102618775	*PBS1*	f: TCCAAAAGAACCAACTGCACAT r: AGCAGCAAGCTCCCGAAAT p: CCGCCCAAACGTTTA	LOC102630702
EDS1	f: GGCTCGAGTATGCCCTGAAG r: CTTGCCCAGAAACATGATTCC p: ATCGGCAGGATCCAG	LOC102618041	*PR1*	f: AAGGAAAGCGGATTGCAAACT r: CTCGCCAAGCTTGAAATTGTC p: CAGCATTCGTTCCCG	LOC102622841
EDS5	f: ATCGAAGTTTGGTAAAGGCAAGA r: AGCGTGGATCCAATGAGAAGA p: TGCTGCTGAAGTCAC	LOC102615350	*RAR1*	f: GTCAGAACGACGACGCTTTG r: GCGTTGCAGCCAATTCG p: AGCGTCTTCGATGCC	LOC102626257
GST1	f: GCCCGTTTGTCTCAGTCCAA r: TGCAAATCGACCAAGGTGAA p: ACTTGGCGTGCGACAG	LOC102614737	*RdRp1*	f: CGGCAGCGCTTTATTGTTC r: ACATCACCTGGATGCAAACAAG p: ACTGGTGGTTGTTGCAA	LOC102608479
ICS1	f: CAGCGCTGGCCTTGGA r: GGAGGTGGGTTGGATTTCAA p: AAACTTCACTCTGCCATTT	LOC102630235	*SGT1*	f: GCTGATGCAGATGAGGACACA r: CCCGTTTGACTCGACGAAAG p: ACGAGCCATGAAAAA	LOC102623505
JAR1	f: AAGGCGATGCAGTCACAATG r: TGGTGGAAATCAGGACCAAAG p: AGCCCTGATGAAGTAA	LOC102611440	*WRKY22*	f: GCGGATTGTCTCGCATGTG r: TTATGGGTTTCTGCCCGTATTT p: AAGTGGGCTTGGCG	LOC102612567
NDR1	f: GCGCCGACCGATCAGA r: CGCCGACCCATTTGCA p: TTTCCCGGGCGGTTT	LOC102630232			

PTI is a broad-spectrum immune response that can protect plants from a wide range of microbes by sensing PAMPs (Ebel and Cosio, 1994). Flagellin, one of the best studied PAMPs, is a protein component of the filament of the bacterial flagellum. Within the N-terminus of flagellin, a conserved 22-amino-acid domain (flg22) is recognized by plants and is capable of initiating PTI (Felix *et al*., 1999). In *Arabidopsis*, flg22 perception results in the induction of defence-associated genes (Navarro *et al*., 2004), reactive oxygen species (ROS) production (Felix *et al*., 1999), salicylic acid (SA) accumulation (Tsuda *et al*., 2008), and local and systemic acquired resistance (SAR) to pathogen infection (Mishina and Zeier, 2007b; Zipfel *et al*., 2004). To investigate PTI in citrus and its role in canker immunity, we used the flg22 derived from *Xcc* (Xflg22) to challenge and compare the expression responses of defence-associated genes in different citrus genotypes, ranging from highly resistant to highly susceptible to canker. The genes selected represent key functional nodes of plant defence (Table 1): PTI and ETI perception and signalling [*EDR1* (*enhanced disease resistance 1*), *EDS1* (*enhanced disease susceptibility 1*), *NDR1* (*non-specific disease resistance 1*), *PBS1* (*avrPphB susceptible 1*), *RAR1* (*required for Mla12 resistance 1*) and *SGT1* (*suppressor of G2 allele of skp1*)] (Azevedo *et al*., 2002; Century *et al*., 1995; Frye and Innes, 1998; Parker *et al*., 1996; Torp and Jorgensen, 1986; Warren *et al*., 1999), including established PTI markers [*WRKY22* transcription factor and *GST1* (*glutathione S-transferase 1*)] (Asai *et al*., 2002); SA metabolism [*EDS5*, *ICS1* (*isochorismate synthase 1*), *PAL1* (*phenylalanine ammonia-lyase 1*) and *AZI1* (*azelaic acid-induced 1*)] (Greenberg *et al*., 2009; Mauch-Mani and Slusarenko, 1996; Nawrath and Métraux, 1999; Wildermuth *et al*., 2001); transcriptional regulators [*NPR1* (*nonexpressor of pathogenesis-related gene 1*), *NPR2* and *NPR3*] (Cao *et al*., 1994; Hepworth *et al*., 2005; Norberg *et al*., 2005; Zhang *et al*., 2006); pathogenesis-related proteins [*PR1* (*pathogenesis-related 1*), *RdRp1* (*RNA-dependent RNA polymerase 1*) and *CHI* (*chitinase*)] (Alexander *et al*., 1993; Samac *et al*., 1990; Xie *et al*., 2001) and jasmonic acid (JA) signalling [*JAR1* (*jasmonic acid-resistant 1*) and *COI1* (*coronatine insensitive 1*)] (Staswick and Tiryaki, 2004; Yan *et al*., 2009). In addition,

we compared the effect of Xflg22 on ROS accumulation and *Xcc* bacterial population growth in different citrus genotypes.

Our results show that Xflg22 induces transcriptional reprogramming in resistant but not susceptible genotypes. The differences in response between genotypes were mostly independent of the Xflg22 concentration used; however, the intensity of the reprogramming correlated with the level of resistance of each citrus genotype. Higher levels of ROS production induced by Xflg22 also correlated with canker resistance. Furthermore, we showed that the genotype with the most intense transcriptional reprogramming (kumquat) exhibited enhanced immunity when pretreated with Xflg22, and was less resistant to a flagellin mutant *Xcc* strain. The role of PTI in resistance against *Xcc* is discussed.

RESULTS

Xflg22 triggers transcriptional reprogramming of defence-associated genes in 'Nagami' kumquat, but not in 'Duncan' grapefruit

To determine the role of Xflg22 in the immune response of citrus against *Xcc*, highly susceptible 'Duncan' grapefruit and highly resistant 'Nagami' kumquat were challenged with the peptide. Subsequently, challenged leaf tissue was collected in a time course from 0 to 120 h post-Xflg22 treatment and subjected to gene expression analysis by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Collection time points were selected on the basis of a previous study (Khalaf *et al*., 2007) and our own empirical data, and aimed to study the extent and long-term defence response. Two concentrations of Xflg22 (10 and 100 μM) were used to rule out the possibility that any lack of response was caused by insufficient peptide. The genes chosen for analysis were categorized as: established markers of PTI (*WRKY22* and *GST1*), PTI/ETI perception and signalling genes (*EDR1*, *EDS1*, *NDR1*, *PBS1*, *RAR1* and *SGT1*), SA metabolism (*EDS5*, *ICS1*, *PAL1* and *AZI1*), transcriptional regulators (*NPR1*, *NPR2* and *NPR3*), pathogenesis-related protein genes (*PR1*, *RdRp1* and *CHI*) and JA signalling genes (*JAR1* and *COI1*). Gene expression levels were considered to be significantly different at a particular time point when their relative quantification (RQ) values were statistically significantly different from pre-inoculation levels (0 h) and the water control at the same time point.

WRKY22 and *GST1* are inducible by flg22 treatment and have been used as early markers of flg22-triggered innate immunity in *Arabidopsis* (Asai *et al*., 2002). In this study, we observed a higher level of *WRKY22* only with 100 μM Xflg22 at 24 h after treatment and no significant induction of *GST1* in susceptible 'Duncan' grapefruit (Fig. 1a,b). However, in 'Nagami' kumquat, *WRKY22* expression was significantly induced at 24 h by 10 μM Xflg22 and at 24 and 72 h by 100 μM Xflg22. In addition, the levels of induction were several orders of magnitude higher than in 'Duncan' grapefruit (Fig. 1c). The other marker gene, *GST1*, was also significantly up-regulated at 24 and 72 h by both Xflg22 treatments (Fig. 1d).

In susceptible 'Duncan' grapefruit, no significant induction of PTI/ETI perception and signalling genes by Xflg22 at either concentration was observed (Figs 2a–e and S1a, see Supporting Information). In contrast, Xflg22 in 'Nagami' kumquat significantly induced the expression of these genes as early as 6 h (*EDS1*, *NDR1*, *RAR1*), at 24 h (*EDS1*, *NDR1*, *PBS1*, *RAR1*, *SGT1*) and up to 72 h (*RAR1*, *SGT1*) after treatment (Fig. 2f–j). The only gene that did not show a significant change in expression was *EDR1* (Fig. S1b). Essentially no difference in the levels of gene expression was observed between the 10 and 100 μM treatments in 'Nagami' kumquat (Fig. 2f–j).

The expression of SA metabolism genes in 'Duncan' grapefruit was no different from that of water controls at any time point and any Xflg22 concentration, except for a significantly lower level of *EDS5* with 10 μM Xflg22 and *ICS1* with 100 μM Xflg22 at 72 h (Fig. 3a-c). In 'Nagami' kumquat, *EDS5* remained unchanged (Fig. 3d). *ICS1* levels were lower than those of water controls at 24, 72 and 120 h, but not significantly lower than the pre-inoculation levels (Fig. 3e). In contrast, *PAL1* expression was significantly induced at 24 h (10 and 100 μM treatments) and 72 h (100 μM treatment) after inoculation (Fig. 3f). Expression of *AZI1* was not significantly induced compared with the controls by any of the treatments in any of the two genotypes (Fig. S2, see Supporting Information). In a separate, replicate experiment, however, significant induction of *ICS1* at 24 and 72 h, and of *AZI1* at 72 h, by Xflg22 was observed in 'Duncan' grapefruit (Fig. S6, j1 and l1, see Supporting Information).

The inoculation of 'Duncan' grapefruit with either 10 or 100 μM of Xflg22 did not change the expression levels of the transcriptional regulators *NPR1*, *NPR2* and *NPR3* significantly (Figs 4a,b and S3a, see Supporting Information). In 'Nagami' kumquat, no significant changes were observed in the expression of *NPR1* by any of the treatments (Fig. S3b). However, *NPR2* and *NPR3* were significantly induced at 6 and 24 h after inoculation. The induction at 6 h of *NPR2* was significantly higher with 100 μM of Xflg22 relative to that with 10 μM of Xflg22 (Fig. 4c,d).

In the case of the *PR* gene responses, we observed higher expression levels of *PR1* in 'Duncan' grapefruit after Xflg22 treatment compared with the water controls; however, the induction of this gene was not significant relative to the levels at 0 h (Fig. 5a), although, in a separate experiment, Xflg22 significantly induced *PR1* at 24 and 72 h (Fig. S6, p1). In 'Nagami' kumquat, Xflg22 induced significantly lower expression of *PR1* at 24 h (Fig. 5b), but did not significantly affect the expression of *RdRp1* (Fig. S4d, see Supporting information). However, *RdRp1* was significantly up-regulated by Xflg22 at 6, 24 and 72 h in a separate experiment

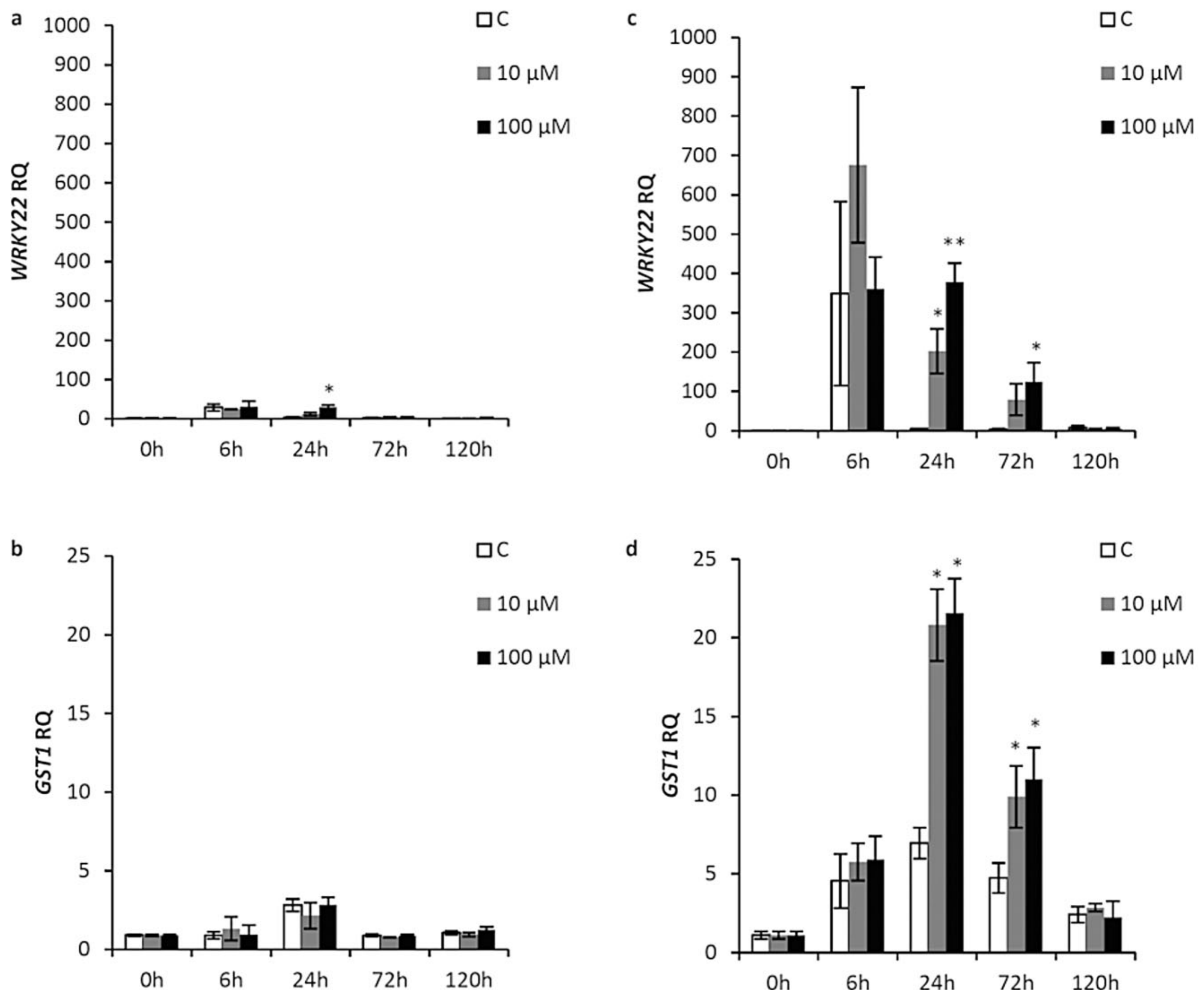

Fig. 1 The effect of Xflg22 on the expression of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) marker genes in 'Duncan' grapefruit (a,b) and 'Nagami' kumquat (c,d). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. A single asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation levels (0 h) and from the water controls at the particular time point. A double asterisk indicates that RQ of the 100 μM Xflg22 treatment is significantly higher ($P < 0.05$) than that of the control and 10 μM Xflg22 at the indicated time point. Bars are means ± standard error ($n = 3$).

(Fig. S6, r2). The expression of *CHI* and *RdRp1* was not affected in 'Duncan' grapefruit (Fig. S4a,b), and *CHI* expression was unaffected in 'Nagami' kumquat, by Xflg22 treatment (Fig. S4c).

In 'Duncan' grapefruit, neither the expression of *JAR1* nor *COI1* was affected by Xflg22 treatment; similar results were also observed in 'Nagami' kumquat, except for the induction of *JAR1* at 24 h by 100 μM of Xflg22 (Fig. S5, see Supporting Information).

In a separate, replicate experiment, Xflg22 treatment (10 μM) consistently induced defence-associated genes *WRKY22*, *GST1*, *EDS1*, *NDR1*, *PBS1*, *RAR1*, *SGT1*, *PAL1*, *NPR2* and *NPR3* in 'Nagami' kumquat, but did not affect the expression of these genes in 'Duncan' grapefruit. However, the expression of *WRKY22*, *EDS5*, *ICS1*, *AZI1* and *PR1* in 'Duncan' grapefruit and *RdRp1* and *JAR1* in 'Nagami' kumquat by Xflg22 (Fig. S6) showed inconsistencies with the results described previously (Figs 1–5).

Thus, our results indicate that Xflg22 induced transcriptional reprogramming of defence-associated genes in 'Nagami' kumquat, but not in 'Duncan' grapefruit. In general, a larger number of genes showed expression levels significantly higher in 'Nagami' kumquat, particularly at 24 h, than those of pre-inoculation and water control levels, compared with 'Duncan' grapefruit. 'Nagami' kumquat responded more rapidly (as early as 6 h post-treatment) than 'Duncan' grapefruit to Xflg22 treatment. Most genes had similar expression levels between the two Xflg22

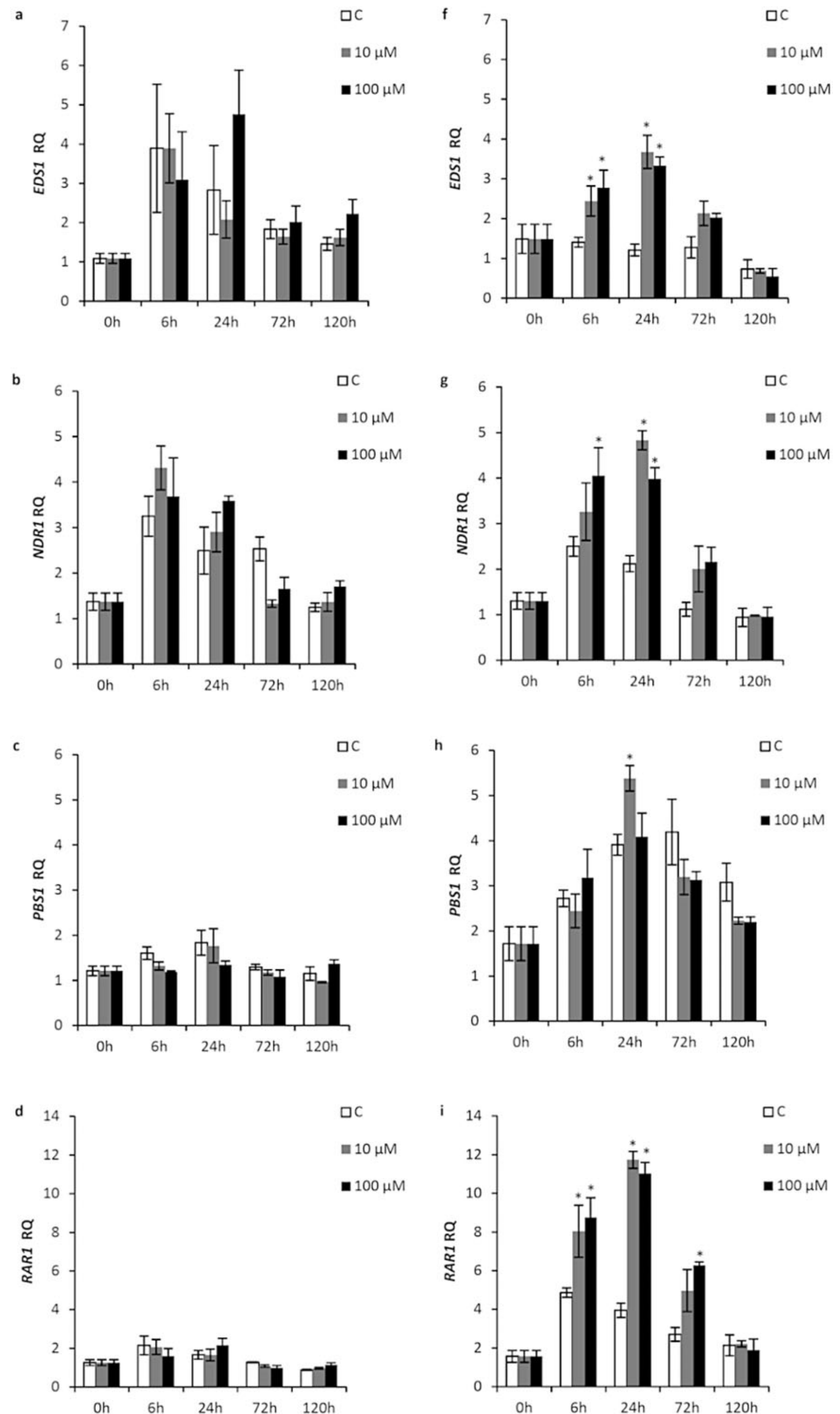

Fig. 2 The effect of Xflg22 on the expression of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI)/effector-triggered immunity (ETI) perception and signalling genes in 'Duncan' grapefruit (a–e) and 'Nagami' kumquat (f–j). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. An asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation levels (0 h) and from the water controls at the particular time point. Bars are means ± standard error ($n = 3$).

concentrations tested (10 and 100 μM) in both citrus genotypes. A few exceptions were *WRKY22* at 24 h and *NPR2* at 6 h in 'Nagami' kumquat, with significantly higher levels in the 100 μM treatment. In addition, 100 μM of Xflg22 resulted in longer lasting induction of *WRKY22*, *RAR1* and *PAL1* than 10 μM of Xflg22 in 'Nagami' kumquat.

The level of Xflg22-induced transcriptional reprogramming is related to the level of *Xcc* resistance/susceptibility in citrus

In citrus, resistance to canker can be divided into five levels: highly resistant, resistant, less susceptible, susceptible and highly

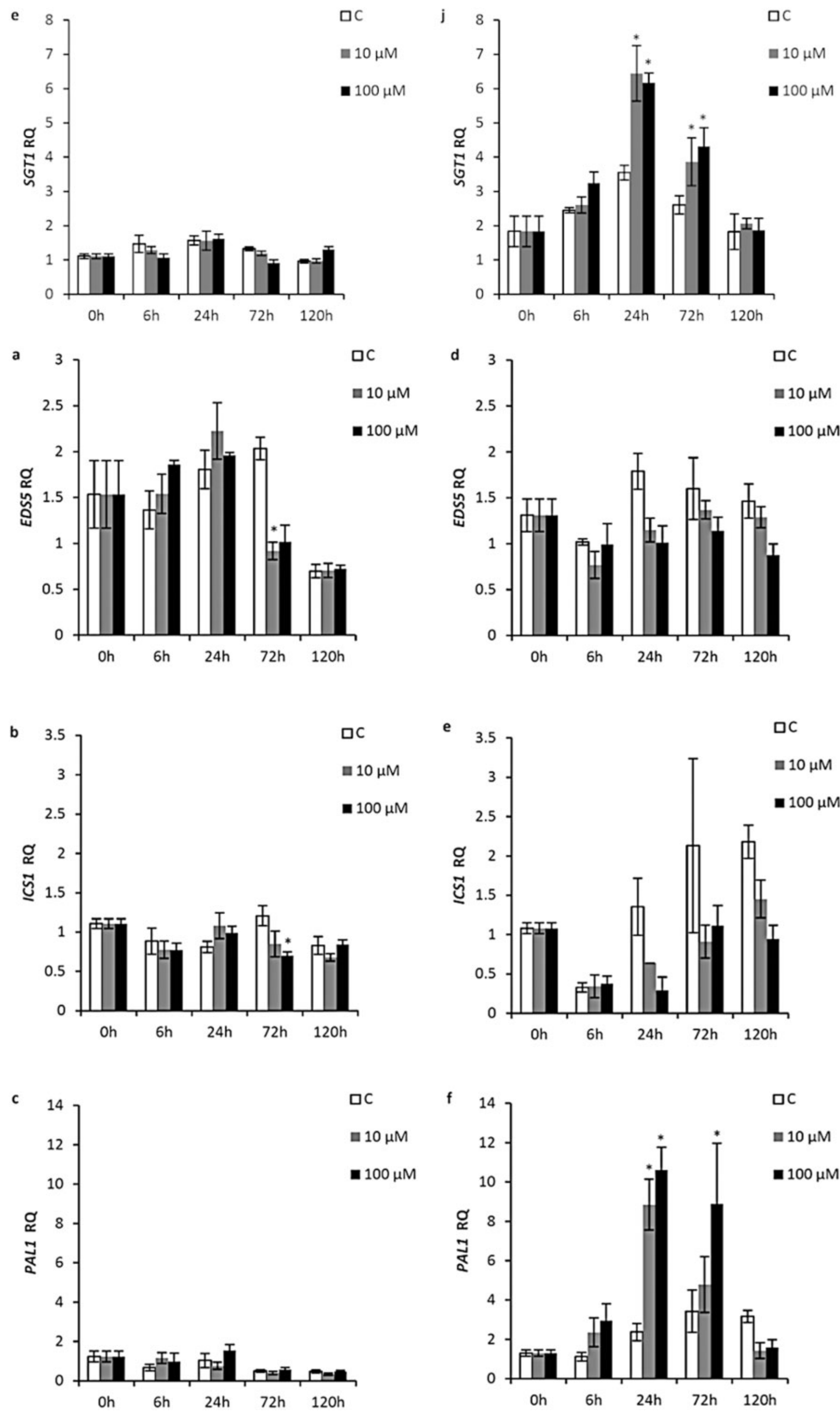

Fig. 2 Continued

Fig. 3 The effect of Xflg22 on the expression of salicylic acid (SA) biosynthesis and signalling genes in 'Duncan' grapefruit (a–c) and 'Nagami' kumquat (d–f). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. An asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation levels (0 h) and from the water controls at the particular time point. Bars are means ± standard error (n = 3).

susceptible (Gottwald *et al*., 2002). 'Nagami' kumquat and 'Duncan' grapefruit represent the highly resistant and highly susceptible levels, respectively (Goto, 1992; Gottwald *et al*., 1993, 2002). In order to determine whether the level of resistance/susceptibility to canker correlated with PTI induced by Xflg22, two further citrus genotypes, susceptible 'Navel' sweet orange and resistant 'Sun Chu Sha' mandarin, were studied. For simplicity, we chose only genes which, in the experiments described above, were differentially expressed after Xflg22 treatment, and we only used the 10 μM Xflg22 concentration.

In susceptible 'Navel' sweet orange, the expression of most genes was not significantly different from pre-inoculation levels

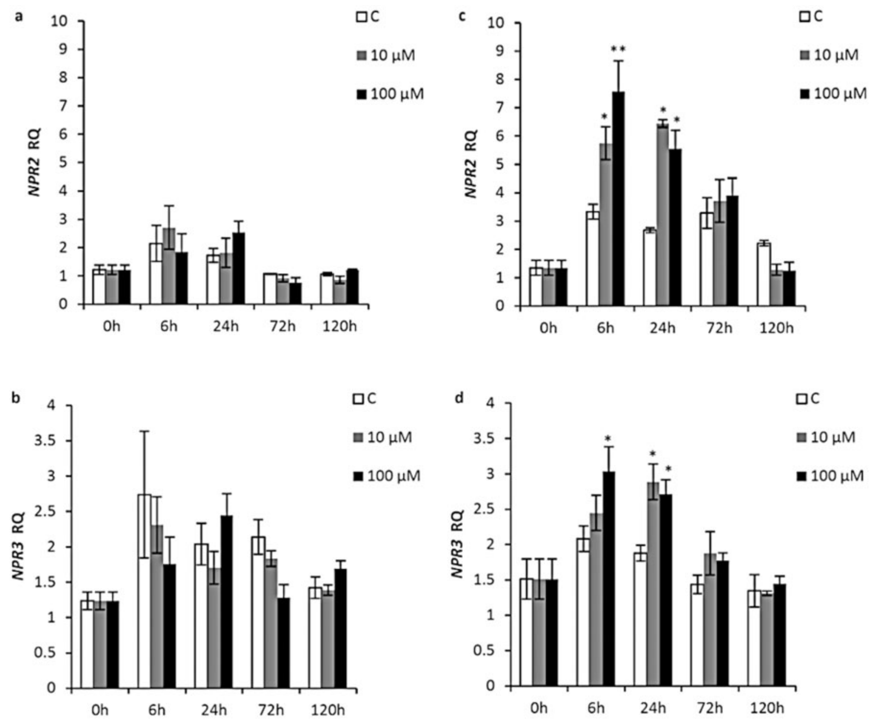

Fig. 4 The effect of Xflg22 on the expression of transcription regulation genes in 'Duncan' grapefruit (a,b) and 'Nagami' kumquat (c,d). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. A single asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation levels (0 h) and from the water controls at the particular time point. The double asterisk indicates that the RQ of the 100 μM Xflg22 treatment is significantly higher ($P < 0.05$) than that of the control and 10 μM Xflg22 at the indicated time point. Bars are means ± standard error ($n = 3$).

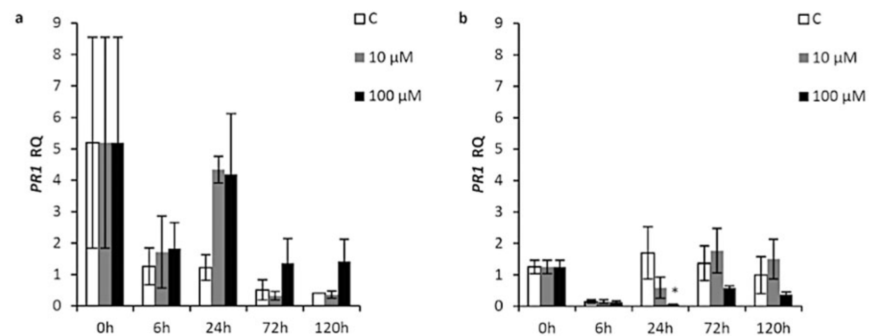

Fig. 5 The effect of Xflg22 on the expression of *PR1* in 'Duncan' grapefruit (a) and 'Nagami' kumquat (b). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. An asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation levels (0 h) and from the water controls at the particular time point. Bars are means ± standard error ($n = 3$).

and water controls. However, the transcript levels of *GST1*, *EDS1*, *NDR1* and *RAR1* were significantly higher and *ICS1* was significantly lower (Fig. 6a). In contrast, resistant 'Sun Chu Sha' mandarin showed significantly higher levels of *GST1*, *EDS1*, *NDR1*, *RAR1*, *SGT1*, *PAL1* and *NPR3* transcripts, and much higher induction of *WRKY22*, than that of 'Navel' sweet orange (Fig. 6b) This pattern was very similar (with the sole exception of *NPR2*) to the observations with the highly resistant 'Nagami' kumquat.

Xflg22 induces a higher oxidative burst in canker-resistant genotypes

flg22 elicits a rapid oxidative burst in plants, one of the earliest observable events in plant immunity (Felix *et al*., 1999). In order to study the early Xflg22 response in citrus and its association with canker resistance, we compared Xflg22-induced ROS accumulation between canker-resistant ('Nagami' kumquat and 'Sun Chu Sha' mandarin) and canker-susceptible ('Duncan' grapefruit and 'Navel' sweet orange) genotypes during the first 60 min of Xflg22 exposure. Xflg22 treatment caused the transient production of ROS in all four genotypes relative to water controls (Fig. 7a-d). In most genotypes, ROS production peaked at around 15 min, quickly declining to pretreatment levels. Interestingly, this was not the case for 'Sun Chu Sha' mandarin (Fig. 7c), in which the decline after the 15-min peak was slower and more variable. Overall, ROS reached higher values in the resistant genotypes ('Nagami' kumquat and 'Sun Chu Sha' mandarin, Fig. 7a,c) compared with the susceptible genotypes ('Duncan' grapefruit and 'Navel' sweet orange, Fig. 7b,d).

Xflg22 pretreatment increases immunity to citrus canker in 'Nagami' kumquat, but not in 'Duncan' grapefruit

Our gene expression analysis indicated that 'Nagami' kumquat was able to induce a series of defence-associated genes in

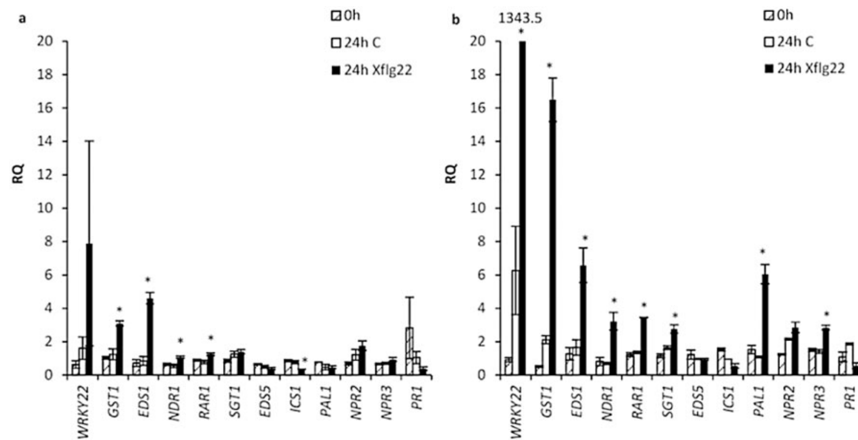

Fig. 6 The effect of 10 μM Xflg22 on the expression of defence-associated genes in (a) 'Navel' sweet orange and (b) 'Sun Chu Sha' mandarin, 24 h after treatment. RQ values 24 h after Xflg22 treatment (black) are shown in comparison with pre-inoculation (shaded) and water control (white) levels. The genes studied are shown on the *x* axis. An asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation (0 h) and control levels. Bars are means ± standard error ($n = 3$).

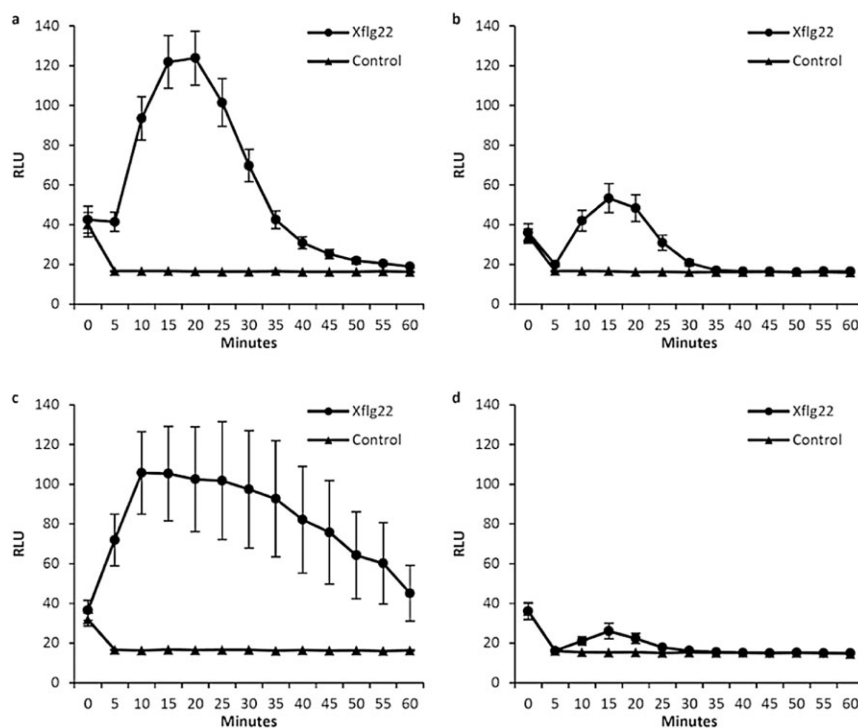

Fig. 7 Xflg22-triggered oxidative burst in (a) 'Nagami' kumquat, (b) 'Duncan' grapefruit, (c) 'Sun Chu Sha' mandarin and (d) 'Navel' sweet orange. Reaction solutions containing 100 nM Xflg22 were added to citrus leaf discs at 0 min. No Xflg22 was added to the control reactions. Relative light unit (RLU) was measured every 5 min for 60 min after treatment. Values are means ± standard error ($n = 40$).

response to Xflg22 treatment. This response was more robust than that observed in 'Duncan' grapefruit. Moreover, we found a correlation between the extent of gene induction by Xflg22 and the level of canker resistance in the four citrus genotypes studied. In order to determine whether the observed Xflg22-triggered defence (gene induction) had an effect on citrus canker resistance, we inoculated 'Duncan' grapefruit and 'Nagami' kumquat leaves with 5×10^5 colony-forming units (CFU)/mL of *Xcc* 24 h after infiltration with Xflg22. The bacterial population growth in each genotype was compared (Fig. 8a,b). In 'Duncan' grapefruit, *Xcc* grew to higher levels than those in 'Nagami' kumquat, and pre-treatment with Xflg22 had little effect on bacterial growth (Fig. 8a). In contrast, 'Nagami' kumquat showed significantly reduced *Xcc* bacterial growth at 2 and 4 days post-inoculation (DPI) (by up to 27-fold at 2 DPI) in leaves pretreated with Xflg22 compared with those pretreated with water. Subsequently, this difference in bacterial populations was reduced (Fig. 8b), but remained higher than that observed in 'Duncan' grapefruit.

A flagellin-deficient *Xcc* mutant strain shows increased growth compared with the wild type in 'Nagami' kumquat, but not in 'Duncan' grapefruit

The previous experiment showed that Xflg22 treatment triggered defence-associated gene induction and *Xcc* growth inhibition in highly resistant 'Nagami' kumquat, but not in highly susceptible 'Duncan' grapefruit, suggesting that Xflg22 perception plays an important role in the immune response against *Xcc*. In order to

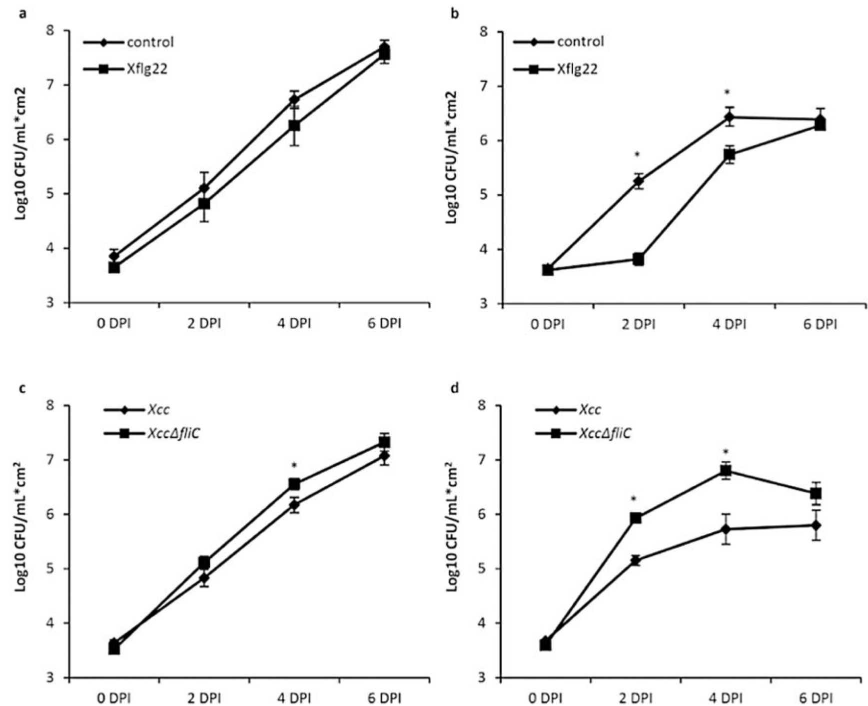

Fig. 8 Role of flagellin in the citrus defence response. Top: effect of Xflg22 pretreatment on *Xanthomonas citri* ssp. *citri* (*Xcc*) population growth in (a) 'Duncan' grapefruit and (b) 'Nagami' kumquat. *Xcc* at a concentration of 5×10^5 CFU/mL was inoculated into leaves that had been pre-treated 24 h before with Xflg22 (10 μM). Leaves pre-treated with water were used as controls. Bottom: comparison of population growth between *Xcc* wild-type and *fliC* mutant (*XccΔfliC*) in (c) 'Duncan' grapefruit and (d) 'Nagami' kumquat. *Xcc* strains at a concentration of 5×10^5 CFU/mL were inoculated into leaves. DPI is days post-*Xcc* inoculation. Values shown are means ± standard error ($n = 9$). An asterisk indicates a significant difference ($P < 0.05$) at the particular time point.

corroborate the role of Xflg22 and, further, flagellin in the initiation of PTI as a component of canker resistance, we used an *Xcc* flagellin mutant (*XccΔfliC*) which contains a deletion in the flagellin-encoding *fliC* gene, including a truncation of the Xflg22 region. We inoculated this mutant in citrus leaves and compared bacterial growth with that in wild-type *Xcc*. In 'Duncan' grapefruit, *XccΔfliC* and *Xcc* populations grew similarly up to 6 DPI (the same time frame studied during the gene expression experiments), with the *XccΔfliC* population significantly higher than that of the wild-type strain (by two-fold) at 4 DPI (Fig. 8c). In 'Nagami' kumquat, *XccΔfliC* growth levels were significantly higher than those of *Xcc* starting at 2 DPI (by six-fold), and the difference in populations between the two bacterial strains reached 12-fold at 4 DPI (Fig. 8d).

DISCUSSION

Previous work comparing the response of 'Nagami' kumquat and 'Duncan' grapefruit has shown that 'Nagami' kumquat exhibits an active defence response against *Xcc*, rendering it highly resistant. This response was found to be associated with physiological (H_2O_2 production), structural (thickening of the cell wall) and molecular (gene expression reprogramming) changes (Khalaf *et al*., 2007). In the present study, we set out to dissect this immune response to better understand which components play a role in citrus defence against canker. One of these components is PTI induced by flg22, a well-characterized epitope in flagellin, the main protein constituent of the bacterial flagellar filament. Is PTI elicited during the *Xcc*–citrus interaction? This is considered an early response. Is it similar in citrus genotypes with different levels of resistance, hinting at adaptation by the pathogen in the form of effectors that would target the plant immune response at later stages of pathogenicity? Or is the level of PTI distinct, indicating genotypic differences in the perception of this important PAMP and suggestive of a more basic adaptation by the pathogen?

In the first part of this study, we compared the expression of 20 genes directly associated with plant defence between highly resistant 'Nagami' kumquat and highly susceptible 'Duncan' grapefruit in the presence and absence of Xflg22. Xflg22 significantly and highly induced the expression of the early PTI marker genes *WRKY22* and *GST1* in 'Nagami' kumquat. In contrast, only *WRKY22* was significantly induced by 100 μM Xflg22 in 'Duncan' grapefruit, and to a fraction of the level observed in 'Nagami' kumquat (Fig. 1). This is suggestive of a robust initiation of PTI by Xflg22 in resistant 'Nagami' kumquat, but not in susceptible 'Duncan' grapefruit, within the studied time course.

In 'Nagami' kumquat, Xflg22 also significantly increased the expression of *EDS1*, *NDR1*, *PBS1*, *RAR1* and *SGT1*, whereas none of these genes were induced in 'Duncan' grapefruit, at the time points analysed (Fig. 2). *EDS1*, *NDR1*, *RAR1* and *SGT1* have been shown to be involved in the early events of pathogen recognition in plants and are important to both *R* gene-mediated resistance (ETI) (Aarts *et al*., 1998; Azevedo *et al*., 2002) and PTI-associated basal or non-host resistance (Fu *et al*., 2009; Peart *et al*., 2002; Yun *et al*., 2003; Zhang *et al*., 2010). Moreover, microarray analysis of *Arabidopsis* seedlings treated with flg22 has shown transcriptional changes in the abundance of genes, including *NDR1* and *EDS1*, as early as 30 min after treatment (Navarro *et al*., 2004;

Zipfel *et al*., 2004). In our study, we observed a similar induction of these genes at 6 and 24 h after treatment during PTI in the resistant citrus genotypes.

Following pathogen recognition, plants accumulate SA, which has been shown to be important in the establishment of the immune response and defence signalling (Malamy *et al*., 1990; Metraux *et al*., 1990). Two separate pathways are involved in SA biosynthesis, each initiated by different catalytic enzymes: ICS1 and PAL1 (Mauch-Mani and Slusarenko, 1996; Wildermuth *et al*., 2001). In model plant systems, it has been proposed that the ICS1 pathway is that which is predominantly involved in SA biosynthesis (in the chloroplast) during plant immunity (Strawn *et al*., 2007; Wildermuth *et al*., 2001). EDS5 is also critical, as it has been shown recently to function as the transporter of chloroplast-accumulated SA into the cytoplasm (Serrano *et al*., 2013). However, AZI1 is involved in the priming of SAR by SA (Greenberg *et al*., 2009). In 'Nagami' kumquat, the expression of *EDS5*, *ICS1* and *AZI1* was mostly unaffected, whereas the expression level of *PAL1* was increased, by Xflg22 treatment (Figs 3d–f and S2b). PAL1 catalyses the production of *trans*-cinnamic acid, a precursor of SA. However, *trans*-cinnamic acid is also a precursor in the biosynthesis of lignin and several antimicrobial compounds (flavonoids and anthocyanins) which are part of the defence response in plants, although they do not play a direct role in signalling and the establishment of immunity (Bate *et al*., 1994; Mauch-Mani and Slusarenko, 1996; Mishina and Zeier, 2007a). Whether our results indicate that the alternative PAL1-mediated SA biosynthetic pathway is preferred in 'Nagami' kumquat or whether the increase in *PAL1* transcripts is merely part of the secondary metabolite defence response, or both, will need to be confirmed in further experiments, but is certainly a compelling idea.

Downstream of SA accumulation, NPR1 regulates plant defence by transcriptional modulation of the expression of *PR1* in an SA-dependent manner (Cao *et al*., 1994), a process negatively controlled by the SA receptors NPR3 and NPR4 (homologous to citrus *NPR2* and *NPR3*, see Experimental procedures) (Fu *et al*., 2012; Zhang *et al*., 2006). In addition to *PR1*, SA can also induce RdRp1 and CHI, two proteins known for their antimicrobial activities against viral and fungal pathogens, respectively (Samac *et al*., 1990; Xie *et al*., 2001). In 'Nagami' kumquat, Xflg22 did not affect the expression of *NPR1* and *CHI*, up-regulated *NPR2* and *NPR3* and down-regulated *PR1*. However, none of these genes was affected by Xflg22 in 'Duncan' grapefruit (Figs 4, 5, S3 and S4), although *PR1* expression increased in another replicate experiment (Fig. S6, p1). In our study, Xflg22 treatment did not change the expression of *NPR1* in either of the genotypes, even when *PR1* was induced, suggesting that, in citrus, this gene may be regulated at the protein level rather than at the mRNA level. In *Arabidopsis*, conversion of the NPR1 protein from an oligomer to a monomer and subsequent translocation from the cytoplasm to the nucleus are required in order to activate *PR1* expression (Kinkema *et al*., 2000; Tada *et al*., 2008). It has been shown recently that *Arabidopsis* NPR3 and NPR4 are important in the regulation of SAR by binding SA and mediating the degradation of NPR1 coordinately (Fu *et al*., 2012). The induction of the citrus orthologues (*NPR2* and *NPR3*) suggests involvement of these two genes during PTI and, perhaps, the activation of SAR. The induction of *PR1* in systemic tissues is frequently used as the marker for SAR. In our study, only local expression of *PR1* was analysed and its induction was not observed in 'Nagami' kumquat or consistently observed in 'Duncan' grapefruit. However, it would be interesting to compare Xflg22-triggered SAR between canker-resistant and canker-susceptible genotypes by measuring *PR1* expression systemically, and to determine whether citrus *NPR1*, *NPR2* and *NPR3* play any role in the induction of SAR in resistant genotypes.

We also studied the expression of *JAR1*, an important enzyme necessary for the activation of JA signalling (Staswick and Tiryaki, 2004), and COI1, a receptor of jasmonate (Yan *et al*., 2009). Neither gene was consistently induced in 'Nagami' kumquat or 'Duncan' grapefruit (Figs S5, S6, s1, t1, s2, t2), suggesting no involvement of JA signalling during Xflg22-initiated PTI in citrus.

We compared changes in gene expression with pre-inoculation (0 h) levels after treatment with Xflg22 and water controls. In certain cases (*EDS1*, *NPR1* and *NPR3* in 'Duncan' grapefruit and *RdRp1* in 'Nagami' kumquat), we observed higher levels for both Xflg22-treated and water controls (Figs 2a, S3a, 4b and S4d) compared with 0 h, suggesting that the inoculation procedure or other factors, such as circadian modulation, may have been the cause of the induction. We also compared the effect of two Xflg22 concentrations (10 and 100 μM). In 'Nagami' kumquat, the treatment with the lower concentration of 10 μM of Xflg22 induced transcriptional reprogramming. For most genes and time points, there was no significant difference in gene expression with a Xflg22 concentration of 100 μM. Only *NPR2* and *NPR3* were induced earlier (6 h) (Fig. 4c,d) and *WRKY22*, *RAR1* and *PAL1* remained induced for longer (72 h) (Figs 1c, 2i and 3f) with the higher Xflg22 concentration in 'Nagami' kumquat. *PR1* was down-regulated at 24 h only by the higher concentration treatment (Fig. 5b). In 'Duncan' grapefruit, we did not observe transcriptional reprogramming even with the higher concentration (100 μM) of Xflg22 (Figs 1–5). These results suggest that the observed absence of a response in 'Duncan' grapefruit is not a result of the application of insufficient Xflg22, but rather of a lack of sensitivity to this PAMP.

We tested two further citrus genotypes with intermediate levels of canker resistance/susceptibility using the Xflg22-responsive genes (*WRKY2*, *GST1*, *EDS1*, *NDR1*, *RAR1*, *SGT1*, *EDS5*, *ICS1*, *PAL1*, *NPR2*, *NPR3* and *PR1*) identified from the first experiment. At 24 h after treatment, the response observed in resistant 'Sun Chu Sha' mandarin was similar to that of the highly resistant 'Nagami' kumquat, whereas susceptible 'Navel' sweet orange responded

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similarly to highly susceptible 'Duncan' grapefruit (Fig. 6). In addition, we studied the oxidative burst induced by Xflg22 among the four citrus genotypes within the first hour of treatment, and found that ROS reached higher levels and lasted longer in the two resistant genotypes ('Nagami' kumquat and 'Sun Chu Sha' mandarin) than in the susceptible genotypes ('Duncan' grapefruit and 'Navel' sweet orange) (Fig. 7). These results, together with the previous ones, indicate a correlation between the levels of resistance in citrus genotypes and the intensity of the flagellin-triggered defence gene reprogramming. Moreover, resistant genotypes, but not susceptible ones, showed a strong early flagellin-induced oxidative burst, an indicator of the establishment of PTI. Although our experiments do not rule out the role of other *Xcc*-derived PAMPs, which were not part of this study, in citrus canker resistance, they underscore the importance of flagellin perception. Reduced gene induction and weak ROS production in susceptible citrus genotypes may indicate their lower sensitivity to flagellin, which renders *Xcc* less 'visible' to the plant defence system. In *Arabidopsis*, perception of flagellin is mediated by the receptor FLS2 (Gomez-Gomez and Boller, 2000), a protein that affects the susceptibility of this plant to adapted and non-adapted *Pseudomonas syringae* strains (Forsyth *et al*., 2010; Hann and Rathjen, 2007; Zipfel *et al*., 2004). In the case of the citrus–*Xcc* pathosystem, characterization of the citrus FLS2 orthologue, such as differences in the protein primary structure and expression levels between genotypes, and how these differences affect canker resistance are interesting future areas of study.

Xcc bacterial growth was inhibited in 'Nagami' kumquat when plants were pretreated with Xflg22 24 h prior to inoculation (Fig. 8a,b). This indicates that the changes in defence gene expression induced by Xflg22 were accompanied by an enhanced immune response against *Xcc* in this genotype. The bacterial growth retardation diminished after 2 DPI. This transient effect suggests that Xflg22-triggered PTI has a role in canker resistance as an early signal that is subsequently amplified into stronger immunity during ETI. The *Xcc* with a mutated flagellin *fliC* gene (*XccΔfliC*) showed markedly higher bacterial growth than wild-type *Xcc* in 'Nagami' kumquat at all time points, but not in 'Duncan' grapefruit (Fig. 8c,d), again indicating that perception of Xflg22 is important to the resistant genotype. This confirms our previous results that Xflg22-induced PTI contributes to canker resistance. It is worth noting that, because natural infection requires flagellum-based motility for bacterial entry, for which *XccΔfliC* is most likely to be compromised, by direct infiltration into the intercellular spaces we can bypass this limitation. The *XccΔfliC* strain contains a 305-amino-acid deletion in *fliC* (a 399-amino-acid protein), including 16 amino acids at the C-terminus of Xflg22. In 'Duncan' grapefruit, negligible gene induction, weak oxidative burst, low bacterial growth inhibition by Xflg22 and little difference in response between *Xcc* and *XccΔfliC* suggest that this genotype has a very low response to Xflg22, which may contribute to its susceptibility. It is well known that virulent pathogens evolve effectors capable of suppressing plant defence, including PTI, rendering plants susceptible (Jones and Dangl, 2006; Nomura *et al*., 2005). However, our study shows evidence that the induction of PTI in citrus plays an important role in defence against *Xcc*, and contributes towards the final outcome in this plant–pathogen interaction.

In conclusion, the *Xcc* PAMP Xflg22 induces PTI in the citrus canker-resistant genotypes but not in susceptible ones, as indicated by the Xflg22-initiated defence gene expression reprogramming and enhanced immune response against *Xcc* bacterial growth. Among the citrus defence-associated genes studied, *GST1*, *EDS1*, *NDR1*, *PBS1*, *RAR1*, *SGT1*, *PAL1*, *NPR2* and *NPR3* were significantly induced by Xflg22 in 'Nagami' kumquat, but not in susceptible 'Duncan' grapefruit. Further, the intensity of defence gene reprogramming (number of genes induced and levels of induction) correlated with the levels of citrus canker resistance observed in the genotypes studied. It will be important to determine the role of these genes in the response to *Xcc* infection, and any promising gene(s) could potentially be used to engineer citrus-susceptible genotypes by genetic transformation to increase disease tolerance or even to achieve resistance. Alternatively, chemical induction of these genes may also be a way to increase resistance in susceptible genotypes.

The flagellin-triggered defence response is widespread among higher plants and is most probably an ancient adaptation (Boller and Felix, 2009). On the basis of our results, there are two possible reasons why Xflg22 does not trigger PTI in susceptible genotypes. One is that there is a deficiency in the detection of flagellin as a result of missing, defective or not fully adapted FLS2-mediated perception. Another possibility is a deficiency in the initiation of PTI caused by mutations in any of the downstream genes, which would render the plant generally insensitive to PAMPs. One way to differentiate between these two possibilities is to study how canker-resistant and canker-susceptible genotypes respond to other PAMPs from *Xcc*. The fact that we observed genotypes with intermediate levels of Xflg22 sensitivity is more in line with different levels of FLS2-mediated adaptation, rather than a more global defect in PTI. Our efforts to further characterize PTI and PAMP perception in citrus will continue.

EXPERIMENTAL PROCEDURES

Plant material

The citrus genotypes used in this study were 'Duncan' grapefruit (*C. paradisi* Macf.), 'Nagami' kumquat [*F. margarita* (L.) Swing.], 'Navel' sweet orange (*C. sinensis* Osb.) and 'Sun Chu Sha' mandarin (*C. reticulalta* Blanco). All citrus plants were grown in pots under glasshouse conditions. Before each experiment, the plants were pruned and fertilized weekly until new flushes were produced (4–6 weeks).

Xflg22 peptide treatment

The *Xcc* flagellin conserved domain (Xflg22: QRLSSGLRINSAKDDAAGLAIS), based on GenBank accession number 21242719, was synthesized by GenScript USA Inc., Piscataway, NJ, USA. The Xflg22 solution was prepared by dissolving the lyophilized peptide in sterile distilled water to a final concentration of either 10 or 100 μM (Zipfel *et al*., 2004). Young, fully expanded leaves were used for the experiments. The Xflg22 solution was infiltrated into the abaxial surface of leaves using a 1-cm^3 insulin syringe with a needle until half of the leaf was saturated. Infiltration with distilled water was used as a control. Leaf tissue was collected at 0 (before infiltration), 6, 24, 72 and 120 h after infiltration, and RNA was subsequently extracted from infiltrated areas. Three different plants of each genotype were used as biological replicates. The experiments were repeated twice with similar results.

RNA extraction and cDNA synthesis

Total RNA was extracted using TriZol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by DNase treatment and clean up with the RNeasy Plant Mini Kit (Qiagen, Gaithersburg, MD, USA). The RNA concentration and purity were determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The cDNA synthesis reaction was performed using 1 μg of RNA and M-MLV reverse transcriptase (Invitrogen) with random decamers.

Gene expression analysis

Gene expression was measured by RT-qPCR using a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA). The reactions were set to comparative C_T ($\Delta\Delta C_T$) with the fast amplification (95 °C for 20 s and 40 cycles of 95 °C for 1 s and 60 °C for 20 s). TaqMan MGB probe (250 nM) labelled with 6-carboxyfluorescein (FAM), primers (900 nM each) and fast universal PCR master mix (Applied Biosystems) were used for target sequence amplification from 5 ng of cDNA (Table 1). Amplification of 5.8S RNA (150 mM of 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC)-labelled probe and 250 mM of each primer) was used as endogenous control. One 0-h sample selected at random from the three biological replicates was used as the reference and to calculate the RQ values. The data obtained (RQ) were first subjected to a Q-test (Rorabacher, 1991) for the evaluation of outliers, and subsequently analysed with JMP Genomics 5.0 (SAS Institute Inc., Cary, NC, USA) for model fitting of standard least-square means (LS means) and Student's *t*-test statistical significance analysis ($P < 0.05$).

ROS production assay

Young, fully expanded citrus leaves were used for the ROS production assay. Leaf discs with a diameter of 3.8 mm were obtained ($n = 8$ per treatment) from at least four plants and kept in 150 μL of sterile water overnight in a 96-well plate at room temperature. The next day, the water was replaced with 100 μL of assay solution (100 μM of luminol, 10 μg/mL of horseradish peroxidase and 100 nM of Xflg22). Assay solution without Xflg22 was used as control. Light emission (relative light unit, RLU) was measured in 5-min intervals for 60 min using a luminescence microplate reader (BioTek, Winooski, VT, USA). Means and standard errors were calculated on the basis of five independent experiments ($n = 40$).

Bacterial population dynamics

Xflg22-pretreated leaves were inoculated with *Xcc* (strain 306) bacterial suspension adjusted to 5×10^5 CFU/mL. Inoculated leaves were collected from three plants (biological replicates) at 0, 2, 4 and 6 DPI. One leaf disc (0.554 cm^2) was sampled from the inoculation area from each leaf and ground in 1 mL of sterile tap water. A total of 50 μL from three serial dilutions (up to 10^{-5}) of suspension was spread on Petri dishes with solid nutrient agar medium and incubated at 28 °C for 48 h. CFU from each sample were counted and converted to $\log_{10}$ CFU/mL cm^2. Means and standard errors were calculated on the basis of three independent experiments with similar results ($n = 9$). Student's *t*-test was used to determine statistical significance ($P < 0.05$).

Xcc fliC mutant

A *fliC* deletion mutant (from nucleotide 106 to 1015) was produced by complementing *Xcc*Δ*fliC*Δ*hrpG* strain 306 with a wild-type *hrpG* from strain A^w (with an *hrpG* identical to strain 306) to generate *Xcc*Δ*fliC*Δ*hrpG::hrpG* (herein referred to as *Xcc*Δ*fliC*).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 The effect of Xflg22 on the expression of *EDR1* in 'Duncan' grapefruit (a) and 'Nagami' kumquat (b). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. Bars are means ± standard error ($n = 3$).

Fig. S2 The effect of Xflg22 on the expression of *AZI1* in 'Duncan' grapefruit (a) and 'Nagami' kumquat (b). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. Bars are means ± standard error ($n = 3$).

Fig. S3 The effect of Xflg22 on the expression of *NPR1* in 'Duncan' grapefruit (a) and 'Nagami' kumquat (b). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. Bars are means ± standard error ($n = 3$).

Fig. S4 The effect of Xflg22 on the expression of pathogenesis-related genes in 'Duncan' grapefruit (a,b) and 'Nagami' kumquat (c,d). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. Bars are means ± standard error ($n = 3$).

Fig. S5 The effect of Xflg22 on the expression of jasmonic acid (JA) signalling genes in 'Duncan' grapefruit (a,b) and 'Nagami' kumquat (c,d). RQ is the relative quantification of gene expression levels after water control (white), 10 μM Xflg22 (grey) or 100 μM Xflg22 (black) infiltration. An asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation levels (0 h) and from the water controls at the particular time point. Bars are means ± standard error ($n = 3$).

Fig. S6 Replicate experiment for the effect of Xflg22 on the expression of defence-associated genes in 'Duncan' grapefruit (a1–t1) and 'Nagami' kumquat (a2–t2). RQ is the relative quantification of gene expression levels after water control (white) or 10 μM Xflg22 (black) infiltration. An asterisk indicates RQ values significantly different ($P < 0.05$) from pre-inoculation levels (0 h) and from the water controls at the particular time point. Bars are means ± standard error ($n = 3$).

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