Artificial *Agrobacterium tumefaciens* strains exhibit diverse mechanisms to repress *Xanthomonas oryzae* pv. *oryzae*-induced hypersensitive response and non-host resistance in *Nicotiana benthamiana*

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SUMMARY

Xanthomonas oryzae pv. *oryzae* (*Xoo*) rapidly triggers a hypersensitive response (HR) and non-host resistance in its non-host plant *Nicotiana benthamiana*. Here, we report that *Agrobacterium tumefaciens* strain GV3101 blocks *Xoo*-induced HR in *N. benthamiana* when pre-infiltrated or co-infiltrated, but not when post-infiltrated at 4 h after *Xoo* inoculation. This suppression by *A. tumefaciens* is local and highly efficient to *Xoo*. The HR-inhibiting efficiency of *A. tumefaciens* is strain dependent. Strain C58C1 has almost no effect on *Xoo*-induced HR, whereas strains GV3101, EHA105 and LBA4404 nearly completely block HR formation. Intriguingly, these three HR-inhibiting strains employ different strategies to repress HR. Strain GV3101 displays strong antibiotic activity and thus suppresses *Xoo* growth. Comparison of the genotype and *Xoo* antibiosis activity of wild-type *A. tumefaciens* strain C58 and a set of C58-derived strains reveals that this *Xoo* antibiosis activity of *A. tumefaciens* is negatively, but not solely, regulated by the transferred-DNA (T-DNA) of the Ti plasmid pTiC58. Unlike GV3101, strains LBA4404 and EHA105 exhibit no significant antibiotic effect on *Xoo*, but rather abolish hydrogen peroxide accumulation. In addition, expression assays indicate that strains LBA4404 and EHA105 may inhibit *Xoo*-induced HR by suppression of the expression of *Xoo* type III secretion system (T3SS) effector genes *hpa1* and *hrpD6*. Collectively, our results unveil the multiple levels of effects of *A. tumefaciens* on *Xoo* in *N. benthamiana* and provide insights into the molecular mechanisms underlying the bacterial antibiosis of *A. tumefaciens* and the non-host resistance induced by *Xoo*.

Keywords: *Agrobacterium tumefaciens*, antibiosis, hydrogen peroxide, hypersensitive response, *Nicotiana benthamiana*, non-host resistance, *Xanthomonas oryzae* pv. *oryzae*.

INTRODUCTION

Non-host resistance is a type of durable and broad-spectrum resistance that is difficult to overcome by the majority of potential pathogens. Therefore, it is attractive for exploitation in plant protection against diseases (Lipka *et al.*, 2008; Niks and Marcel, 2009; Schulze-Lefert and Panstruga, 2011; Senthil-Kumar and Mysore, 2013; Thordal-Christensen, 2003). Reactive oxygen species (ROS), well-known key molecules that regulate host resistance (Torres *et al.*, 2006), also play important roles in non-host resistance in some pathosystems (Rojas *et al.*, 2012; Senthil-Kumar and Mysore, 2012; Zurbriggen *et al.*, 2009). In addition, the hypersensitive response (HR) is a marker of the majority of bacterial pathogen-induced non-host resistance, which is triggered by type III secretion system (T3SS) effectors of the bacterial pathogens (Senthil-Kumar and Mysore, 2013). The relative position of ROS and HR in the pathway leading to non-host resistance is controversial (Torres *et al.*, 2002; Yoshioka *et al.*, 2003).

Xanthomonas oryzae pv. *oryzae* (*Xoo*) causes bacterial blight disease in rice (*Oryza sativa*), but is a non-adapted pathogen of many other plant species, including *Nicotiana benthamiana*. Great progress has been made in understanding the rice–*Xoo* interaction (White and Yang, 2009), yet, the non-host resistance to *Xoo* has been less well studied. The T3SS of *Xoo*, which is encoded by a hypersensitive response and pathogenicity (*hrp*) gene cluster, is necessary for both pathogenicity in susceptible hosts and HR induction in non-host plants. A set of *Xanthomonas* effectors causing HR and non-host resistance in non-host plants has been identified. Among them are HrpD6 (Guo *et al.*, 2010) and Hpa1 (Li *et al.*, 2004; Zhu *et al.*, 2000). However, the functional mechanisms of these effectors remain unclear. In addition, the oxidative burst and calcium-dependent signalling pathways are involved in *Xoo*-induced HR and non-host resistance (Li *et al.*, 2012, 2015). Collectively, the mechanisms underlying *Xoo*-induced HR and non-host resistance are still largely unknown.

Agrobacterium tumefaciens is a plant pathogen that induces tumours on most dicots and causes disease by integrating transferred-DNA (T-DNA) of the bacterium into the plant genome

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(McCullen and Binns, 2006). The capacity for DNA transfer has made this bacterium the workhorse of gene transformation and transient gene expression studies in plants (Choi *et al.*, 2003; Untergrasser *et al.*, 2012). The interactions between plants and *A. tumefaciens* are increasingly being understood (Gohlke and Deeken, 2014; Hwang *et al.*, 2015). Nevertheless, the role of *A. tumefaciens* in antibiosis to other pathogens inside the host plant is rarely documented. *Agrobacterium tumefaciens* biotype 1 and strain GV3101 locally inhibit the *Pseudomonas syringae* pv. *phaseolicola* (Pph)- and *P. syringae* pv. *tomato* (Pst) DC3000-induced HR, respectively, in their non-host plant *N. tabacum* (Rico *et al.*, 2010; Robinette and Matthyse, 1990). *Agrobacterium tumefaciens* strain ASE/Pp211 induces local resistance in *N. tabacum* to Tobacco mosaic virus (TMV) (Pruss *et al.*, 2008). However, whether the role of *A. tumefaciens* in HR inhibition and local plant resistance induction can be attributed to its antibiosis capacity remains unknown.

In a study using *A. tumefaciens*-mediated transient transformation to uncover gene functions involved in resistance to *Xoo*, we found that *A. tumefaciens* strain GV3101 strongly represses *Xoo*-induced HR in *N. benthamiana*. We then further explored the nature and mechanism of this phenomenon. Here, we demonstrate that a series of artificial *A. tumefaciens* strains suppress *Xoo*-induced HR and non-host resistance in *N. benthamiana* by diverse mechanisms. *Agrobacterium tumefaciens* strain GV3101 exhibits antibiotic properties, partially and negatively affected by the T-DNA in pTiC58, that directly kill *Xoo*, whereas strains LBA4404 and EHA105 inhibit the *Xoo*-induced HR and non-host resistance by blocking hydrogen peroxide (H₂O₂) accumulation.

RESULTS

Agrobacterium tumefaciens GV3101 blocks *Xoo*-induced HR in *N. benthamiana*

The infiltration of *Xoo* strain PX099 resulted in rapid HR in infiltrated areas of its non-host plant *N. benthamiana* (Fig. 1a, left), as reported previously for strain YN-1 (Li *et al.*, 2012). This HR was blocked by co-infiltration of *Xoo* with *A. tumefaciens* strain GV3101 (Fig. 1a, right; Table 1). In contrast with *Xoo*, *A. tumefaciens* GV3101 alone did not trigger any HR at 24 h post-inoculation (hpi) (Fig. 1b, right). Expression analysis showed that expression of the HR marker gene *HIN1* and two pathogenesis-related (PR) genes *PR1* and *PR4* was dramatically down-regulated in leaves co-infiltrated with *Xoo* and *A. tumefaciens* GV3101 when compared with *Xoo* inoculation alone at 12 hpi (Fig. S1, see Supporting Information). These data demonstrate that *A. tumefaciens* GV3101 has the ability to suppress HR induction by *Xoo* in *N. benthamiana*.

To examine whether *Xoo*-induced HR was specifically repressed by GV3101, *Escherichia coli* strain TG1 and *Xoo* PX099

Fig. 1 *Agrobacterium tumefaciens* strain GV3101 suppresses *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)-induced hypersensitive response (HR) in *Nicotiana benthamiana*. (a–d) Effect of GV3101 on *Xoo*-induced HR. The left halves of the leaves were infiltrated with *Xoo*, whereas the right halves were infiltrated with both *Xoo* and GV3101 (a), solely GV3101 (b), both *Xoo* and *Escherichia coli* strain TG1 (c) and both *Xoo* and its $\Delta hrcU$ mutant (d). The photographs were taken at 24 h post-inoculation (hpi). (e, f) Effect of GV3101 on the *Pseudomonas syringae* pv. *tomato* (Pst) DC3000-induced HR. (e) Infiltration of Pst DC3000 induced typical HR at 24 hpi. (f) Co-infiltration with GV3101 did not inhibit the Pst DC3000-induced HR. The photographs were taken at 48 hpi. At least 20 leaves were tested for each treatment.

$\Delta hrcU$ mutant, which cannot cause HR in *N. benthamiana* (Li *et al.*, 2012), were tested for their ability to suppress *Xoo*-induced HR. Co-infiltration of *Xoo* with either bacterium had no effect on *Xoo*-induced HR (Fig. 1c,d). These results suggest that *A. tumefaciens* strain GV3101 suppresses *Xoo*-induced HR *in planta* not through a general mechanism, such as space and nutrition competition, but rather by specific mechanisms.

To check whether GV3101 can repress HR induction by a bacterial pathogen other than *Xoo*, the effect of GV3101 on HR

Table 1 Effect of diverse *Agrobacterium* strains on *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)-induced hypersensitive response (HR) at 2 days after they were co-infiltrated with *Xoo* into *Nicotiana benthamiana* leaves.

Strain	Experiment	HR leaves/total treated leaves (%)	Average percentage of HR-producing leaves (%)
GV3101	1	0/20 (0%)	0 ± 0
	2	0/20 (0%)	
	3	0/20 (0%)	
EHA105	1	1/20 (5%)	3.3 ± 2.9
	2	0/20 (0%)	
	3	1/20 (5%)	
LBA4404	1	1/20 (5%)	1.7 ± 2.9
	2	0/20 (0%)	
	3	0/20 (0%)	
C58C1	1	19/20 (95%)	95 ± 5
	2	20/20 (100%)	
	3	18/20 (90%)	
NT1RE	1	20/20 (100%)	98.3 ± 2.9
	2	19/20 (95%)	
	3	20/20 (100%)	
NT1RE::pMP90	1	0/20 (0%)	0 ± 0
	2	0/20 (0%)	
	3	0/20 (0%)	
NT1RE::pGV2260	1	19/20 (95%)	93.3 ± 2.9
	2	19/20 (95%)	
	3	18/20 (90%)	
C58	1	13/20 (65%)	63.3 ± 2.9
	2	13/20 (65%)	
	3	12/20 (60%)	
C58::pTiC58Δ <i>virB2</i>	1	11/20 (55%)	58.3 ± 5.8
	2	13/20 (65%)	
	3	11/20 (55%)	

induced by another non-adapted pathogen *Pst* DC3000 was analysed. Leaves inoculated with *Pst* DC3000 alone in its non-host *N. benthamiana* displayed obvious HR at 24 hpi (Fig. 1e). Leaves co-infiltrated with *Pst* DC3000 and GV3101 also formed clear HR, although 24 h later (at 48 hpi) (Fig. 1f), demonstrating that *A. tumefaciens* GV3101 is not able to block, but rather only delay, *Pst* DC3000-induced HR. These data indicate that repressing profundity of pathogen-induced HR by *A. tumefaciens* is distinguishable between different non-adapted pathogens.

Temporal and spatial characteristics of *A. tumefaciens* GV3101-induced repression of *Xoo*-induced HR

To investigate whether GV3101 can repress *Xoo*-induced HR when it is infiltrated before *Xoo* inoculation as efficiently as when it is co-infiltrated with *Xoo*, a time-course analysis was conducted. The leaves that were inoculated with GV3101 at 3–48 h prior to *Xoo* inoculation did not show any HR symptoms, as observed for those that were co-inoculated with the two pathogens (Fig. 2a), indicating that prior inoculation of *A. tumefaciens* as far in

advance as 48 h can block *Xoo*-induced HR as efficiently as co-infiltration treatment.

To examine the maximum lag time for *A. tumefaciens* infiltration after *Xoo* inoculation that is allowed to achieve HR suppression, the leaves were first infiltrated with *Xoo*. The same areas were then infiltrated with GV3101, 1–6 h later. The results showed that GV3101 could efficiently suppress HR when infiltrated at 2 h after *Xoo* inoculation, but failed when infiltrated at 4 h after *Xoo* inoculation (Fig. 2b). These findings indicate that GV3101 blocks *Xoo*-induced HR by targeting an early event leading to this HR in *N. benthamiana*.

To determine whether GV3101 can suppress *Xoo*-induced HR in systemic leaves, GV3101 was inoculated in the lower leaves of plants, and *Xoo* was challenge inoculated in the upper leaves 5 h later. A typical HR appeared in *Xoo*-inoculated leaves at 24 hpi (Fig. 2c, left). We further performed a similar investigation in different halves of the same leaf, and obtained similar results (Fig. 2c, right). These data demonstrate that GV3101 executes its role in the suppression of *Xoo*-induced HR only locally in the GV3101-infiltrated areas, and imply that the repertoire of molecules of GV3101 required for HR repression is most probably produced and located within the GV3101-infiltrated areas.

Agrobacterium tumefaciens GV3101 strongly suppresses the growth of *Xoo* both *in vitro* and *in vivo*

The temporal and spatial characteristics of GV3101-induced repression of *Xoo*-induced HR suggest that GV3101 may inhibit the development of HR in response to *Xoo* simply by the direct repression of *Xoo* growth in leaves. To investigate this possibility, we performed co-culture plate assays. *Xoo* was co-cultured with GV3101 in liquid medium. After growth for 0–48 h, the bacterial numbers of both bacteria were counted when bacterial colonies were visible, taking advantage of the obvious difference in colony colour and growth speed between the two bacteria. As shown in Fig. 3a, *Xoo* numbers were decreased dramatically by 3.3 orders of magnitude at 48 hpi in the co-culture of *Xoo* and GV3101 relative to the pure culture of *Xoo*. This result reveals that GV3101 strongly represses the growth of *Xoo in vitro*. To confirm this result, *Xoo* bacterial numbers in leaves that were co-infiltrated with *Xoo* and GV3101 were counted. Our results showed that, in leaves infiltrated solely with *Xoo*, *Xoo* numbers were reduced by nearly four orders of magnitude at 48 hpi when tissues were desiccated as a result of HR. Interestingly, at the same time point, in leaves co-infiltrated with *Xoo* and GV3101, *Xoo* numbers were strongly reduced by nearly five orders of magnitude to a concentration that was not sufficiently high to induce HR, although tissues did not exhibit any necrosis (Fig. 3b), whereas GV3101 numbers did not change significantly (Fig. S2, see Supporting Information).

Fig. 2 The temporal and spatial characteristics of *Agrobacterium tumefaciens* GV3101-induced repression of the *Xanthomonas oryzae* pv. *oryzae* (Xoo)-induced hypersensitive response (HR). (a) Effect of prior infiltration of GV3101 on Xoo-induced HR. Leaves were infiltrated first with GV3101 and buffer as control and then with Xoo at the same sites at 3, 12, 24 and 48 h later. Photographs were taken at 48 h post-inoculation (hpi). (b) Effect of post-infiltration of GV3101 on Xoo-induced HR. Leaves were infiltrated first with Xoo and then with GV3101 and buffer as control at the same sites at 1, 2, 4 and 6 h later. Photographs were taken at 3 days post-inoculation (dpi). (c) Systemic effect of GV3101 on Xoo-induced HR. One half of the leaves was infiltrated with GV3101. The remaining half and upper non-infiltrated leaves were challenge inoculated with Xoo 5 h later. Photographs were taken at 24 hpi. At least 20 leaves were tested for each treatment.

To examine the possibility that GV3101 induces plant defences, thereby resulting in the suppression of Xoo growth, we analysed the dynamics of Xoo bacterial numbers when GV3101 was infiltrated at 24 and 48 h prior to Xoo inoculation. Our results demonstrated that, under these conditions, the Xoo bacterial number was similar to that for co-infiltration with GV3101 and Xoo, except that, at 12 hpi, Xoo numbers were reduced slightly more dramatically when GV3101 was infiltrated 24 h prior to Xoo inoculation in comparison with the other two types of GV3101 treatment (Fig. 3c). Collectively, these results reveal that *A. tumefaciens* GV3101 possesses strong antibiotic activity towards Xoo, thereby resulting in an absence of HR in GV3101 and Xoo co-infiltrated leaves.

Efficiency of *A. tumefaciens* in suppressing Xoo-induced HR is strain dependent

Considering the specificity of *A. tumefaciens* strain GV3101 to repress Xoo-induced HR, we were curious to determine whether other *A. tumefaciens* strains also possessed a similar HR suppression ability to GV3101. The tested strains included EHA105, LBA4404 and C58C1 (Table 2). As described above, all leaf areas infiltrated with Xoo alone displayed strong HR as early as 1 day post-inoculation (dpi) (Figs 1a and 4). However, none of the leaf areas co-infiltrated with Xoo and *A. tumefaciens* strain GV3101 showed an HR (Fig. 4a, Table 1). Similarly, only 3.3% and 1.7% of leaf areas exhibited HR within 3 dpi when co-infiltrated with Xoo and EHA105 (Fig. 4b, Table 1) or LBA4404 (Fig. 4c, Table 1), respectively. However, 95% of the leaf areas co-infiltrated with Xoo and *A. tumefaciens* strain C58C1 showed strong HR as early as 1 dpi, similar to infiltration with Xoo alone (Fig. 4d, Table 1). These data reveal that *A. tumefaciens* strains can be distinguished with respect to their ability to repress Xoo-induced HR; GV3101 completely abolished, EHA105 and LBA4404 strongly inhibited, whereas C58C1 had almost no effect on Xoo-induced HR.

Agrobacterium tumefaciens strains adopt distinct mechanisms to repress Xoo-induced HR

To check whether *A. tumefaciens* strains LBA4404 and EHA105 show antibiotic ability against Xoo, as does GV3101, we performed similar *in vitro* co-culture assays as for GV3101. As shown in Fig. 5a, Xoo numbers were decreased weakly (by less than 0.6 orders of magnitude within 48 hpi) when co-cultured with the HR-inhibitory strains LBA4404 and EHA105, as well as the non-HR-inhibitory strain C58C1. This was distinctly different from the HR-inhibitory strain GV3101, which caused a dramatic reduction in Xoo bacterial numbers by 3.3 orders of magnitude at 48 hpi (Fig. 3a). The bacterial numbers of these *Agrobacterium* strains were not altered obviously in these co-culture assays (Fig. S3, see Supporting Information). This result reveals that strains LBA4404 and EHA105, similar to the non-HR-inhibitory strain C58C1,

Fig. 3 *Agrobacterium tumefaciens* strain GV3101 strongly suppresses *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) growth both *in vitro* and *in vivo*. (a) *In vitro* assays. *Xoo* [10^8 colony-forming units (cfu)/mL] was cultured alone or co-cultured with GV3101 at the same concentration in nutrient agar (NA) medium at 28 °C. *Xoo* bacterial numbers were counted at 0, 6, 12, 24 and 48 h post-culture. (b) *In vivo* assays for co-infiltration treatments. *Xoo* was infiltrated alone or co-infiltrated with GV3101 in leaves of *Nicotiana benthamiana*, and bacterial numbers inside the leaf tissues were measured at 0, 6, 12, 24 and 48 h post-inoculation (hpi) by plate-counting assays after bacterial extraction. (c) *In vivo* assays for GV3101 prior infiltration treatments. *Xoo* was infiltrated alone or at 1 and 2 days after GV3101 inoculation in the GV3101 prior-infiltrated leaf areas, and then subjected to *Xoo* bacterial number counting assays as in (b). Data represent the mean $\pm$ standard deviation (SD) of three independent experiments. Significant differences between bacterial numbers at various time points and that at the zero time point (control) are indicated by an asterisk ($P < 0.05$) or double asterisk ($P < 0.01$).

possess only very weak antibiotic activity towards *Xoo*. Therefore, unlike GV3101, LBA4404 and EHA105 must adopt mechanisms other than antibiosis to inhibit *Xoo*-induced HR in *N. benthamiana*. To further test this hypothesis, we measured *Xoo* numbers in leaves that were co-infiltrated with both *Xoo* and the three other *A. tumefaciens* strains (Fig. 5b). *Xoo* numbers in C58C1 + *Xoo* co-infiltrated leaf areas were strongly reduced by 3.3 orders of magnitude at 48 hpi when tissues were desiccated as a result of HR, which was similar to the observations in *Xoo* alone-infiltrated areas. However, *Xoo* numbers in LBA4404 + *Xoo* and EHA105 + *Xoo* co-infiltrated areas were reduced by only 1.3 and 1.1 orders of magnitude, respectively, at 48 hpi (Fig. 5b), being dramatically higher than those in *Xoo* alone-infiltrated areas (Fig. 3b). Meanwhile, the bacterial numbers of these *Agrobacterium* strains did not change

significantly in these co-infiltration analyses (Fig. S3). This result of *in vivo* analyses confirmed the observations *in vitro* and, together, revealed that *A. tumefaciens* adopts strain-specific mechanisms to repress *Xoo*-induced HR in *N. benthamiana*: antibiosis to *Xoo* for GV3101, but not for LBA4404 and EHA105.

pMP90 and the T-DNA of pTiC58 affect GV3101-conferred growth inhibition against *Xoo*

Strains GV3101 and C58C1 can be distinguished by differences in their ability to cause antibiosis to *Xoo* and suppression of *Xoo*-induced HR (Figs 3–5). The main genetic difference between the two strains is that GV3101 carries pMP90 (pTiC58ΔT-DNA), whereas C58C1 is cured for this Ti plasmid, but harbours

Fig. 4 Comparison of the inhibitory effect of four *Agrobacterium tumefaciens* strains on *Xanthomonas oryzae* pv. *oryzae* (Xoo)-induced hypersensitive response (HR) in *Nicotiana benthamiana*. Xoo and four *A. tumefaciens* strains were both infiltrated alone and co-infiltrated into different areas of the same leaves. Photographs were taken at 3 days post-inoculation (dpi) for GV3101 (a), EHA105 (b) and LBA4404 (c) and at 1 dpi for C58C1 (d). At least 20 leaves were tested for each treatment.

Table 2 *Agrobacterium tumefaciens* strains used in this study.

<i>Agrobacterium</i> strain	Chromosome		Ti plasmid		Reference/source
	Background	Marker gene*	Background	Marker gene*	
GV3101	C58	Rm ^R	pMP90 (pTiC58ΔT-DNA)	Gm ^R	Koncz and Schell (1986)
LBA4404	Ach5	Rm ^R	pAL4404 (pTiAch5ΔT-DNA)	Sp ^R and St ^R	Hoekema <i>et al.</i> (1983)
EHA105	C58	Rm ^R	pEHA105 (pTiB6S3ΔT-DNA)		Hood <i>et al.</i> (1993)
C58C1	C58	Rm ^R	pGV2260 (pTiB6S3ΔT-DNA)	Cb ^R	Deblaere <i>et al.</i> (1985)
C58	C58		pTiC58, pAtC58		Hamilton and Fall, (1971)
NT1RE	C58	Rm ^R , Em ^R			Kao <i>et al.</i> (1982)
NT1RE::pMP90	C58	Rm ^R , Em ^R	pMP90 (pTiC58ΔT-DNA)	Gm ^R	Erh-Min Lai
NT1RE::pGV2260	C58	Rm ^R , Em ^R	pGV2260 (pTiB6S3ΔT-DNA)	Cb ^R	Erh-Min Lai
C58::pTiC58Δ <i>virB2</i>	C58		pTiC58Δ <i>virB2</i> , pAtC58		Wu <i>et al.</i> (2014)

*Rm^R, Gm^R, Sp^R, St^R, Cb^R and Em^R indicate resistance to rifampicin, gentamicin, spectinomycin, streptomycin, carbenicillin and erythromycin, respectively.

pGV2260 (pTiB6S3ΔT-DNA) (Table 2). This implies that the antibiosis and HR-inhibitory ability of GV3101 is conferred by the Ti plasmid pMP90, but not pGV2260. To verify this hypothesis, we tested three strains, including NT1RE, which derives from wild-type strain C58, but is cured for pTiC58, and its two transgenic strains, NT1RE::pMP90 and NT1RE::pGV2260, which were gener-

ated by conjugal transfer of pMP90 from GV3101 and pGV2260 from C58C1, respectively, into NT1RE (Table 2). These strains were compared for their ability to suppress Xoo-induced HR and Xoo growth. Our results showed that 98.3% and 93.3% of leaf areas that were co-infiltrated with Xoo and NT1RE and NT1RE::pGV2260, respectively, formed strong HR, whereas none

Fig. 5 *Agrobacterium tumefaciens* strains LBA4404, EHA105 and C58C1 do not exhibit strong antibiosis to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). (a) *In vitro* bacterial number counting assays. *Xoo* [10^8 colony-forming units (cfu)/mL] was cultured alone or co-cultured with LBA4404, EHA105 or C58C1 with the same concentration at 28 °C. *Xoo* bacterial numbers were counted at 0, 6, 12, 24 and 48 h post-culture. (b) *In vivo* bacterial number counting assays. *Xoo* was infiltrated alone or co-infiltrated with LBA4404, EHA105 or C58C1 into leaves of *N. benthamiana*, and bacterial numbers inside the leaf tissues were measured at 0, 6, 12, 24 and 48 h post-inoculation (hpi). Data represent the mean $\pm$ standard deviation (SD) of three independent experiments. Significant differences between bacterial numbers at various time points and that at the zero time point (control) are indicated by an asterisk ($P < 0.05$) or double asterisk ($P < 0.01$).

of the leaf areas which were co-infiltrated with *Xoo* and NT1RE::pMP90 developed any HR (Table 1). This result demonstrates that pMP90, but not pGV2260, confers the ability to inhibit *Xoo*-induced HR in *N. benthamiana*. Moreover, *in vitro* bacterial counting assays showed that, 2 days after co-culture of *Xoo* with these strains, *Xoo* bacterial numbers decreased by approximately two orders of magnitude when co-cultured with NT1RE and NT1RE::pGV2260, but decreased by about four orders of magnitude when co-cultured with NT1RE::pMP90 (Fig. 6), which is similar to co-culture with GV3101 (Fig. 3a). This result reveals that pMP90, but not pGV2260, possesses the capacity for *Agrobacterium*'s antibiosis against *Xoo*. Collectively, our results confirm that the antibiosis and HR-inhibitory ability of GV3101 is conferred by the Ti plasmid pMP90.

The T-DNA is deleted in the Ti plasmid pMP90. To understand the effect of this deletion on the antibiosis and HR-inhibitory ability of GV3101, the wild-type strain C58 and two derived strains GV3101 and NT1RE::pMP90 were compared for their ability to suppress *Xoo*-induced HR and *Xoo* growth. The essential difference between the three strains is that C58 contains T-DNA,

whereas GV3101 and NT1RE::pMP90 do not possess T-DNA in their Ti plasmid pTiC58 (Table 2). Plant co-infiltration assays showed that, unlike co-infiltration with *Xoo* and GV3101 or NT1RE::pMP90, which caused no HR in any infiltrated area, 63.3% of the leaf areas that were co-infiltrated with *Xoo* and C58 developed HR at 2 dpi (Table 1). In addition, bacterial co-culture assays showed that *Xoo* numbers did not change significantly when co-cultured with C58 (Fig. 6), which could be distinguished from co-culture with GV3101 and NT1RE::pMP90, which resulted in a dramatic decrease in *Xoo* numbers (Figs 3 and 6). Furthermore, we conducted *in vitro* co-culture experiments using C58::pTiC58 Δ *virB2*, a C58 mutant lacking *virB2* and thus defective in T-DNA transfer. Co-culture of *Xoo* with C58::pTiC58 Δ *virB2* resulted in a reduction in *Xoo* bacterial numbers by about one order of magnitude at 48 hpi, which is significantly greater than with co-culture with the wild-type C58, but obviously less than with co-culture with GV3101 and NT1RE::pMP90 (Figs 3 and 6). Further examination showed that C58::pTiC58 Δ *virB2* exhibited a similar HR inhibitory percentage to the wild-type C58 (Table 1), indicating that a defect in T-DNA transfer does not significantly affect the HR-inhibitory ability in host plant leaves. Collectively, these results demonstrate that the T-DNA of pTiC58 is a negative, but not sole, regulator of the ability of strain GV3101 to inhibit *Xoo* growth, which consequently suppresses HR.

***Agrobacterium tumefaciens* strains LBA4404 and EHA105 block *Xoo*-induced HR by suppression of the *Xoo*-induced H₂O₂ burst**

As the H₂O₂ burst is an early and essential event regulating *Xoo*-dependent HR and non-host resistance (Li *et al.*, 2015), we hypothesized that the non-antibiotic *A. tumefaciens* strains LBA4404 and EHA105 may block *Xoo*-induced HR by suppression of the *Xoo*-induced H₂O₂ burst. To test our hypothesis, *Xoo* was infiltrated alone or together with one of the three *A. tumefaciens* strains LBA4404, EHA105 or C58C1. Subsequently HR symptoms were observed and H₂O₂ accumulation was detected by 3,3'-diaminobenzidine (DAB) staining analysis. Our results showed that H₂O₂ in leaves that were co-infiltrated with *Xoo* and LBA4404 or EHA105 within 24 hpi was completely abolished, which coincided with the lack of *Xoo*-induced HR in these leaves (Fig. 7a,b). In contrast, H₂O₂ in leaves that were co-infiltrated with *Xoo* and C58C1 accumulated strongly as early as 12 hpi, identical to our observations in control leaves infiltrated with *Xoo* alone (Fig. 7c). As a result, *Xoo*-induced HR formed as strongly in these leaves as in controls (Fig. 7c). Furthermore, we performed quantitative analyses to determine the H₂O₂ accumulation level in infiltrated leaf areas. H₂O₂ in leaf areas that were co-infiltrated with *Xoo* and C58C1 accumulated strongly to over 1000 μ mol/g fresh weight (FW) at 12 hpi, which is similar to that detected in control leaves that were infiltrated with *Xoo* alone (Fig. 7d); however, H₂O₂

Fig. 6 The Ti plasmid pTiC58 confers the growth inhibitory capacity of GV3101 against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), and its transfer-DNA (T-DNA) negatively, but not uniquely, regulates this antibiosis. *Xoo* was cultured alone as control and co-cultured with four strains of *Agrobacterium*. The growth dynamics of *Xoo* in each culture were detected at 0, 1 and 2 days post-culture. The experiments were repeated three times with three replications for each culture in each experiment. Data represent the mean $\pm$ standard deviation (SD) of three independent experiments. Significant differences between *Xoo* + *Agrobacterium* co-cultures and *Xoo* culture alone are indicated by an asterisk ($P < 0.05$) or double asterisk ($P < 0.01$). cfu, colony-forming unit.

accumulation in leaf areas that were co-infiltrated with *Xoo* and LBA4404 or EHA105 was lower than 15 $\mu\text{mol/g}$ FW at 12 hpi, which is 66 times lower than that in control leaves (Fig. 7d). This result confirms the observations based on qualitative DAB staining data. Together, these findings clearly show that *A. tumefaciens* strains LBA4404 and EHA105 block *Xoo*-induced HR by suppression of the *Xoo*-induced H_2O_2 burst.

Suppression of H_2O_2 accumulation during blocking of *Xoo*-induced HR by *A. tumefaciens* strains LBA4404 and EHA105 most probably does not result from agrobacterial H_2O_2 catabolism

To address the mechanism by which strains LBA4404 and EHA105 repressed H_2O_2 accumulation during the blocking of *Xoo*-induced HR, we first examined whether it resulted from direct H_2O_2 detoxification by *A. tumefaciens*, as it has been reported that *A. tumefaciens* carries catalase genes to catabolize H_2O_2 (Xu and Pan, 2000). Plate zone-of-inhibition tests were conducted to compare the three HR-suppressing strains GV3101, EHA105 and LBA4404 and the non-HR-suppressing strain C58C1 of *A. tumefaciens* for their ability to catabolize H_2O_2 . All four tested strains exhibited similar size of zone of inhibition in plates supplied with H_2O_2 (Fig. S4, see Supporting Information). These data show that the tested HR-suppressing and non-HR-suppressing strains of *A. tumefaciens* are similar in their ability to catabolize H_2O_2 , and indicate that the suppression of H_2O_2 accumulation during blocking of *Xoo*-induced HR by *A. tumefaciens* most probably does not result from direct H_2O_2 catabolism by *A. tumefaciens* itself.

The expression of *Xoo* T3SS genes is altered moderately after co-infiltration of *Xoo* with *A. tumefaciens* strains LBA4404 and EHA105

The T3SS is required for *Xoo*-induced HR in its non-host plant (Fig. 1). Thus, we were interested to check whether *A. tumefaciens*

represses the function of T3SS, thereby blocking *Xoo*-induced HR. To this end, we conducted comparative time-course analysis on the expression of three *Xoo* T3SS genes in leaves infiltrated with *Xoo* alone or co-infiltrated with both *Xoo* and *A. tumefaciens* strains LBA4404, EHA105 or C58C1. The monitored genes included the T3SS core component gene *hrcU* and the secreted essential HR-inducer genes *hrpD6* and *hpa1* (Tampakaki *et al.*, 2010). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) gene expression analysis revealed that, compared with infiltration with *Xoo* alone, co-infiltration with *Xoo* and the non-HR-suppressing strain C58C1 transiently repressed the expression of *hrcU* by 2.8-fold at 3 hpi and consistently induced the expression of *hpa1* and *hrpD6* by 2.7- and 4.8-fold, respectively at 6 hpi; however, co-infiltration with *Xoo* and the HR-suppressing strain EHA105 exhibited the opposite effect on the expression of these genes to C58C1. It induced the expression of *hrcU* by 2.8-fold at 3 hpi and consistently repressed the expression of *hpa1* and *hrpD6* by 7.3- and 4.1-fold, respectively, at 6 hpi. Co-infiltration with *Xoo* and another HR-suppressing strain LBA4404 showed a similar effect on the expression of these genes to EHA105, but with much lower magnitude (Fig. 8). Considering that the alteration in expression of all three tested T3SS genes by the *A. tumefaciens* strains was not dramatic (less than eight-fold), whether T3SS indeed affects *Xoo*-induced HR and non-host resistance awaits further verification.

DISCUSSION

The effect of *Agrobacterium* on pathogen-induced HR and/or resistance in plants has rarely been documented. The available examples (Table S1, see Supporting Information) all concern the inhibitory effects of *A. tumefaciens* on the HR induced by *P. syringae* or TMV in tobacco (Pruss *et al.*, 2008; Rico *et al.*, 2010; Robinette and Matthysse, 1990). The molecular basis of the effect of *Agrobacterium* on pathogen-induced HR and non-host

Fig. 7 *Agrobacterium tumefaciens* strains LBA4404 and EHA105 suppress H₂O₂ accumulation and thereby block the *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)-induced hypersensitive response (HR). Leaves were solely infiltrated with *Xoo* or co-infiltrated with *Xoo* and LBA4404 (a), EHA105 (b) or C58C1 (c). 3,3'-Diaminobenzidine (DAB) staining was conducted at 12 and 24 h post-inoculation (hpi). H₂O₂ levels at 12 hpi were also quantitatively measured (d). Photographs of HR symptoms were taken before DAB staining. At least 20 leaves were analysed for each treatment. FW, fresh weight.

resistance in plants is largely unknown. We found, in this study, that four strains of *A. tumefaciens* exhibit diverse abilities to inhibit *Xoo*-induced HR on co-infiltration of *N. benthamiana* leaves. C58C1 cannot inhibit HR, whereas the remaining three strains, GV3101, LBA4404 and EHA105, strongly repress *Xoo*-induced HR. To our surprise, these three strains adopt distinct strategies to suppress *Xoo*-induced HR. GV3101 possesses strong antibiotic activity and thus kills *Xoo* cells directly. However, strains LBA4404 and EHA105 only show very weak antibiotic activity *in vivo*, but rather suppress *Xoo*-induced HR by the repression of defence mechanisms, such as ROS accumulation. The antibiotic role of *A. tumefaciens* towards other pathogens has not been documented until recently, when *A. tumefaciens* was found to compete *in planta* with *Pseudomonas aeruginosa*, an opportunistic pathogen of humans and plants (Ma *et al.*, 2014). This study provides a new example to demonstrate *A. tumefaciens*-conferred antibiosis against an agriculturally important crop bacterial pathogen.

Previously, the *A. tumefaciens* wild-type strain C58 has been reported to be able to suppress the *Pph*-elicited HR by auxin biosynthesis conferred by the *tms* genes in the T-DNA (Robinette and Matthyse, 1990). However, in this study, we observed that C58 can only partially (36.7%) repress the *Xoo*-induced HR (Table 1) and full repression requires the removal of the T-DNA (Fig. 6). This discrepancy in HR suppression efficiency and mechanism may reflect the difference in the strength and/or developmental mechanisms of HR elicited by the two pathogens. Moreover, the experimental conditions, such as the inoculation systems, including the applied concentration of C58, plant species and stages, as well as growth conditions, would also have affected the phenotype.

The detailed molecular mechanism of antibiosis of *A. tumefaciens* to *Xoo* remains unclear. A comparison of the genotype and *Xoo* antibiosis activity of all eight *A. tumefaciens* strains listed in Table 2 reveals that the Ti plasmid pTiC58 with deletion of T-DNA (pTiC58ΔT-DNA/pMP90) confers the antibiosis of

A. tumefaciens. Interestingly, a comparison between wild-type C58 and two derived strains, GV3101 and NT1RE::pMP90, demonstrates that the T-DNA of pTiC58 most probably plays a negative role in antibiosis but only partially accounts for this effect (Figs 3

Fig. 8 Effect of *Agrobacterium tumefaciens* strains LBA4404, EHA105 and C58C1 on the expression of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) type III secretion system (T3SS) genes. The expression of three *Xoo* T3SS genes (*hrcU*, *hpa1* and *hrpD6*) in leaves infiltrated solely with *Xoo* and co-infiltrated with *Xoo* and *A. tumefaciens* strains EHA105, LBA4404 or C58C1 was comparatively analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The relative expression levels of sole *Xoo* infiltration to co-infiltration with *Xoo* and an *A. tumefaciens* strain at 0, 3 and 6 h post-inoculation (hpi) are shown. Data represent the mean $\pm$ standard deviation (SD) of three independent experiments.

and 6; Table 1). However, the T-DNA of pTiB6S3 seems to have no obvious role in antibiosis (Figs 4–6; Table 1). This is probably because the T-DNA deleted in pMP90 (but existing in pTiC58) and that deleted in pGV2260 (but existing in pTiB6S3) may differ, and the key genetic factor in the T-DNA deleted in pMP90 (but existing in pTiC58), which confers antibiosis capability, does not exist in the T-DNA that is deleted in pGV2260 (but existing in pTiB6S3). Notably, a C58 *virB2* deletion mutant (C58::pTiC58 Δ *virB2*) possessed only weak HR inhibitory ability and antibiosis towards *Xoo*, as was observed for the wild-type C58 (Figs 3 and 6; Table 1), implying that the T-DNA does not have to be delivered to the host to prevent the antibiosis exerted by *Agrobacterium* carrying pTiC58. However, although the antibiosis towards *Xoo* of both the wild-type C58 and the C58::pTiC58 Δ *virB2* mutant is weak compared with that of GV3101 and NT1RE::pMP90, the C58::pTiC58 Δ *virB2* mutant exhibits approximately 10-fold higher inhibition of *Xoo* than the wild-type C58 (Fig. 6). This indicates that some, but not all, of the negative influence of the T-DNA is dependent on a functional T4SS (or at least *VirB2*).

From the *Xoo* perspective, the T-DNA of pTiC58 seems to benefit *Xoo*, as co-culture of *Xoo* with *Agrobacterium* strains with deletion of this T-DNA, such as GV3101 and NT1RE::pMP90, causes a dramatic decrease in *Xoo* (Figs 3 and 6; Table 1). Moreover, transfer of this T-DNA may benefit *Xoo*, as co-culture of *Xoo* with the C58::pTiC58 Δ *virB2* mutant results in about a 10-fold decrease in *Xoo* (Fig. 6). However, how the transfer of T-DNA benefits *Xoo* is unknown. *In planta*, the T-DNA of pTiC58 may be transferred into the plant genome and consequently inhibit the antibiosis towards *Xoo*, as it is well known that T-DNA is transferred and integrated into the plant genome during infection (McCullen and Binns, 2006). In addition, we observed that the *in vitro* co-culture of *Xoo* with the C58::pTiC58 Δ *virB2* mutant, which does not involve a plant host, leads to an obvious decrease in *Xoo* (Fig. 6). Thus, it will be interesting to examine the possibility that the T-DNA of pTiC58 is transferred into *Xoo* and thereby suppresses the antibiosis towards *Xoo*.

It is unclear what part of the T-DNA in pTiC58 prevents the antibiosis towards *Xoo*. According to the sequence deposited in the National Center for Biotechnology Information (NCBI) (NC_003065), the T-DNA region of pTiC58 contains 19 open

reading frames (ORFs). Among them, three encode hypothetical proteins, whereas all the remaining ORFs encode proteins involved in the biosynthesis and metabolism of opines and two hormones auxin and cytokinin. We examined the effect of indole-3-acetic acid (IAA) and zeatin on *Xoo*-induced HR and *Xoo* growth. As shown in Fig. S5 (see Supporting Information), co-infiltration of *Xoo* with 100 μ M IAA (Robinette and Matthysse, 1990) and 2 nM zeatin (Smigocki and Owens, 1988) did not alter the *Xoo*-induced HR in its non-host plant *N. benthamiana*. Likewise, *in vitro* culture experiments showed that incubation with these hormones did not affect *Xoo* growth. Nonetheless, assays to block the biosynthesis of these hormones are required to further clarify their role in the promotion of HR and *Xoo* growth. Moreover, three functionally unknown proteins of small molecular weight [35 amino acids (aa) for Atu8054, 88 aa for Atu8061 and 101 aa for Atu8062] in the T-DNA may act as ligands, which are recognized by receptors of *Xoo*, resulting in suppression against *Agrobacterium* carrying pTIC58. Further genetic analyses are required to verify these possibilities. Of course, we cannot say with certainty that the presence or absence of the T-DNA of the Ti plasmid pTIC58 is the only difference between C58 and GV3101 and NT1RE::pMP90, as we have not sequenced the chromosomes of the strains used in this study. Therefore, it is also possible that a certain sequence in the chromosomes is responsible for the negative role in antibiosis.

In addition, the differences among the *Agrobacterium* strains in their ability to inhibit *Xoo* growth may well reflect differences in the type VI secretion system (T6SS)-mediated production and/or delivery of the antibiotic compounds that are highly toxic to *Xoo*. The growth-suppressing strains (GV3101 and NT1RE::pMP90) may promote the biosynthesis and/or delivery of these antibiotic compounds, whereas the non-growth-suppressing strains (EHA105, C58C1, C58, NT1RE and NT1RE::pGV2260) may not. Therefore, the elucidation of how the growth-suppressing strains (GV3101 and NT1RE::pMP90) promote the biosynthesis and/or delivery of these antibiotic compounds will be the next challenge. The role of T6SS in bacterial recognition and targeting of heterologous cells during bacterial cell–cell interactions has been reported (Coulthurst, 2013; Russell *et al.*, 2014). A typical example is that of *P. aeruginosa*, which employs the T6SS to arrest the growth of a variety of prokaryotic and eukaryotic organisms, including *Vibrio cholerae* and *Acinetobacter baylyi* (Basler *et al.*, 2013). The T6SS is present in the genome of *A. tumefaciens* (Boyer *et al.*, 2009) and is functional (Lin *et al.*, 2014; Wu *et al.*, 2012). The role of T6SS in *Xoo* repression by *A. tumefaciens* GV3101 and NT1RE::pMP90 awaits further study.

Surprisingly, unlike GV3101, *A. tumefaciens* strains LBA4404 and EHA105 have no strong antibiotic effect on *Xoo* either *in vivo* or *in vitro*. Rather, they block *Xoo*-induced HR through the abolition of the *Xoo*-induced H₂O₂ burst (Fig. 7), which is essential to HR development in response to *Xoo* (Li *et al.*, 2015). How they

repress H₂O₂ accumulation is unclear. It has been reported that *A. tumefaciens* carries catalase genes, enabling it to catabolize H₂O₂ (Xu and Pan, 2000). However, our plate zone-of-inhibition tests revealed that both HR-suppressing and non-HR-suppressing strains of *A. tumefaciens* show a similar ability to catabolize H₂O₂ (Fig. S4), thus ruling out the possibility that LBA4404 and EHA105 remove the H₂O₂ produced, thereby preventing accumulation in plants. Therefore, the remaining question is how LBA4404 and EHA105 repress H₂O₂ production. We found that EHA105 and LBA4404 suppressed, whereas the non-HR-suppressing strain C58C1 induced, the expression of *hpa1* and *hrpD6* (Fig. 8). Considering that Δ *hrpD6* fails to induce H₂O₂ and HR and Δ *hpa1* delays the generation of H₂O₂ and HR in *N. benthamiana* (Li *et al.*, 2015), our results indicate that the suppression of *hpa1* and *hrpD6* may play a role in the inhibition of *Xoo*-induced HR by LBA4404 and EHA105. However, the significance of this suppression remains to be confirmed, as the suppression of *hpa1* and *hrpD6* gene expression by LBA4404 and EHA105 is not dramatic (less than eight-fold). Alternatively, LBA4404 and EHA105 may target other T3SS effector gene(s) or other types of gene(s) to repress *Xoo*-induced HR. Moreover, the interactions between plants and *Agrobacterium* are very complex (Gohlke and Deeken, 2014; Hwang *et al.*, 2015). *Agrobacterium* is able to suppress diverse plant defences at various stages during infection (Gohlke and Deeken, 2014; Hwang *et al.*, 2015; Veena *et al.*, 2003). Therefore, it is also possible that these *Agrobacterium* strains compromise plant pathways responsible for the generation of the *Xoo*-induced ROS burst, thereby blocking the *Xoo*-induced HR in *N. benthamiana*. The identification of key regulators of plant ROS accumulation and HR development that are targeted by these *Agrobacterium* strains will be an interesting study for the future.

In summary, our findings provide an insight into the interactions between *A. tumefaciens* and other pathogens inside plants, and the molecular mechanisms underlying the non-host resistance induced by *Xoo*. In addition, *Agrobacterium* strains, such as GV3101 and EHA105, are frequently used for gene functional transient expression analysis. Our finding that these strains are antibiotic to some pathogens and/or inhibit HR and non-host resistance should remind researchers that caution should be taken when explaining the results of functional analyses of genes on disease resistance employing *Agrobacterium*-mediated transient transformation assays in plants.

EXPERIMENTAL PROCEDURES

Strains of *A. tumefaciens*

The strains of *A. tumefaciens* used in this study are listed in Table 2.

Plant growth and pathogen inoculation

The growth of *N. benthamiana* and the preparation of inocula of *Xoo* and its Δ *hrpD6*, Δ *hpa1* and Δ *hrcU* mutants followed previous procedures (Li

et al., 2012). *Agrobacterium tumefaciens* was grown at 28 °C in YEB medium (0.5% tryptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sucrose and 0.05% MgSO₄) until the optical density at 600 nm (OD₆₀₀) reached about 0.8. *Agrobacterium* cells were collected and resuspended in MMAi buffer (0.44% Murashige and Skoog (MS) powder, 2% sucrose, 10 mM 2-(N-Morpholino)ethanesulfonic acid (MES), 20 µM acetosyringone, pH 5.8) to an OD₆₀₀ of 1.5 and recovered by slow shaking of the culture at 60 rpm for 2 h before use. *Pst* DC3000 and *E. coli* strain TG1 were grown in KB (1% protease peptone, 0.15% anhydrous K₂HPO₄, 1.5% glycerol and 5 mM MgSO₄, pH 7.0) and LB (1% tryptone, 0.5% yeast extract and 1% NaCl) media, respectively. The bacterial cells were collected and resuspended in 10 mM MgCl₂ to 10⁷ colony-forming units (cfu)/mL. The prepared bacterial inocula were infiltrated into leaves with a sterilized needleless syringe. The inoculated plants were grown at 28 °C under a light/dark cycle of 16 h/8 h. At least 30 leaves were used for each treatment.

Bacterial number counting assays

Bacterial number counting of *Xoo* and *A. tumefaciens* in co-culture plate assay and co-infiltration *in vivo* assay was conducted as described previously (Li *et al.*, 2012). For the *in vitro* co-culture plate assay, 100 µL of bacterial suspension were taken and serial dilutions were prepared with sterilized distilled water and plated in triplicate on nutrient agar (NA)/YEB solid plates with appropriate antibiotics. Plates were incubated at 28 °C until the single colonies could be counted. Bacterial numbers were counted taking advantage of the obvious difference in colony colour and growth speed between the two bacteria. Colonies of *Xoo* and *Agrobacterium* are yellow and greyish, respectively. Moreover, colonies of *Agrobacterium* appear about 12 h earlier than those of *Xoo*. All the experiments were repeated three times. For the assay to study the effect of IAA and zeatin on *Xoo* growth, IAA at a final concentration of 100 µM (Robinette and Matthysse, 1990) and zeatin at 2 nM (Smigocki and Owens, 1988) were added, and *Xoo* growth was monitored by bacterial number counting assay as described above.

Detection of H₂O₂ in leaves

DAB staining was performed to detect H₂O₂ *in situ* in plant leaves as described previously (Li *et al.*, 2012). At least 20 leaves for each time point of each treatment were analysed. For the quantitative analysis of H₂O₂ in plant leaves, we followed the protocol described previously (Gay *et al.*, 1999). Briefly, leaf samples were collected and homogenized in liquid nitrogen with cooled acetone. After centrifugation at 10 000 *g* at 4 °C, the suspension was mixed with extraction buffer (CCl₄–CHCl₃–H₂O, 3 : 1 : 5, v/v/v), and then centrifuged at 10 000 *g* at 4 °C to obtain H₂O₂ in the water-soluble layer. For H₂O₂ concentration detection, the assay solution was freshly prepared by mixing solutions A (100 µM xylenol orange, 100 µM sorbitol) and B [3.3 mM FeSO₄, 3.3 mM (NH₄)₂SO₄, 412.5 mM H₂SO₄] in a ratio of 10 : 1 (v/v). The H₂O₂-containing solution was mixed with a two-fold amount of assay solution and incubated at 30 °C for 30 min. H₂O₂ in the solution was detected by measuring the absorbance at 560 nm. A standard curve was prepared using five H₂O₂ concentrations of 2, 4, 6, 8 and 10 µM, and the concentrations of H₂O₂ in plant leaves were calculated according to the standard curve.

Gene expression analyses

To understand the plant molecular response to inoculation with *Xoo* or co-inoculation with *Xoo* and GV3101, we monitored the expression of a set of *PR* genes, the pivotal defence regulatory gene *NPR1* and the HR marker genes *HSR203J* and *HIN1*. The transcription of genes was quantified by qRT-PCR analyses as described previously (Li *et al.*, 2012). The PCR primers used in this study are listed in Table S2 (see Supporting Information). *Xoo80* and *Nb18s-rDNA* genes were used as loading reference genes for *Xoo* and *N. benthamiana*, respectively (Lang *et al.*, 2010).

Inhibition zone assays to reveal the ability of *A. tumefaciens* to catabolize H₂O₂

Agrobacterium tumefaciens was grown as described above. Top agar medium was prepared as described by Xu and Pan (2000). Sterilized paper discs (0.6 cm in diameter) were placed on the surface of the plates; 10 µL of H₂O₂ solution at concentrations of 2, 20, 200 and 1000 mM were loaded on the paper discs. The zone of inhibition of bacterial growth was visualized after overnight incubation at 28 °C.

Experimental repetition and statistical analysis of the experimental data

All experiments were conducted three times independently. The significance of the difference between treatments and control was analysed by Student's *t*-test. Results are shown as the mean ± standard deviation (SD). Significant difference is indicated by an asterisk (*P* < 0.05) or double asterisk (*P* < 0.01).

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

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

Fig. S1 Effect of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Agrobacterium tumefaciens* strain GV3101 on the expression of pathogenesis-related (*PR*) and hypersensitive response (*HR*) marker genes in *Nicotiana benthamiana*.

Fig. S2 Bacterial number dynamics of *Agrobacterium tumefaciens* strain GV3101 inside the leaves of *Nicotiana benthamiana* after co-inoculation with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). cfu, colony-forming unit.

Fig. S3 Bacterial number dynamics of *Agrobacterium tumefaciens* strains LBA4404 and EHA105 inside the leaves of *Nicotiana benthamiana* after co-inoculation with *Xanthomonas oryzae* pv. *oryzae* (Xoo) (a) and during co-culture *in vitro* (b). cfu, colony-forming unit.

Fig. S4 Inhibition zone test to manifest the ability of *Agrobacterium tumefaciens* strains to catabolize H₂O₂. *Agrobacterium tumefaciens* was grown overnight in YEB solid medium. Sterilized filter paper discs containing 10 µL of H₂O₂ at diverse concentrations were placed on the surface of the plates.

The inhibition zones appeared after overnight culture. Three independent experiments were conducted with similar results.

Fig. S5 Effect of indole-3-acetic acid (IAA) and zeatin on *Xanthomonas oryzae* pv. *oryzae* (Xoo)-induced hypersensitive response (HR) in its non-host plant *Nicotiana benthamiana* (a, b) and on Xoo growth in culture (c).

Table S1 Reported pathosystems in which hypersensitive response (HR) and resistance are inhibited by *Agrobacterium*.

Table S2 Polymerase chain reaction (PCR) primers used in this study.