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Hexanoic acid is a resistance inducer that protects tomato plants against *Pseudomonas syringae* by priming the jasmonic acid and salicylic acid pathways

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SUMMARY

Hexanoic acid-induced resistance (Hx-IR) is effective against several pathogens in tomato plants. Our study of the mechanisms implicated in Hx-IR against *Pseudomonas syringae* pv. *tomato* DC3000 suggests that hexanoic acid (Hx) treatment counteracts the negative effect of coronatine (COR) and jasmonyl-isoleucine (JA-Ile) on the salicylic acid (SA) pathway. In Hx-treated plants, an increase in the expression of jasmonic acid carboxyl methyltransferase (*JMT*) and the SA marker genes *PR1* and *PR5* indicates a boost in this signalling pathway at the expense of a decrease in JA-Ile. Moreover, Hx treatment potentiates 12-oxo-phytodienoic acid accumulation, which suggests that this molecule might play a role *per se* in Hx-IR. These results support a positive relationship between the SA and JA pathways in Hx-primed plants. Furthermore, one of the mechanisms of virulence mediated by COR is stomatal re-opening on infection with *P. syringae*. In this work, we observed that Hx seems to inhibit stomatal opening *in planta* in the presence of COR, which suggests that, on infection in tomato, this treatment suppresses effector action to prevent bacterial entry into the mesophyll.

INTRODUCTION

In response to a wide variety of microbial pathogens and insect herbivores, plants have developed numerous defence mechanisms, and many of these defences are induced by pathogen attack. The appropriate regulation of defence responses is important for plant fitness, as the activation of defence responses has deleterious effects on plant growth. Plants partly protect themselves against pathogen challenges by means of constitutive preformed barriers (constitutive resistance). When these barriers fail to prevent the entry of pathogens, plants generally activate another chain of defence responses (pathogen-induced resistance), such as reinforced barriers and stomatal closure control. These systems are activated when plants detect microbial elicitors, named pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). Moreover, many plant hormones are involved in these responses, including jasmonates, salicylates and ethylene (ET). Generally, salicylic acid (SA) is part of the defence responses against biotrophic pathogens, whereas jasmonic acid (JA) participates in defence responses against necrotrophs and insects (Beckers and Spoel, 2006). SA induces the expression of a set of defence-related genes, such as genes encoding some of the pathogenesis-related (PR) proteins. Although SA has been shown to play an important role in signal transduction, other molecules are also involved directly in plant defence. For example, methyl salicylate (MeSA) has recently been identified as a mobile signal for systemic acquired resistance (SAR) in *Tobacco mosaic virus* (TMV)-infected tobacco (*Nicotiana tabacum*) (Park *et al*., 2007).

Furthermore, several molecules from the JA synthesis pathway may be involved in plant defence processes. Jasmonates are synthesized in plants via the octadecanoid pathway. JA is regarded as the primary intracellular transducer of this pathway because of its similarity to prostaglandin of animals (Cheong and Choi, 2003; Kazan and Manners, 2008). However, depending on the biological system studied, a number of biosynthetic intermediates, isomers, derivatives and metabolites of the octadecanoid pathway have been observed to be powerful cellular regulators. For instance, the octadecanoid precursors of JA, including linolenic acid, 13(*S*)-hydroperoxylinolenic acid and 12-oxo-phytodienoic acid (OPDA), have known biological activities in the jasmonate-regulated responses of potato plants against *Pseudomonas syringae* pv. *maculicola*. These activities suggest that OPDA is important in the SAR of potato plants (Avanci *et al*., 2010; Landgraf *et al*., 2002) The *Arabidopsis opr3* mutant does not produce an isoform of the enzyme 12-oxophytodienoate reductase (12-OPR), which is essential for JA biosynthesis (Stintzi and Browse, 2000), but displays increased resistance to the insect *Bombus impatiens* and the fungus *Alternaria brassicicola*. The analysis of the transcriptional profile of *opr3* indicates that genes formerly characterized as exhibiting JA-dependent expression after wounding are also expressed in this mutant, which suggests that cyclopentenone jasmonate derivatives can fulfil some JA tasks *in vivo* (Stintzi *et al*., 2001).

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JA, a hormone, can also be catabolized to form its volatile counterpart methyl jasmonate (MeJA) and numerous conjugates, such as jasmonyl-leucine (JA-Leu) and jasmonyl-isoleucine (JA-Ile). Amino acid conjugation is necessary for JA activation, and (–)-JA-L-Ile has been demonstrated recently to be the bioactive form of JA (Staswick, 2008).

Previous studies have suggested that MeJA formation is one of the important control points for jasmonate-regulated plant responses. MeJA may be a candidate for intra- and intercellular signal transducers that mediate jasmonate-responsive plant responses because it can diffuse through membranes. One important aspect of this molecule in plant defences against *P. syringae* is that MeJA affects plant transpiration (Wang, 1999) by promoting stomatal closure (Gehring *et al*., 1997; Suhita *et al*., 2003), which induces alkalinization in the cytosol of guard cells before the production of reactive oxygen species (Suhita *et al*., 2004). This aspect might affect the plant–bacterium interaction by inhibiting the bacterial suppression of stomatal closure, which was observed in a *Pseudomonas*–plant interaction.

Pathogens have also evolved complex mechanisms to evade plant defences. *Pseudomonas syringae* pv. *tomato* is a hemibiotrophic microorganism, which causes bacterial speck disease in tomato, *Arabidopsis thaliana* and *Brassica* spp. One mechanism of *P. syringae* pv. *tomato* pathogenesis is to produce the effector coronatine (COR), a JA-Ile mimic that activates JA-Ile synthesis. Both molecules suppress SA-mediated host responses and therefore increase bacterial virulence (Brooks *et al*., 2005; Katsir *et al*., 2008). COR also facilitates the entry of *P. syringae* into *A. thaliana* tissue by suppressing the closure of stomata, a basal defence response induced on microbial attack (Melotto *et al*., 2006). Moreover, COR is also required for the growth and persistence of this pathogen in plant tissue (Brooks *et al*., 2004; Elizabeth and Bender, 2007).

In addition to pathogen attack, several types of induced resistance, such as SAR and induced systemic resistance (ISR), can be produced by other microorganisms or by treatment with certain natural or synthetic compounds (Beckers and Conrath, 2007). During induced resistance, the sensitization of stress responsiveness, also called priming, is not only observed in plants, but also in animals (Jung *et al*., 2009; Pastor *et al*., 2012; Pham *et al*., 2007; Prime-A-Plant Group *et al*., 2006). Priming is the phenomenon that enables cells to respond to low levels of a stimulus in a more rapid and robust manner than is found in nonprimed cells (Conrath, 2011; Conrath *et al*., 2002). Thus, when plants primed by treatments inducing resistance are subsequently challenged by pathogens or abiotic stresses, these plants show a faster and/or stronger activation of defence responses (Prime-A-Plant Group *et al*., 2006). One chemical inducer of priming, β-aminobutyric acid (BABA), is a potent inducer of resistance against a wide range of pathogens, and uses a signalling mechanism that differs from SAR and ISR (Ton and Mauch-Mani, 2004).

Recently, we have demonstrated that hexanoic acid (Hx) induces resistance in *Solanum lycopersicum* and *A. thaliana* plants against *Botrytis cinerea* (Kravchuk *et al*., 2011; Vicedo *et al*., 2009). This natural compound acts as an inducer of plant defences by means of a priming mechanism. On infection, the oxylipin OPDA and the bioactive molecule JA-Ile were significantly induced in treated plants. In addition, abscisic acid (ABA) acted as a positive regulator of Hx-induced resistance (Hx-IR) by enhancing callose accumulation (Vicedo *et al*., 2009).

In this study, we performed assays to determine both the mode of action and the effectiveness of Hx as an inducer of resistance against the hemibiotrophic pathogen *P. syringae*. We present evidence that Hx primes SA signalling through the inhibition of COR-mediated bacterial manipulation of the SA pathway. We also show that Hx alters the oxylipin pathway by reducing the accumulation of the active molecule JA-Ile and by increasing OPDA accumulation. OPDA could participate in the defence against this pathogen, although the involvement of other JA derivatives in the mechanism of Hx-IR cannot be excluded. These results reveal a positive interaction between the JA and SA pathways in Hx-primed plants.

RESULTS

Hx enhances tomato resistance against *P. syringae* infection

To evaluate and confirm the efficacy of Hx against *Pseudomonas* in tomato plants, Hx treatments were applied in hydroponic culture, as described in Vicedo *et al*. (2009), and by soil drench. Four-week-old tomato plants (cv. Ailsa Craig) were treated with different Hx concentrations for 48 h under hydroponic conditions prior to *P. syringae* pv. *tomato* DC3000 (*Pst*) inoculation. Statistically significant reductions in disease symptoms (Fig. 1A) and in the size of the bacterial population (Fig. 1B) were observed at 72 h post-inoculation (hpi), which demonstrated that the inducer effect is concentration dependent. We also checked the efficacy of Hx treatment when Hx was applied by soil drench. In these experiments, a treatment with 0.6 mM Hx at 72 h before inoculation also protected tomato plants by reducing significantly both the symptoms of disease (by approximately 50%) and the bacterial population (Fig. 1C,D).

In this work, the analyses of the inducer effect were performed by treating plants in hydroponic conditions with 0.6 mM Hx. This treatment reduced the symptoms by 50%, and the observed decrease in the bacterial population was similar to that described previously by other authors (Jung *et al*., 2009).

Hx-IR does not alter callose deposition

We analysed callose deposition, which is a commonly used marker of basal defence activation. Four-week-old tomato plants (cv. Ailsa

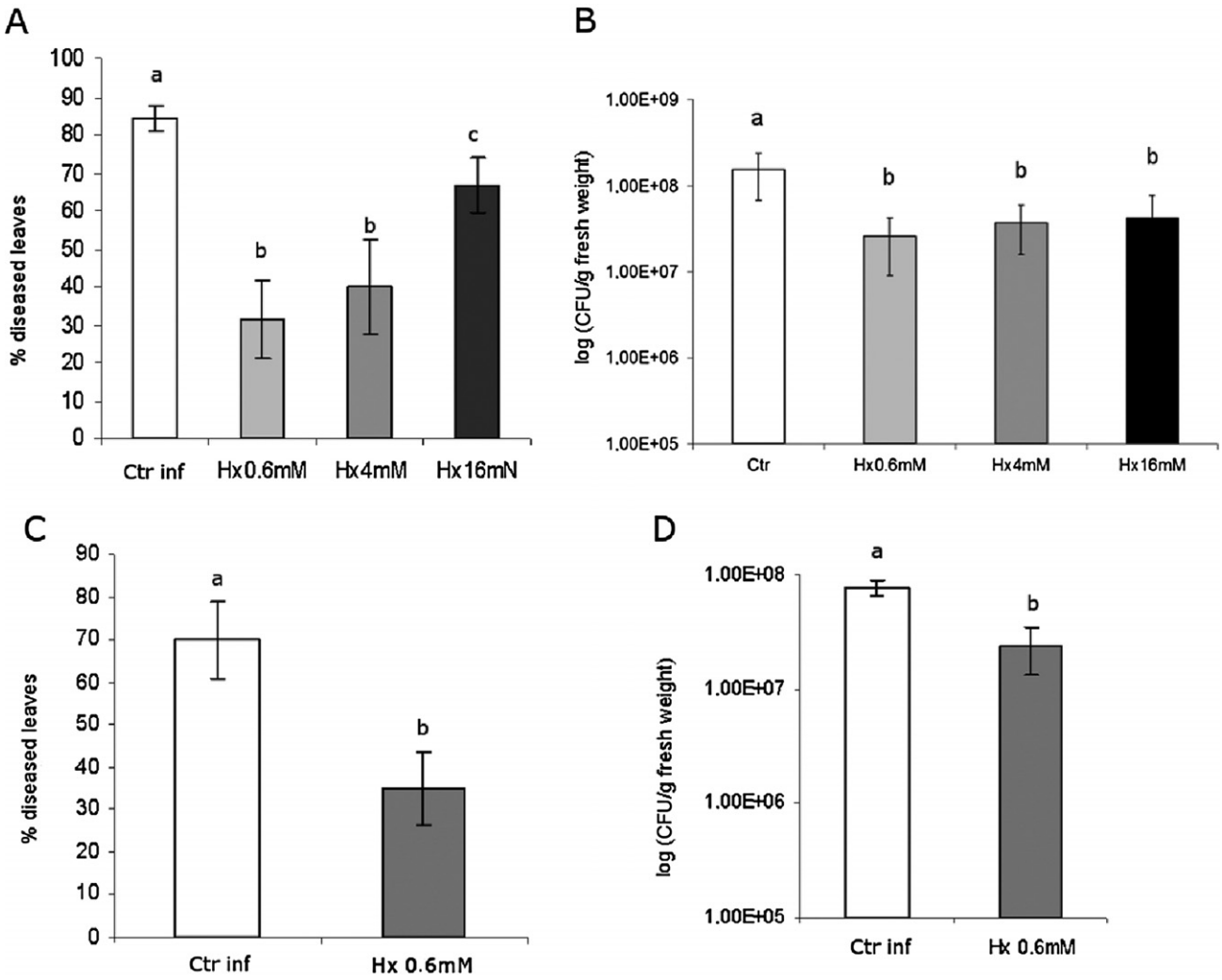

Fig. 1 Hexanoic acid (Hx)-induced resistance against *Pseudomonas syringae*. Four-week-old tomato plants were treated with Hx under hydroponic conditions (A,B) and after soil drench treatment (C,D). After 72 h of inoculation, the disease rating was scored by measuring the percentage of infected leaves in relation to the total number of analysed leaves (A,C) and by recounting of bacterial populations by plating in agar–King's B medium (B,D). Data show average values ± standard error ($n = 20$). Different letters represent statistically significant differences ($P < 0.05$; least-significant difference test). Ctr, untreated and uninoculated plants; Ctr inf, untreated and inoculated plants.

Craig) were treated with Hx as described previously, and callose deposition was analysed by cytological observations at the infection sites at two time points (48 and 72 hpi). As expected, untreated plants with an advanced infection state accumulated callose, but the more resistant Hx-treated plants presented a similar callose accumulation on infection (Fig. S1, see Supporting Information).

Hx-IR against *P. syringae* alters the hormonal profile of tomato plants

To determine whether Hx-IR involves signalling pathways, the main hormones and metabolites were analysed simultaneously in the leaves of control and Hx-treated tomato plants at 24, 48 and 72 hpi. On infection with *P. syringae*, no changes in the SA content were observed in treated plants during the experiment (Fig. 2A). ABA levels were increased slightly at 48 hpi in treated plants compared with untreated plants (Fig. 2B). Interesting results were obtained with regard to the components of the JA pathway. A significant increase in the oxylipin OPDA, a precursor of JA, was found only in Hx-treated plants at 48 hpi, whereas no changes were found in the JA levels of treated plants relative to the levels of control plants (Fig. 2C,D). Moreover, the peak of the bioactive JA conjugate, JA-Ile, was observed at 24 hpi and was not produced in Hx-treated plants. This finding supports a priming effect of the inducer that interferes with the plant response to *P. syringae* (Fig. 2E).

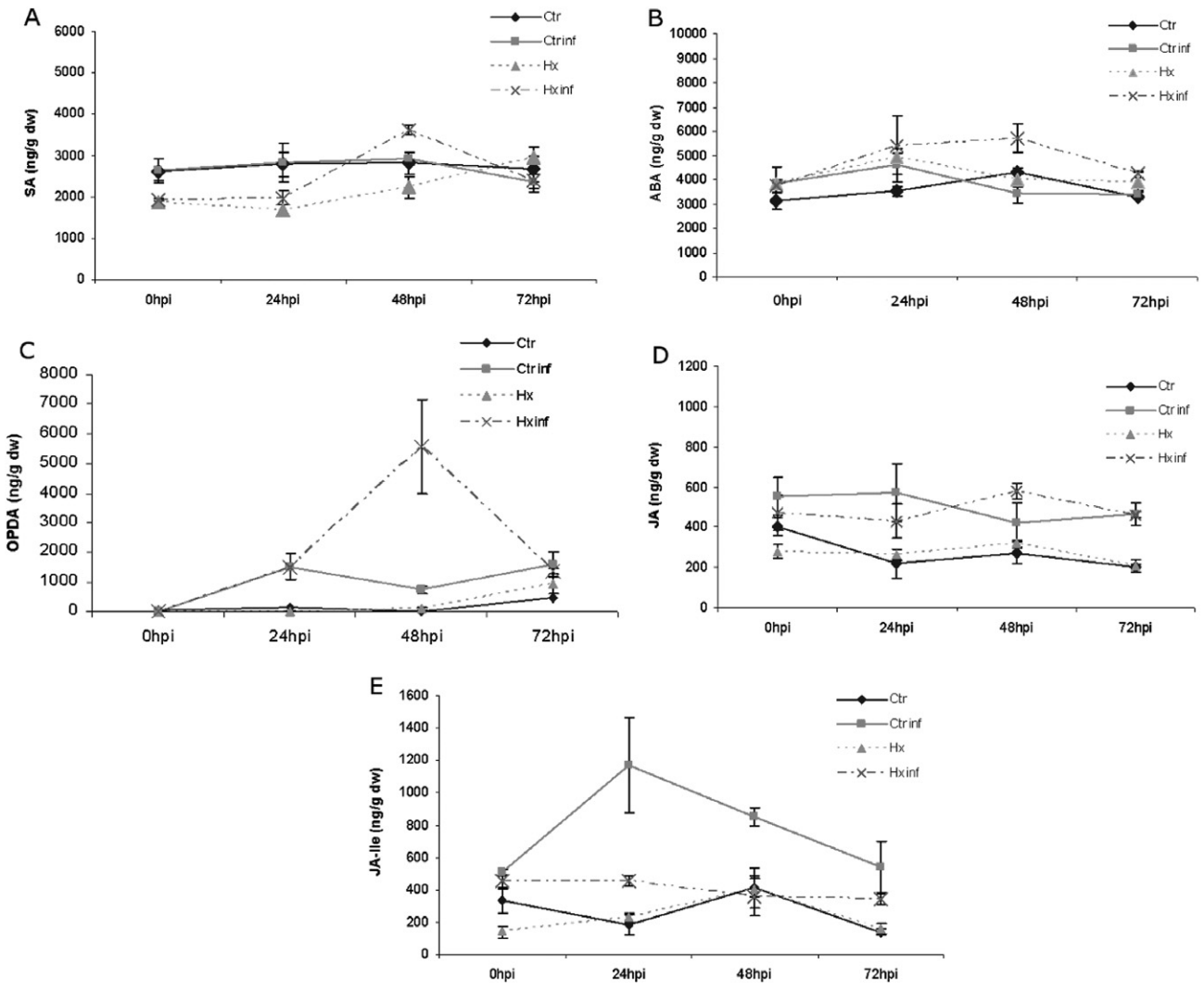

Fig. 2 Hormone levels in water- and hexanoic acid (Hx)-treated tomato plants (cv. Ailsa Craig) on *Pseudomonas syringae* infection. Leaves were collected at various time points and salicylic acid (SA) (A), abscisic acid (ABA) (B), 12-oxo-phytodienoic acid (OPDA) (C), jasmonic acid (JA) (D) and jasmonyl-isoleucine (JA-Ile) (E) levels were determined by high-performance liquid chromatography (HPLC)-mass spectrometry. Data show a representative experiment that was repeated three times; each point is the average of a pool of 10 plants ± standard error. Ctr, untreated and uninoculated plants; Ctr inf, untreated and inoculated plants; hpi, hours post-inoculation; Hx, treated plants; Hx inf, treated and inoculated plants.

Hx-IR involves SA and JA signalling pathways

Because the inducer changed the hormonal balance, we next analysed the expression pattern of the marker genes for SA (*PR-1*, *PR-5* and *SAMT*), JA (*LoxD*, *OPR3*, *JMT*, *JAR*, *PIN2* and *JAZ1*), ET (*AccOx*) and ABA (*Asr1*) signalling pathways in leaf samples harvested from treated and untreated plants at 0, 24, 48 and 72 hpi.

Although Hx did not increase the SA levels on infection, as described previously, it potentiated the early expression of the SA-inducible marker genes *PR1* (Fig. 3A), *PR5* (Fig. S2A, see Supporting Information) and *SAMT*, which acts downstream of the SA synthetic pathway (Fig. 3B). No differences in the expression levels of the ABA- and ET-related genes were observed in treated relative to untreated plants on infection (Fig. S2B,C). However, consistent with the hormonal analysis, Hx treatment induced transcript accumulation at 48 hpi of *LoxD* (Fig. 3C) and *OPR3* (Fig. 3D), which are involved in the biosynthesis of OPDA and jasmonates. Interestingly, Hx increased the expression level of *JMT* (Fig. 3E) and reduced the levels of *JAR* transcripts (Fig. 3F), which might indicate the formation of MeJA (Fig. 4). To test this hypothesis, we further monitored the expression of the MeJA-response gene, *PIN2* (Fig. 3G). We observed elevated mRNA levels of *PIN2* in treated plants within 24 h of treatment.

We extended our analysis to *JAZ1* (Fig. 3H), a repressor of the JA signalling pathway, as its SCF^{COI1}-dependent ubiquitination is required for the activation of JA-responsive gene expression (Chini

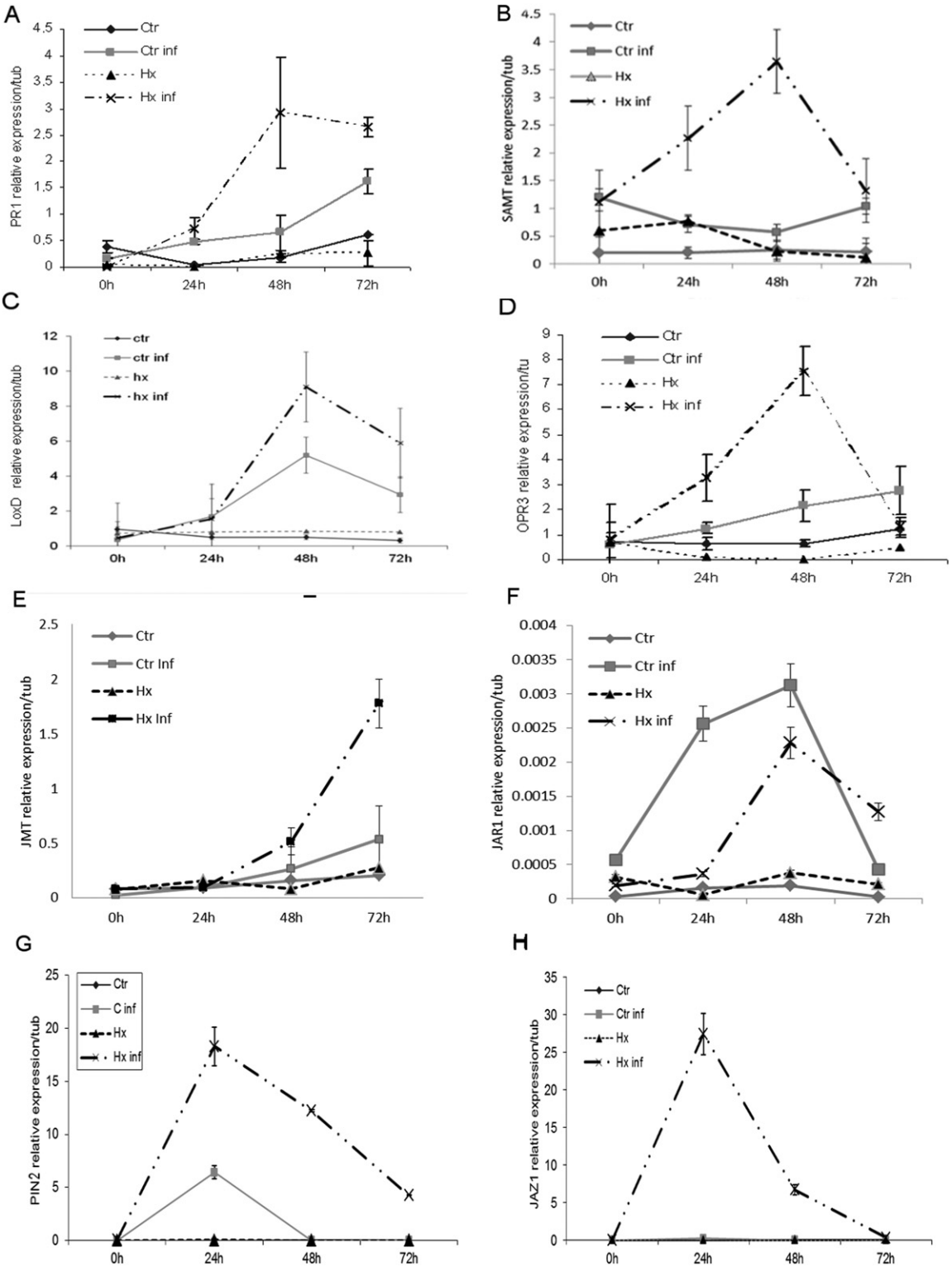

Fig. 3 Effect of hexanoic acid (Hx) on the gene expression levels of tomato plants on *Pseudomonas syringae* infection. Tomato plants were grown, treated and inoculated as described in Experimental procedures. The expression of the *PR1* (A), *SAMT* (B), *LoxD* (C), *OPR3* (D), *JMT* (E), *JAR* (F), *PIN2* (G) and *JAZ1* (H) genes was analysed in cDNA from untreated and uninoculated plants (Ctr), untreated and inoculated plants (Ctr inf), treated plants (Hx) and treated and inoculated plants (Hx inf) at different time points (0, 24, 48, 72 h post-inoculation). Results show average values of three independent experiments with similar results ± standard error ($n = 3$).

et al., 2007). Notably, *JAZ1* expression was induced only in treated and infected plants, which might prevent MYC2 activation of JA-inducible genes. Taken together, these results suggest that Hx treatment prevents the *JAZ1* increase in JA-Ile levels, the physical interaction between JAZ1 and SlCOI1, which is dependent on the presence of (–)-JA-L-Ile (Fonseca *et al*., 2009), and the further ubiquitination and proteosomal degradation of JAZ, which would subsequently release MYC2 and activate transcription. These changes were only observed in treated plants on infection, which supports a priming effect for the inducer Hx.

We also analysed tomato mutants impaired in the SA, ABA or JA pathways. Hx treatment did not protect transgenic *NahG* plants against infection because reduced SA levels were observed (Fig. 5A,B); this indicates a requirement for this hormonal pathway in Hx-IR against *P. syringae*.

When we tested the ABA-deficient mutant *flacca*, an enhanced basal resistance to *P. syringae* was observed (Fig. 5C,D). This mutant displayed intact Hx-IR, which supports the notion that ABA does not play an important role in Hx-IR. The mutant *acx1*, which is deficient in JA biosynthesis (Li *et al*., 2005) and acts downstream of OPDA formation, showed an enhanced basal resistance to *P. syringae* (Fig. 5E,F) and a functional Hx-IR. This result points to a possible role for OPDA in the protection mediated by the inducer Hx.

When the response of the *jai1* mutant, a jasmonic-insensitive *coi1* homologue (Li *et al*., 2004), to Hx treatment was analysed, both treated and untreated plants exhibited similar phenotypes with few detectable disease symptoms (Fig. 5G). This result might be caused by the high basal resistance of *jai1* to *P. syringae* infection (Zhao *et al*., 2003). However, when bacterial growth was monitored (Fig. 5H), Hx-IR seemed to be unaffected. This reduction in bacterial multiplication might indicate that, in addition to plant defence responses, changes in the plant caused by Hx treatment also have an effect on bacterial behaviour. Further studies need to be performed to investigate this possibility.

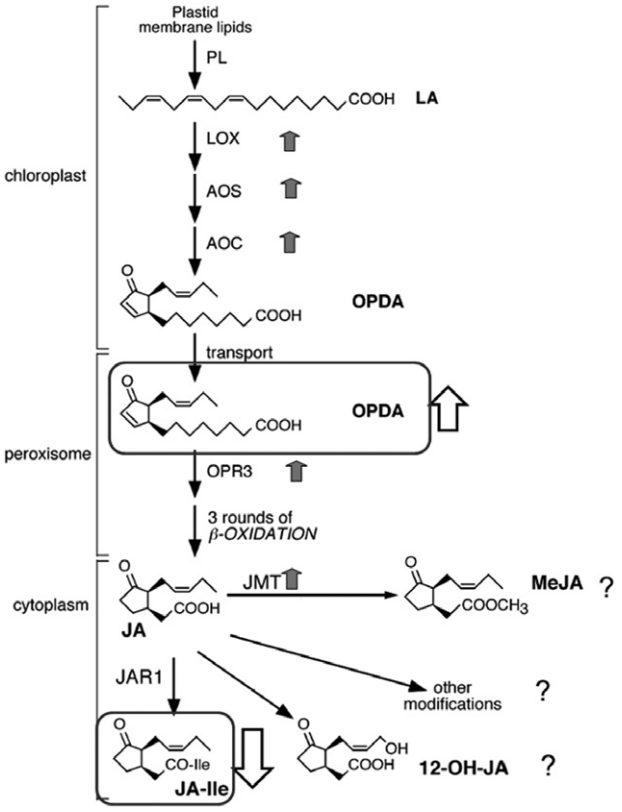

Fig. 4 Induction of the oxylipin biosynthethic pathway in hexanoic acid (Hx)-treated and infected plants. Shaded arrows indicate changes in gene expression: *AOC*, allene oxide cyclase; *AOS*, allene oxide synthase; *LOX*, lipoxygenase; *JMT*, jasmonic acid carboxyl methyltransferase; *OPR3*, oxo-phytodienoic acid reductase. White arrows show the metabolites analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). JA, jasmonic acid; JA-Ile, jasmonyl-isoleucine; JAR1, jasmonate resistant 1; MeJA, methyl jasmonate; LA, linoleic acid; 12-OH-JA, 12-hydroxy jasmonic acid; OPDA, 12-oxo-phytodienoic acid; PL, phospholipids.

The absence of COR compromises Hx-IR

After we had demonstrated a priming effect of Hx on plant defences, we tested the relevance of the virulence factor COR, a JA-Ile mimic, on Hx-IR. Using *Pst* as a control, Hx-treated plants (cv. Ailsa Craig) were infected with the *P. syringae* strain *cmaA*, which lacks COR (Uppalapati *et al*., 2007). A reduction in bacterial growth was observed in plants infected with the mutant strain, as reported by Brooks *et al*. (2004). Hx treatment did not confer resistance to the mutant bacteria (Fig. 6), which supports the hypothesis that the inducer counteracts the effects of COR.

Hx inhibits stomatal opening mediated by COR

In our previous studies, we observed that, when Hx-treated plants were inoculated by infiltration, no effect of the treatment was observed (data not shown). The fact that treatment with Hx is effective when *P. syringae* is inoculated by dipping might indicate that this treatment affects the early stages of infection, such as bacterial entry into the mesophyll. On infection with *P. syringae*, one of the mechanisms of virulence controlled by COR is stomatal re-opening (Melotto *et al*., 2006). To study the possible effect of Hx treatment on this process, we developed an *in situ* method to measure stomatal opening. Hx- and water-treated tomato plants were sprayed with COR, and the effect on stomata was determined in leaves. Under our experimental conditions, COR induced stomatal opening 1 h after application, which is probably a result of its direct application to the leaf mesophyll and the absence of PAMPs/MAMPs. Interestingly, in plants treated with Hx, the COR-mediated opening of stomata was prevented (Fig. 7). This effect supports the interference of the inducer with the bacterial virulence mechanisms because, even in the absence of COR, Hx

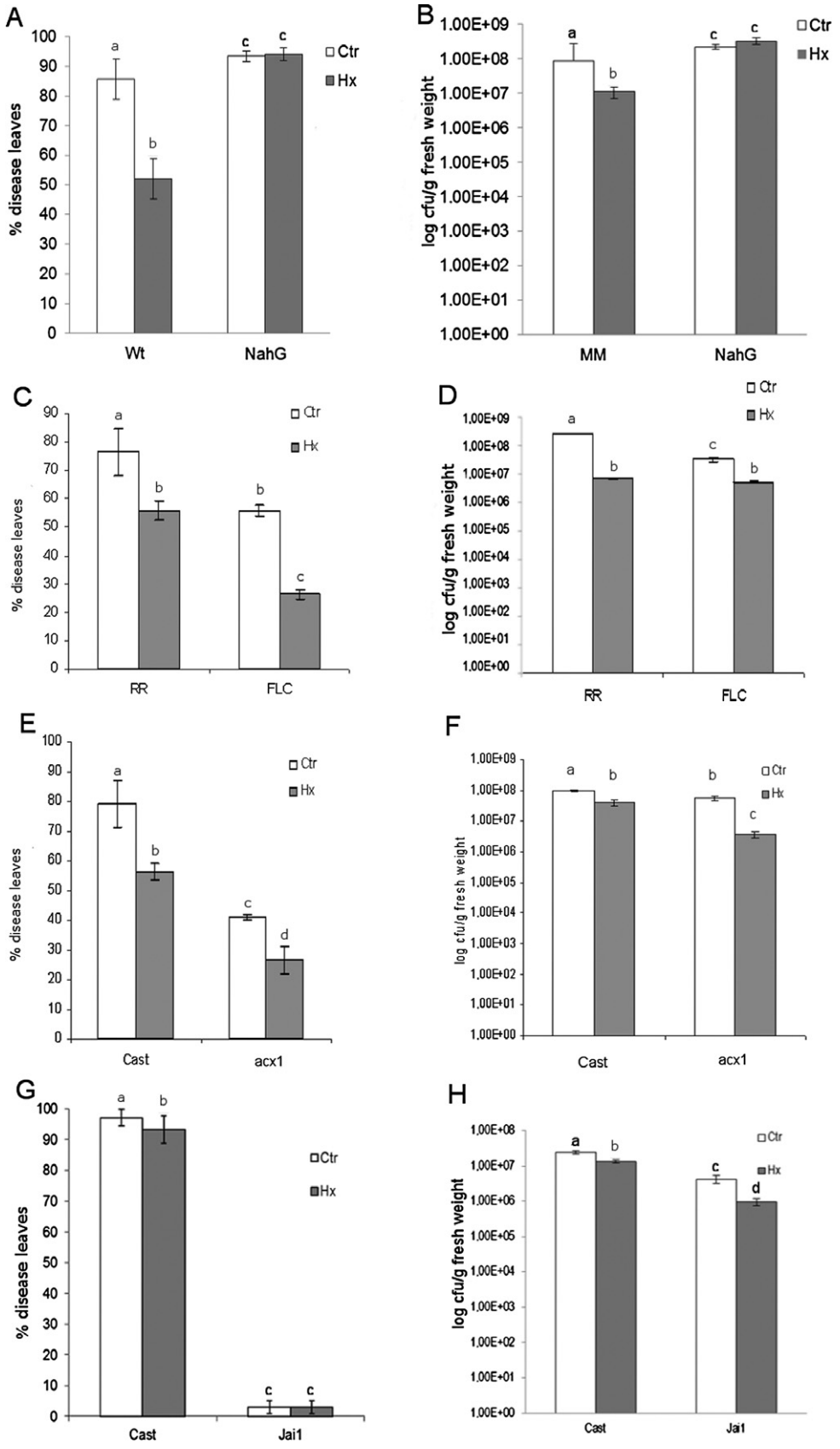
A
% disease leaves
Ctr
Hx
Wt
NahG
B
log cfu/g fresh weight
MM
NahG
C
RR
FLC
D
E
Cast
acx1
F
G
Jai1
H

Fig. 5 Influence of salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA) signalling pathways on hexanoic acid (Hx)-induced resistance against *Pseudomonas syringae*. After 72 h of inoculation, the disease rating was scored for wild-type tomato plants of cv. Moneymaker (MM), Rheinlands Ruhm (RR) and Castlemart (Cast) and their respective SA-impaired mutant *NahG* (A,B), ABA-impaired mutant *flacca* (C,D) and JA-impaired mutants *acx1* (E,F) and *jai1* (G,H). Data show the average values ± standard error ($n = 20$). Different letters represent statistically significant differences ($P < 0.05$; least-significant difference test).

treatment caused stomatal opening. This finding confirmed our previous results showing that Hx enhanced the photosynthetic rate, transpiration rate and stomatal conductance, which were obtained using a portable, open-system infrared gas analyser LCpro+ (ADC Bioscientific Ltd, Hoddesdon, Hertfordshire, UK) for the analysis of physiological parameters (data not shown).

The expression patterns of *PR1*, *LoxD* and *JMT* were analysed under these conditions, and demonstrated that COR application altered plant responses. We also observed that Hx induced *PR1* expression and *JMT* induced mRNA accumulation after COR treatment. However, after COR application, no differences in the transcript levels of *LoxD* were observed between water- and Hx-treated plants (Fig. 8).

These results suggest that Hx treatment represses the COR-mediated stomatal aperture, the inhibition of PR protein synthesis and the alteration of the JA pathway.

DISCUSSION

In this work, we observed a putative interaction between the JA and SA signalling pathways in the priming mechanism of Hx against *P. syringae* in tomato plants. Although the antagonistic action between JA and SA in plant defence responses is well documented (Koornneef *et al*., 2008; Li *et al*., 2004), synergistic effects have also been observed in response to pathogens (Halim *et al*., 2009; Mur *et al*., 2006) and fungal elicitors (Xui *et al*., 2009).

Previous investigations by our research group have shown that Hx protects *S. lycopersicum* plants against the necrotroph *B. cinerea*, a pathogen that is difficult to control (Leyva *et al*., 2008; Vicedo *et al*., 2009). Furthermore, its efficacy was demonstrated in *A. thaliana* (Kravchuk *et al*., 2011). We also observed an approximately 50% reduction in disease symptoms in tomato plants in hydroponic culture, which indicated that Hx induced resistance against *P. syringae* (Vicedo *et al*., 2009)

Here, we have demonstrated that Hx treatment protects tomato plants in a concentration-dependent manner in hydroponic conditions in all the tomato cultivars analysed, and when Hx is applied by soil drench. Taken together, these data demonstrate the versatility of the inducer treatment and the future practical applications in different culture conditions to protect tomato plants against *P. syringae*.

Although we have demonstrated previously that Hx treatment results in enhanced callose deposition after *B. cinerea* inoculation (Vicedo *et al*., 2009), here we have shows that tomato plants display Hx-IR on bacterial infection without enhanced callose deposition. This result suggests that the mechanism involved in

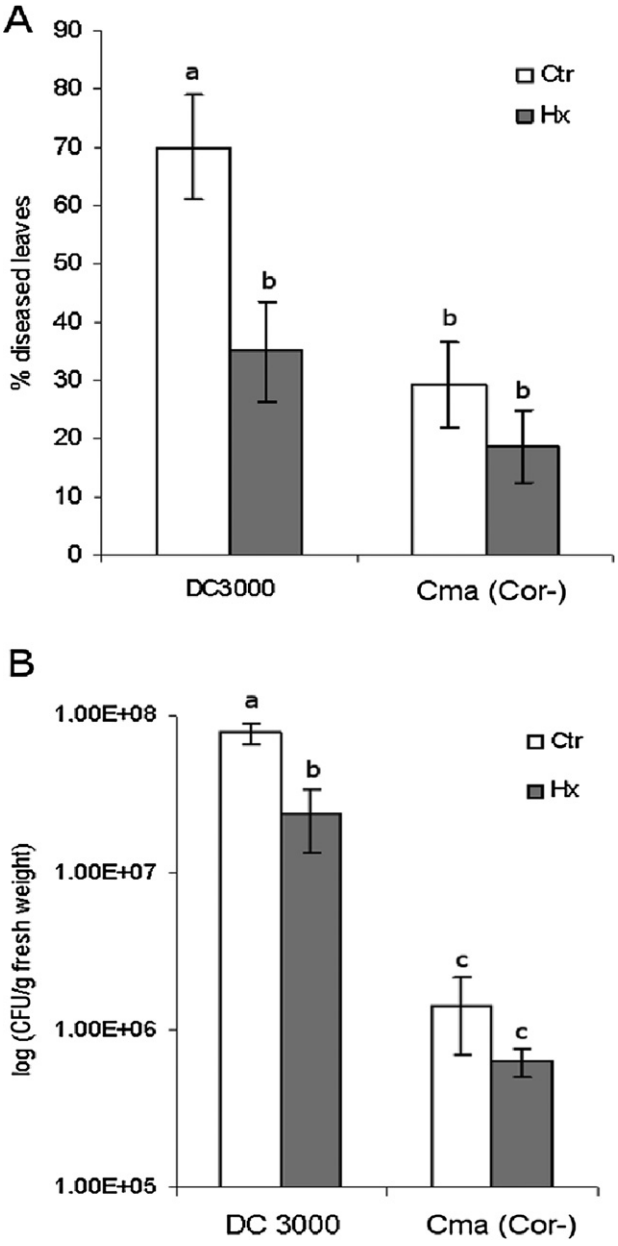

Fig. 6 Hexanoic acid priming is absent in tomato plants infected with the coronatineless strain of *Pseudomonas syringae* (*CmaA*). Tomato plants were grown and treated as described in Experimental procedures and infected with *Pseudomonas syringae* pv. *tomato* DC3000 and with the coronatineless strain of *Pseudomonas syringae* (*CmaA*). The percentage of infected leaves (A) and bacterial growth (B) were evaluated 4 days after bacterial dip inoculation. Data show average values ± standard error ($n = 20$). Different letters represent statistically significant differences ($P < 0.05$; least-significant difference test). Ctr, untreated and uninoculated plants; Hx, treated plants.

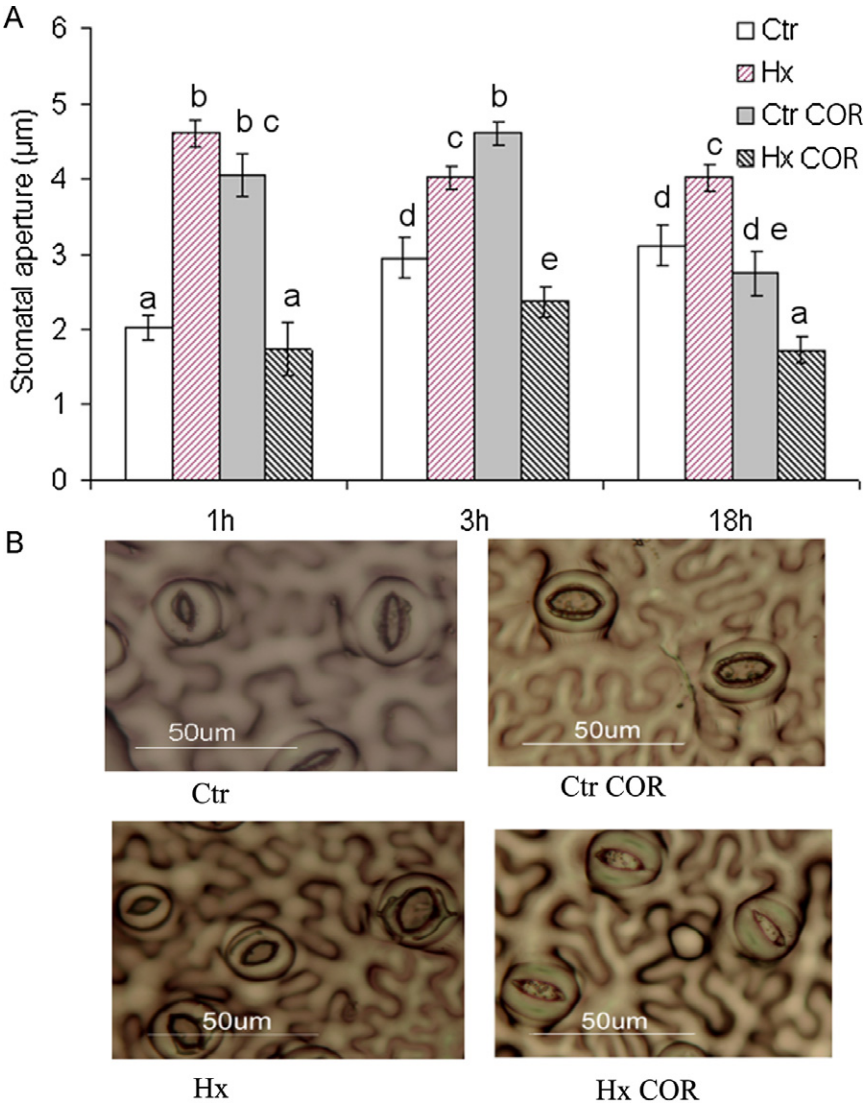

Fig. 7 Hexanoic acid inhibits stomatal opening after coronatine (COR) treatment. (A) Stomatal apertures were analysed '*in situ*' in leaflets of water (Ctr)- and hexanoic acid (Hx)-treated tomato plants at 1, 3 and 18 h after COR treatment. (B) Representative photographs of stomatal aperture taken after COR treatment. Results are means ± standard error (SE) ($n >$ 50 stomata). Different letters represent statistically significant differences ($P < 0.05$; least-significant difference test).

Hx-IR varies depending on the pathogen. This finding is further confirmed by the results obtained in the analysis of gene expression and hormonal content.

We further investigated whether the main signalling pathways are involved in Hx-IR. It is widely accepted that the SA pathway is induced against biotrophs and hemibiotrophs, such as *P. syringae*. Plants activate SA-dependent defences that are effective against this pathogen and are able to neutralize some of the immune-suppressive effects, such as the effector-induced auxin response described by Wang *et al*. (2007). However, some pathogens are also able to suppress SA-dependent defences by injecting different virulence effectors into the host cell via a type III protein secretion system. For instance, the virulence factor HopI1 is able to suppress SA accumulation. In concrete terms, *P. syringae* produces the phytotoxin COR and promotes the synthesis of JA-Ile, the bioactive molecule that inhibits the expression of SA-responsive genes (Pieterse *et al*., 2009). COR functions as a JA-Ile mimic by suppressing SA-dependent defences (Brooks *et al*., 2005; Uppalapati *et al*., 2007), which thereby promotes plant susceptibility to this pathogen. An intact COR molecule is required for both the suppression of SA-mediated defence responses and full disease symptom development in tomato (Uppalapati *et al*., 2007).

The efficacy of Hx suggests that it might activate the SA pathway. Indeed, on infection of Hx-treated plants, Hx treatment primed the induction of *PR1* and *PR5* and potentiated the expression of *SAMT* without changes in SA levels. These results may reflect control of this pathway which is downstream of SA production. Considering that the conversion of SA into MeSA is required for SAR in tobacco and that the silencing of *SAMT* leads to the loss of SAR (Park *et al*., 2007), it is possible that MeSA is involved in the SAR of tomato plants. Although the role of SA/MeSA in local defence seems to be conserved between Arabidopsis and tomato (Amet *et al*., 2010), it is currently unclear whether this role also extends to systemic responses, because, in

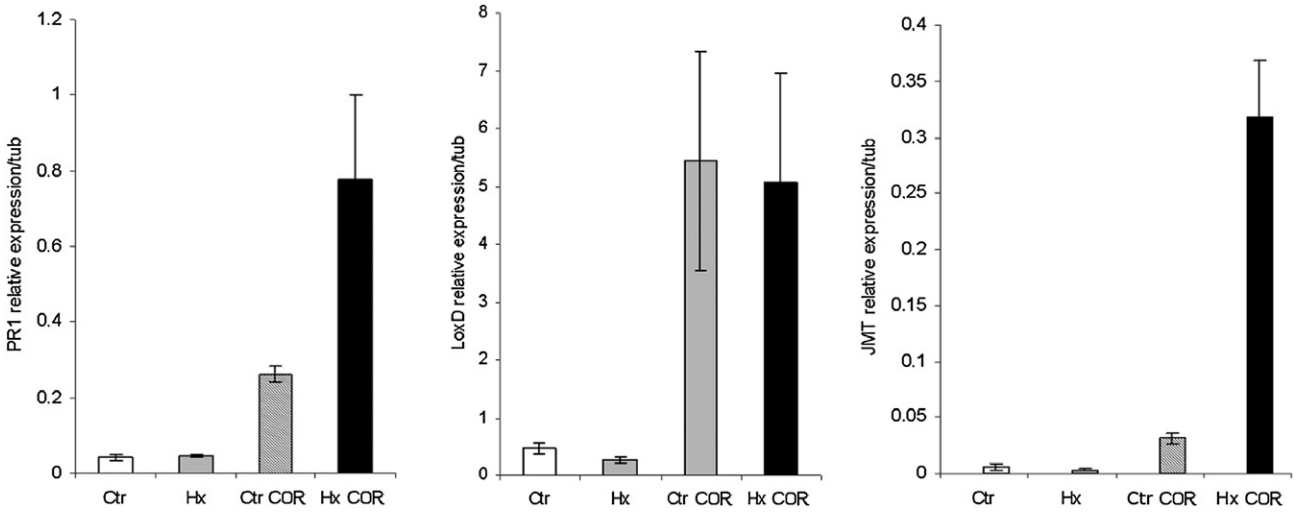

Fig. 8 Hexanoic acid treatment represses coronatine (COR)-mediated inhibition of pathogenesis-related (PR) protein synthesis. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed for *PR1*, *LoxD* and *JMT* genes. RNAs were extracted from leaves of 4-week-old tomato plants, 18 h after treatment with 0.5 μM purified COR. Error bars are the standard deviation (SD) ($n = 3$).

Arabidopsis, MeSA production was not found to be essential for SAR; instead, the mobile metabolite azelaic acid seems to be involved (Attaran *et al*., 2009; Jung *et al*., 2009).

The role of the SA pathway in Hx-IR was confirmed in SA-deficient *NahG* plants. These results confirm that the SA pathway is required for Hx-IR and support the hypothesis that SA levels present in treated plants on infection before its conversion to MeSA are sufficient for the early activation of PR proteins involved in defence.

Interestingly, Hx also induced the JA pathway. In treated plants, a faster and stronger accumulation of OPDA was produced 48 h after *P. syringae* infection. However, this accumulation was not accompanied by an increase in JA and JA-Ile, which was observed in water-treated plants on infection. These results suggest that Hx treatment prevents the increase in JA-Ile associated with bacterial infection in the pathogenesis system (Pieterse *et al*., 2009). However, we cannot discard the possibility that OPDA or other JA derivatives are involved in the mechanism of Hx-IR. The oxylipin OPDA, which is a regulator of plant defences and is also active against microorganisms, including *B. cinerea* (Prost *et al*., 2005; Vellosillo *et al*., 2007), might play a role *per se* in Hx-IR or may remain an inactive conjugate in chloroplasts.

At the transcriptional level, the expression of genes in the JA pathway supports the previously described results. Interestingly, Hx induced the expression of *JMT* after infection, whereas the synthesis of JA-Ile decreased. This result may indicate that MeJA is released into the environment to diminish intracellular JA levels and may prevent the synthesis of the bioactive signal JA-Ile. Furthermore, the increase in *PIN2* and *JAZ1* transcripts in treated plants on infection reinforces this idea. The low levels of JA-Ile in these plants may prevent the formation of JAZ–COI1 complexes; thus, JAZ proteins may not be ubiquitinated and degraded, and MYC2 may not be released to activate the transcription of JA-inducible genes.

The Hx-IR against *B. cinerea* (Vicedo *et al*., 2009) also involves the JA-dependent defence pathway, but, in this case, it primes the accumulation of JA-Ile.

Here, we have shown that Hx counteracts the action of COR in tomato plants. Previous studies have demonstrated that COR facilitates the entry of *P. syringae* into *A. thaliana* tissue by suppressing the closure of stomata, a basal defence response that is induced on microbial attack (Melotto *et al*., 2006). To confirm that Hx inhibits the response of tomato plants to COR, we analysed the stomatal aperture in treated and untreated plants after the application of COR by spraying. We confirmed that Hx inhibited the COR-dependent stomatal aperture. In our experimental conditions, COR did not induce stomatal closure 1 h after spraying, as reported previously (Melotto *et al*., 2006; Tsai *et al*., 2011). This result may be a result of the direct application of COR on leaf mesophyll and the direct observation of the stomatal aperture on the surface of intact leaves. In these conditions, we observed that COR opens the stomata. However, Hx seems to inhibit stomatal opening in the presence of COR, which suggests that it represses the action of COR in infected tomato plants by preventing the entry of bacteria into the mesophyll. This finding may indicate that bacteria enter the mesophyll of treated plants more slowly than in untreated plants and that quorum sensing (QS) may not take place. Many species of bacteria use QS to coordinate gene expression according to the density of their local population (Miller and Bassler, 2001). Many traits, including the production of virulence factors, are regulated by QS and presumably play important and context-dependent roles in the lifestyles of microbes that utilize

QS. The attainment of a bacterial concentration needed for QS establishment may be prevented by Hx treatment and, with a faster induction of plant defences, Hx treatment may block disease development. However, a larger amount of *PR1* in Hx-treated relative to untreated plants after COR application suggests that the inducer treatment alters the negative effect of COR on the SA pathway. It is tempting to speculate that priming in these circumstances is a consequence of the inhibition of COR action on stomatal opening and on the inactivation of the SA pathway, which are both important aspects in the pathogenesis of *P. syringae*.

In conclusion, we have shown that Hx-IR alters the content of several phytohormones, which correlates with changes in the expression of the genes involved in their biosynthesis and the responses they regulate. This study suggests that Hx primes the SA signalling pathway through the inhibition of COR-mediated bacterial manipulation of this pathway. Hx treatment also increases the accumulation of OPDA in infected plants and prevents the accumulation of JA-Ile promoted by bacterial infection. Notably, the Hx priming of the JA biosynthesis pathway does not inhibit the SA-dependent signalling pathway or the expression of SA-responsive genes on *P. syringae* infection. This unusual complementary relationship between the JA and SA pathways supports previous observations suggesting that a synergistic interaction exists between these pathways. Moreover, we have observed that changes in bacterial populations are not correlated with changes in disease development; this observation might point to a response of the bacterium to new conditions encountered in plants.

EXPERIMENTAL PROCEDURES

Microbial strains, growth conditions and plant material

Pst was grown in King's B medium (KB) (King *et al*., 1954) at 28 °C. Rifampicin was added to KB at 50 μg/mL. The *CmaA* mutant (Brooks *et al*., 2004) was grown in KB with rifampicin (50 μg/mL) and kanamycin (25 μg/mL).

The following tomato (*S. lycopersicum* Mill) genotypes were used in our studies: wild-type Ailsa Craig, Rheinlands Ruhm, Moneymaker and Castlemart. We are grateful to Jonathan Jones (John Innes Centre, Norwich, Norfolk, UK) for seeds of the SA-deficient *NahG* tomato plant in the background Moneymaker, and to G. Howe (Michigan State University, East Lansing, MI, USA) for the JA pathway mutants, *acx1* and *jai1*, in the background Castlemart. The *jai1-1* homozygous plants were selected from an F2 population, as described by Li *et al*. (2004).

The ABA pathway mutant used was the ABA-deficient mutant *flacca* in the background Ailsa (LA3613), which was provided by the Tomato Genetics Resource Center (TGRC), University of California, Davis, CA, USA.

Seeds were germinated in either vermiculite (for hydroponic culture) or jiffy peat pellets. Plants were grown at day and night temperatures of 24 °C and 18 °C, respectively, with 16 h of light and 8 h of darkness, and 60% relative humidity (RH).

Pseudomonas syringae bioassays

Four-week-old tomato plants of *S. lycopersicum* Mill. cv. Ailsa Craig were treated with Hx using two different methods: application in the nutritive solution of hydroponic culture or in a soil drench application.

For experiments using Hx application in the nutritive solution of hydroponic culture, 3-week-old plants grown in vermiculite were transferred 1 week prior to treatment into hydroponic conditions in tanks containing Hoagland solution. Plants were treated with water or Hx to a nonbactericide concentration (0.6 mM) at pH 6 in the nutrient solution 48 h before inoculation (Leyva *et al*., 2008). The disease rate was scored at 72 hpi by determining the percentage of dark-brown spots on the leaf surface. At least three samples for colony counting and 20 samples for disease rate scoring were taken for each treatment over a 3-day period. For molecular and hormonal analyses, the samples were taken at 0, 24, 48 and 72 hpi. At least three samples for colony counting and 20 samples for disease rate scoring were taken for each treatment over a 3-day period. Each experiment was independently conducted at least three times.

For Hx treatments applied by soil drench, Hx was added to 4-week-old plants grown in jiffy peat pellets at the same concentration as described previously, but 72 h before bacterial inoculation. The disease rate and colony counting were performed as described previously.

In all experiments, plants were then maintained at 24 °C with 16 h of light and 8 h of darkness and 100% RH.

For inoculation, *Pst* was grown in KB at 28 °C for 24 h. Bacterial suspensions were adjusted to 5×10^5 colony-forming units (CFU)/mL in sterile $MgSO_4$ (10 mM) containing 0.01% of the surfactant Silwet L-77 (Osi Specialties, Danbury, CT, USA), as described previously (Katagiri *et al*., 2002). Pathogen inoculation was performed by dipping the third and fourth leaves into the bacterial suspension.

Each experiment was independently conducted at least three times. When Hx was applied in the nutritive solution of hydroponic culture or by soil drench, similar results were obtained.

Quantification of callose deposition

Samples of 10 leaves were collected for callose staining at 48 and 72 hpi, and callose deposition was determined as described by Flors *et al*. (2007).

Analysis of gene expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

RNA was extracted from tomato leaves with the Total Quick RNA Cells and Tissues Kit (Talent, Trieste, Italy) at the specified time points post-inoculation. Leaf tissue was collected from five treated and five untreated plants. Samples for qRT-PCR were obtained from the experiments described previously. To assess differential gene expression, the differences in the cycle numbers during the linear amplification phase between samples containing cDNA from infected and uninfected plants were used.

The expression detected from the tomato *actin* and *tubulin* genes was used as an internal reference. The primers used for *actin*, *PR1* and *LoxD*

have been described by Flors *et al*. (2007); the primers *Letub* and *LeACCOx* have been described by Vicedo *et al*. (2009); the primers *LeAOC* and *LeOPR3* have been described by Uppalapati *et al*. (2005); *LeAOS1* has been described by López-Ráez *et al*. (2009). In addition, in this work, we used the following primers: *LeAsr1* forward (fw) primer 5′-ACACCA CCACCACCTGT-3′ and *LeAsr1* reverse (rev) primer 5′-GTGTTTGTGT GCATGTTGTGGA-3′; *LeJMT* fw primer 5′-GGTTCAAAGTGCATGAGAGCT-3′ and *LeJMT* rev primer 5′-TACACCACACACTGAAGGAAA-3′; *LePR5* fw primer 5′-GAGGTTCATGCCAAACTGGTC-3′ and *LePR5* rev primer 5′-CCG TCAACCAAAGAAATGTCC-3′; *LeSAMT* fw primer 5′-TCAATATACACCATC ACAAGGAGAAG-3′ and *LeSAMT* rev primer 5′-GCTCTCATGCACTTTGA CACATTG-3′; *LePIN2* fw primer 5′-CGTTCACAAGGAAAATCGTTAAT-3′ and *LePIN2* rev primer 5′-CTTGGGTTCATCACTCTCTCCTT-3′; and *LeJAZ1* fw primer 5′-CGTCCGTTGAAACAAATCCT-3′ and *LeJAZ1* rev primer 5′-GGGGTTCTGTTTGTTGGCTA-3′. Triplicate analyses were performed on all reactions using the cDNA samples derived from three independent experiments.

Stomatal aperture analysis *in planta*

Tomato plants were maintained in the same culture conditions and treated as described for the *P. syringae* bioassays (24 °C with 16 h of light and 8 h of darkness and 100% RH). The third and fourth leaves were sprayed with water or 0.5 μM of purified COR. Leaflets were placed on glass slides with the adaxial epidermis in contact with dental resin (Delgado *et al*., 2011; Geisler *et al*., 2000). For stomatal aperture analysis, images of random regions were taken using a Leica IRB microscope equipped with a Leica DC300F camera (Leica Microsystems CMS GmbH, Wetzlar, Germany). The stomatal aperture was analysed using the Eclipse-Net software of the Laboratory imaging program (http://www.laboratory-imaging.com). Approximately 50 stomata from each leaflet were measured.

Chromatographic analysis

For hormonal analysis, fresh material was frozen in liquid nitrogen, ground and freeze dried. Dry tissue (0.05 g) was immediately homogenized in 2.5 mL of ultrapure water, and a mixture of internal standards was added prior to extraction (100 ng of [^{2}H6]-ABA, 100 ng of prostaglandin B1 (Pinfield-Wells *et al*., 2005), dihydrojasmonic acid (Flors *et al*., 2007) and propylparaben). After extraction, a 20-μL aliquot was injected directly into the high-performance liquid chromatography (HPLC) system. Analyses were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, USA) with a nucleosil ODS reverse-phase column (100 mm × 2 mm i.d.; 5 μm) (Scharlab, Barcelona, Spain). The chromatographic system was interfaced to a Quatro liquid chromatography (LC) (quadrupole–hexapolequadrupole) mass spectrometer (Micromass, Manchester, UK).

Statistical analysis

Statistical analysis was carried out using a one-way analysis of variance in the Statgraphics-plus software of Windows V.5 (Statistical Graphics Corp., Rockville, MD, USA). The means were expressed with standard errors and compared using a Fisher's least-significant difference test at the 95% confidence interval. All the experiments were repeated at least three times.

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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 Callose deposition in untreated (Ctr) and hexanoic acid-treated (Hx) plants on infection with *Pseudomonas syringae*. Leaves of tomato plants were sampled, stained with calcofluor/aniline blue and analysed by epifluorescence microscopy. Quantification was performed by determining the number of yellow pixels/million corresponding to pathogen-induced callose on digital photographs of infected leaves. Data show average valuesn $\pm$ standard error (SE) ($n = 10$). Different letters represent statistically significant differences ($P < 0.05$; least-significant difference test). Higher magnification views are shown of callose deposition in tomato leaves infected with *P. syringae* visualized with bright light and UV.

Fig. S2 Expression analyses of marker genes *PR5*, *Asr1* and *AccOx* for salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) signalling pathways, respectively, in priming by hexanoic acid against *Pseudomonas syringae*. Tomato plants were grown, treated and inoculated as described in Experimental procedures. Genes were analysed in cDNA from untreated and uninoculated plants (Ctr), untreated and inoculated plants (Ctr inf), treated plants (Hx), and treated and inoculated plants (Hx inf) at different time points. Results show average values of three independent experiments with similar results ± standard error (SE) ($n = 3$).