Hexanoic Acid-Induced Resistance Against *Botrytis cinerea* in Tomato Plants

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Submitted 16 December 2008. Accepted 18 June 2009.

We have demonstrated that root treatment with hexanoic acid protects tomato plants against *Botrytis cinerea*. Hexanoic acid-induced resistance (Hx-IR) was blocked in the jasmonic acid (JA)-insensitive mutant *jai1* (a *coi1* homolog) and in the abscisic acid (ABA)-deficient mutant *flacca* (*flc*). Upon infection, the *LoxD* gene as well as the oxylipin 12-oxo-phytodienoic acid and the bioactive molecule JA-Ile were clearly induced in treated plants. However, the basal ABA levels were not altered. Hexanoic acid primed callose deposition against *B. cinerea* in a cultivar-dependent manner. Treated plants from Ailsa Craig, Moneymaker, and Rheinlands Ruhm showed increased callose deposition but not from Castlemart. Hexanoic acid did not prime callose accumulation in *flc* plants upon *B. cinerea* infection; therefore, ABA could act as a positive regulator of Hx-IR by enhancing callose deposition. Furthermore, although hexanoic acid protected the JA-deficient mutant *defensless1* (*def1*), the priming for callose was higher than in the wild type. This suggests a link between JA and callose deposition in tomato. Hence, the obtained results support the idea that callose, oxylipins, and the JA-signaling pathway are involved in Hx-IR against *B. cinerea*. Moreover our data support the relevance of JA-signaling for basal defense against this necrotroph in tomato. Hexanoic acid also protected against *Pseudomonas syringae*, indicating a broad-spectrum effect for this new inducer.

Plants defend themselves from pathogens with a variety of chemical and physical defenses. Upon infection, plants respond by activating basal resistance in an attempt to prevent disease. This process is regulated by a complex network of signal molecules and transcriptional regulators. The main players in the regulation of signaling pathways are the plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and, more recently, abscisic acid (ABA) (Thomma et al. 1998; Flors et al. 2005; Mauch-Mani and Mauch 2005). Crosstalk between signaling pathways leads to the expression of different responses in accordance with the challenging pathogens (Glazebrook 2005).

In addition to basal resistance, plants are able to develop an induced resistance (IR), this being a state of enhanced defense

capacity developed by a plant when it is properly stimulated (van Loon et al. 1998). IR allows the plant to resist future attacks by virulent pathogens and can be activated by different biotic and abiotic stresses. The best-characterized IR is systemic acquired resistance (SAR) (Durrant and Dong 2004). On the one hand, SAR is mediated by the accumulation of SA that leads to the expression of the hypersensitive response (HR) and is mostly effective against biotrophic pathogens (Mettraux et al. 1990; Thomma et al. 1998). On the other hand, non-pathogenic rhizobacteria are able to activate an IR that is effective against necrotrophic pathogens known as induced systemic resistance (ISR). In *Arabidopsis*, this response is mediated by the JA and ET hormones (Thomma et al. 1998; Pieterse and van Loon 1999). ISR can also be induced in tomato by *Pseudomonas aeruginosa* TNSK2 but, in this case, it leads to the stimulation of SA-dependent defense responses (Audenaert et al. 2002b).

Apart from biological stimuli, IR can also be triggered by chemicals. Some of the best characterized are 2,6-dichloro isonicotinic acid (INA), benzo-(1,2,3)-thiadiazole-7-carbotionic acid S-methyl ester (BTH), and β -aminobutyric acid (BABA) (Oostendorp et al. 2001; Conrath et al. 2002). The mode of action of these compounds remains unclear but they probably act at different points on the specific signal transduction pathways that mediate the defense responses. Direct antibiotic activity has been ruled out for them all. There are compounds that act as inducers but which also present antimicrobial activity. That is the case of chitosan (Amborabé et al. 2008), oxylipins (Prost et al. 2005), and plant volatiles (VOC) (Matsui 2006).

BTH is effective against *Botrytis cinerea* but it does not protect against *Oidium neolycopersici* in tomato. However, it induced resistance against this biotroph in tobacco but had no effect on *B. cinerea* (Achuo et al. 2004), which reflects the divergence in the resistance pathways within plant species. In contrast, BABA induces resistance against a broad spectrum of biotrophic and necrotrophic pathogens in *Arabidopsis* by activating different signaling pathways depending on the challenging pathogen (Zimmerli et al. 2000, 2001; Ton et al. 2005). Several IR processes are associated with an enhanced capacity to express specific defense responses upon attack by a pathogen, which is called priming (Conrath et al. 2002). BABA-induced resistance (BABA-IR) is based on different priming mechanisms than activate SA-dependent defense mechanisms, callose deposition, or ABA-dependent defense mechanisms (Zimmerli et al. 2000; Mauch-Mani and Mauch 2005).

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Today, the development of novel resistance-inducing chemicals represents an attractive alternative to protect crops against pathogens. Fungicide application is a common practice to control fungi but growing public health and environmental concerns mean that alternative strategies need to be developed (Osborn 1996).

We previously reported that derivatives of adipic acid were effective to control several plant diseases. A glycoside derivative of adipic acid monoethyl ester (AAME) and 1,2,3,4-tetra-O-acetyl- β -D-glucopyranose, named TOGE, protected tomato plants against *Alternaria solani* and *Phytophthora citrophthora* (Flors et al. 2003). In addition, three novel amides of adipic acid induced resistance against *A. solani* in pepper plants (Flors et al. 2003). These compounds induced the phenylpropanoid pathway and the production of antioxidant compounds. AAME also displayed direct antimicrobial activity and controlled *B. cinerea* in tomato (Vicedo et al. 2006). *B. cinerea* is a pathogen with a broad host range that causes huge losses during the storage of fruit and vegetables (Elad and Evensen 1995). An effective control requires several chemical treatments and often leads to unsatisfactory results (Farettra and Pollastro 1991; Leroux 1996; Lamondia and Douglas 1997). The defense mechanisms of tomato plants against this pathogen remain unclear but a complex interplay of the SA, JA, ET, and ABA signaling pathways is apparently implicated (Díaz et al. 2002; Audenaert et al. 2002b; Achuo et al. 2004; Anderson et al. 2004; Asselbergh and Höfte 2007).

Very recently, hexanoic acid was found to protect tomato plants against *B. cinerea* (Leyva et al. 2008). This natural short-chain monocarboxylic acid displayed antimicrobial activity. However, these studies suggested that it could play a dual function not only by acting directly on fungal growth but also by activating protective processes in plants in a concentration-dependent manner. Interestingly, and as previously mentioned, the green leafy volatiles (GLV), a group of chemicals which are structurally close to hexanoic acid, are effective on both the plant and the pathogen (Matsui 2006). They are usually formed from linolenic and linoleic acids as one of the branches of the oxylipin pathway, and the exposure of plants to them results in various plant defense responses (Hatanaka 1993). GLV treatments enhanced the resistance of *Arabidopsis* and *Citrus* spp. against *B. cinerea* and *A. alternata*, probably by inducing a wide range of defense reactions (Kishimoto et al. 2005). Treatment with different GLV can prime *Arabidopsis* plants for a faster and enhanced JA synthesis, thus increasing protection against necrotrophic pathogens (Engelberth et al. 2004; Sha 2005). GLV also show antibacterial activities against both gram-negative and gram-positive bacteria (Lanciotti et al. 2003), and also display fungicidal activity (Kubo et al. 2003).

In this work, we demonstrate that hexanoic acid acts as an inducer of resistance against the necrotrophic pathogen *B. cinerea* in tomato. We have investigated its mode of action, and we provide evidence that hexanoic acid-induced resistance (Hx-IR) is based on priming callose deposition and on enhanced JA-dependent defenses. We also demonstrate that hexanoic acid is effective against *Pseudomonas syringae* in tomato.

RESULTS

Hexanoic acid enhances tomato resistance to *B. cinerea* infection.

To determine whether hexanoic acid protection of tomato plants at nonfungicidal concentrations was due to an IR, 4-week-old tomato plants (cv. Ailsa Craig) were treated under hydroponic conditions with 0.06, 0.6, and 4 mM hexanoic acid for 48 h, as previously described (Leyva et al. 2008), and were

subsequently challenged with *B. cinerea*. The time of plant conditioning was initially based on previous reports of known resistance inducers (Ton and Mauch-Mani 2004). Compared with untreated control plants, all the hexanoic acid treatments resulted in a statistically significant reduction of lesion diameter at 72 h after challenge inoculation (Fig. 1A), which confirms that hexanoic acid could act on both the pathogen and the plant defenses in a concentration-dependent manner. Further analysis of the inducer effect of this chemical was performed by treating plants with 0.6 mM hexanoic acid.

To establish the optimal time window for plant conditioning, tomato plants (cv. Ailsa Craig) were challenge inoculated with *B. cinerea* at 0, 12, 24, and 48 h after treatment with 0.6 mM hexanoic acid. It was confirmed that 48 h was optimal for plant conditioning after this treatment (Fig. 1B to E). Longer periods of incubation did not improve hexanoic-induced protection (data not shown).

Hexanoic acid does not accumulate in treated plants.

To assess the possibility that hexanoic acid accumulates in the aerial parts of the plant, leading to a direct effect on the pathogen, 0.6 mM ^{13}C -hexanoic acid was supplied to plants under hydroponic conditions for 48 h. Leaf samples were analyzed at different time points by high-performance liquid chromatography (HPLC) interfaced with a triple quadrupole mass spectrometer (QhQ) (experimental details provided below). Isotopic measurements allowed us to distinguish between applied hexanoic acid and the compound already present in the plant. The chromatographic analysis revealed no detectable trace of either hexanoic acid or ^{13}C -hexanoic acid above the detection limits ($10\text{ }\mu\text{g liter}^{-1}$) (Supplementary Fig. 1).

Efficacy of the Hx-IR compared with BABA- and SA-IR against *B. cinerea*.

In order to assess the effectiveness of Hx-IR against *B. cinerea*, tomato plants (cv. Ailsa Craig) were treated for 48 h with 0.6 mM hexanoic acid, 0.5 mM SA, and 0.5 mM BABA, and were subsequently challenged with the fungus. Compared with water-treated control plants, treatment with hexanoic acid induced a statistically significant reduction in lesion size at 72 h after challenge inoculation (Fig. 1F). Under these conditions, the level of Hx-IR was comparable with that of BABA-IR, whereas treatment with SA resulted in a relatively slight reduction in lesion size compared with control plants.

Hexanoic acid treatment primed callose deposition against *B. cinerea*.

In order to assess whether the Hx-IR against *B. cinerea* was associated with an increase in callose accumulation, cytological observations were performed at the infection sites. Four-week-old tomato plants (cv. Ailsa Craig) were treated as described above and callose deposition was analyzed after 72 h of infection. Hexanoic acid significantly induced callose accumulation upon infection when compared with water-treated control plants (Fig. 2A). Interestingly, hexanoic acid-treated plants showed no callose accumulation in the absence of infection, thus suggesting a priming mechanism for Hx-IR against *B. cinerea* in tomato (data not shown). BABA-treated tomato plants also primed callose deposition after *B. cinerea* infection (Fig. 2A), as previously demonstrated for BABA-IR against other necrotrophs in *Arabidopsis* (Ton et al. 2005; Flors et al. 2008). However, SA slightly induced callose when compared with the other chemicals. It is noteworthy that Ailsa Craig untreated plants hardly accumulated any callose upon infection in spite of previous reports demonstrating that other cultivars, such as T5 (Flors et al. 2007) and Moneymaker (Asselbergh and Höfte 2007), responded to *B. cinerea* by callose deposition at

the infection site. Thus, the participation of callose in basal defense mechanisms appears to be cultivar-dependent in tomato.

Microscopic observation of the infected leaves showed that Hx- and BABA-IR were associated with a primed deposition of callose-rich papillae around the area of infection (Fig. 2B). Double staining with calcofluor and aniline blue showed a radial growth of *B. cinerea* mycelia in water-treated plants, whereas nonorganized hyphal growth was observed in both

BABA- and hexanoic acid-treated plants (Fig. 2B). A detailed observation of the area of infection revealed the presence of wet-brown necrosis in water- and SA-infected plants with non-defined limits 72 h after inoculation. Conversely, BABA- and hexanoic acid-induced plants showed dry necrosis with well-defined limits (data not shown).

To establish whether this primed callose deposition contributes to Hx-IR against *B. cinerea*, 4-week-old tomato plants

Fig. 1. Effect of hexanoic acid on tomato plants (cv. Ailsa Craig) infected with *Botrytis cinerea*. **A**, Lesion diameter was measured after 72 h of inoculation. The data show the lesion diameter (mm) $\pm$ standard error (SE) ($n = 20$). **B** through **E**, Optimal hexanoic acid treatment conditions of tomato plants. Four-week-old tomato plants were treated with 0.6 mM hexanoic acid under hydroponic conditions. Inoculation was performed at **B**, 0; **C**, 12; **D**, 24; and **E**, 48 h after treatment. The lesion diameter was measured at 48, 72, and 96 h after inoculation. The data show the lesion diameter (mm) $\pm$ SE ($n = 20$). **F**, Comparative effectiveness of hexanoic acid-induced resistance to salicylic acid (SA) and β -aminobutyric acid (BABA) induced resistance in tomato plants infected with *B. cinerea*. Four-week-old tomato plants were treated with 0.6 mM hexanoic acid, 0.5 mM SA, and 0.5 mM BABA under hydroponic conditions. Lesion diameter was measured at 72 h after inoculation. Data show the lesion diameter (mm) $\pm$ SE ($n = 20$). Different letters represent statistically significant differences (A and F) ($P < 0.05$; least significant difference test).

treated with either water or hexanoic acid for 48 h were treated locally with the callose inhibitor 2-deoxy-D-glucose (2-DDG) (Jakab et al. 2001). At 48 h after incubation, leaves were challenged with *B. cinerea*. Callose accumulation was quantified microscopically (Fig. 2C) and the level of IR was estimated by determining the average lesion diameters at 72 h after inoculation (Fig. 2D). The leaves from hexanoic acid-treated plants showed a reduction in lesion size of 48% when compared with leaves from water-treated plants (Fig. 2D). As previously shown, this enhanced resistance correlated with increased callose accumulation upon infection (Fig. 2C). Treatment with 2-

DDG significantly reduced the hexanoic acid effects after fungal infection (Fig. 2C and D), which correlates callose deposition with Hx-IR against *B. cinerea*.

It was also demonstrated that tomato plants (cv. Ailsa Craig) responded to *B. cinerea* by accumulating caffeic acid, a phenolic component of papillae, as occurs in cotton plants infected by *Xanthomonas campestris* (Dai et al. 1996). Hexanoic acid treatment increased the caffeic acid levels in relation to control plants 72 h after fungal infection (Fig. 3). This observation supports the involvement of cell wall reinforcement in the Hx-IR.

Fig. 2. Contribution of callose deposition to hexanoic acid-induced resistance. **A**, Callose deposition was measured in tomato plants (cv. Ailsa Craig) treated with hexanoic acid, salicylic acid (SA), and β -aminobutyric acid (BABA) upon infection with *Botrytis cinerea*. Tomato plant leaves were sampled 72 h after *B. cinerea* inoculation. Leaves were stained with calcofluor and aniline blue and analyzed by epifluorescent microscopy. Quantification was performed by determining the number of yellow pixels per million corresponding to pathogen-induced callose on digital photographs of infected leaves. Data show average values $\pm$ standard error (SE) ($n = 10$). **B**, Higher-magnification views of callose depositions around the *B. cinerea* infection site 72 h after inoculation. i, Water-treated plants (arrow points radial growth of mycelium); ii, hexanoic acid-treated plants; and iii, BABA-treated plants. Fungal structures are stained bright blue and callose is stained yellow (scale bar = 200 μ m). **C**, Callose deposition in hexanoic acid-treated plants after 2-deoxy-D-glucose (2-DDG) treatment. Plants were treated locally with 2-DDG and inoculated with *B. cinerea*. Callose was measured at 72 h after inoculation. Data show average values $\pm$ SE ($n = 10$). **D**, Effect of callose inhibition in hexanoic acid-induced resistance against *B. cinerea*. Lesion diameter was measured 72 h after inoculation in water- and hexanoic acid-treated plants treated locally with the callose inhibitor 2-DDG. Data show average values $\pm$ SE ($n = 20$). Different letters represent statistical significant differences (A, C, and D) ($P < 0.05$; least significant difference test).

Study of the main signaling pathways in hexanoic acid-treated plants.

In order to establish whether the enhanced resistance of hexanoic acid is mediated by the induction of SA-, JA-, and ET-signaling pathways, the expressions of the *PR1*, *LoxD*, and *ACCOx* marker genes were analyzed by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). These genes have been previously reported to be pathogen inducible in tomato (Benito et al. 1998; Cohn and Martin 2005). BABA- and SA-treated plants were also analyzed. Treatment with hexanoic acid induced *LoxD* mRNA accumulation 72 h after *B. cinerea* infection but no differences were observed in the absence of infection (Fig. 4). Hexanoic acid reduced the levels of *PR1* and *ACCOx* transcript in noninoculated plants but, upon infection, both reached the same level of that in water-treated plants (Fig. 4). SA treatment induced the *PR1* expression, as expected, and BABA treatment also induced *PR1* mRNA accumulation upon infection, although no increase in *LoxD* mRNA was detected (Fig. 4). Therefore, BABA treatment primes for SA-dependent defense responses in tomato against *B. cinerea*, as occurs in *Arabidopsis* (Zimmerli et al. 2001).

Hence, hexanoic acid could enhance JA-dependent defense responses upon infection by *B. cinerea*. In order to further confirm this possibility, the jasmonate-deficient tomato mutants *defenseless1* (*defl*) (Howe et al. 1996) and the *coil* homolog *jasmonic insensitive1* (*jail*) (Li et al. 2004) were tested. Wild-type and mutant plants (cv. Castlemart) were treated with 0.6 mM hexanoic acid and, 48 later, were infected with *B. cinerea*. Hexanoic acid increased resistance to the pathogen in wild-type plants (Fig. 5A) but had no effect on *jail* plants, suggesting that COI1-dependent JA signaling is involved in Hx-IR against *B. cinerea* in tomato. Interestingly, *jail* plants were more susceptible than wild-type plants to this necrotroph, which supports the idea that JA signaling is relevant for basal defense in tomato, as has been shown in *Arabidopsis* (Thomma et al. 1998).

Although the expression of Hx-IR was unaffected in *defl* plants, they showed an augmented callose accumulation upon fungal infection with respect to wild-type plants (Fig. 5A and B). This mutant is defective in biosynthesis of JA and is considered a leaky mutation that does not completely abolish JA signaling (Howe et al. 1996). The fact that *defl* plants show an

Fig. 3. Quantification of caffeic acid in tomato plants (cv. Ailsa Craig) upon infection. Leaves were collected at various time points, and caffeic acid levels were determined in freeze-dried material by high-performance liquid chromatography tandem mass spectrometry. Data show the average of two independent experiments of a pool of 10 plants $\pm$ standard error. The experiment was repeated three times with similar results.

increased perception of the hexanoic acid priming for callose suggests a JA link between hexanoic acid perception and callose deposition. In addition, the fact that Castlemart wild-type plants showed Hx-IR with no enhanced callose accumulation associated suggests that both callose and JA are relevant for Hx-IR. A similar experiment was performed with *defl* in the Ailsa Craig background. In this cultivar, in which the Hx-IR is associated with an increased callose accumulation, the mutant was also protected by hexanoic acid and showed enhanced callose deposition upon infection (Fig. 5C and D). In this case, the mutant plants were slightly more resistant to *B. cinerea* in the absence of treatment (Fig. 5C and D), which could be related to its ability to produce more callose than the wild-type plants (Ailsa Craig). These results further support a link between JA and callose deposition.

ABA-deficient mutant *flacca* is impaired in Hx-IR.

The plant hormone ABA appears to be involved in plant-pathogen interactions by acting as both a negative regulator of

Fig. 4. Effect of hexanoic acid (Hx), β -aminobutyric acid (BABA), and salicylic acid (SA) treatments on the gene expression upon *Botrytis cinerea* infection. Expressions of *PR-1*, *LoxD*, and *ACCOx* genes were analyzed in Hx-, BABA-, and SA-treated tomato plants (cv. Ailsa Craig) upon *B. cinerea* infection. Total RNA was isolated from leaves at 72 h after inoculation, converted to cDNA, and subjected to reverse-transcription quantitative polymerase chain reaction analysis. Results were normalized to the actin gene expression measured in the same sample and expressed logarithmically. Results show the average of three independent experiments with similar results $\pm$ standard error ($n = 3$).

disease resistance by interfering with different signal transduction pathways (Audenaert et al. 2002a; Flors et al. 2005; Mauch-Mani and Mauch 2005), and a positive regulator of disease resistance by enhancing callose deposition in the case of BABA-IR against oomycetes and fungi (Ton and Mauch Mani 2004; Ton et al. 2005; Flors et al. 2008). We tested the ABA-deficient mutant *flacca* in order to investigate the possible relationship between ABA and Hx-IR against *B. cinerea*. To avoid possible differences due to the genetic background, mutant plants from different cultivars (Rheinlands Ruhm, Moneymaker, and Ailsa Craig) were tested. In all cases, hexanoic acid treatment induced resistance in the wild-type plants. The Hx-IR was associated with an increase in callose deposition in those cultivars that respond to *B. cinerea* accumulating callose as a basal response (Rheinlands Ruhm and Moneymaker) as well as in those that do not (Ailsa Craig). (Fig. 6A and B).

Interestingly, *flacca* plants from all the backgrounds analyzed failed to show Hx-IR (Fig. 6A and B), which was correlated with the absence of additional callose accumulation upon infection with respect to water-treated plants. It is interesting to note that the mutant plants (water-treated) in all the investigated backgrounds showed no differences in the susceptibility to *B. cinerea* with respect to wild-type plants but accumulated reduced amounts of callose upon infection (Fig. 6B). These results confirmed the relevance of callose accumulation in Hx-IR and also the ABA mediation of this accumulation upon infection.

Study of the hormonal pattern during Hx-IR.

In order to further confirm the possible role of the different signaling pathways in Hx-IR, we analyzed the hormonal levels in control and hexanoic acid-treated tomato plants (cv. Ailsa Craig) at several time points after *B. cinerea* infection. The

basal ABA levels did not differ between control and hexanoic acid-treated plants after *B. cinerea* infection and ranged between 4,000 and 6,000 ng g⁻¹ dry weight (Fig. 7A). These results show that ABA does not accumulate in response to *B. cinerea* in tomato plants and that Hx-IR is not mediated by an increase in ABA accumulation.

However, the fact that Hx-IR was absent in the ABA-deficient mutant *flacca*, independently of the genetic background tested, supports the idea that an intact ABA signaling is required, at least to induce callose accumulation upon fungal infection.

Interestingly, water-treated plants showed a significant increase in JA accumulation 72 h after *B. cinerea* inoculation that was not observed in hexanoic acid-treated plants upon infection (Fig. 7B). However, water-treated plants responded to fungal infection, accumulating the JA-conjugate JA-Ile 48 h after inoculation that was further increased by hexanoic acid treatment. This reinforces the relevance of this signaling pathway in basal resistance and IR against *B. cinerea* in tomato (Fig. 7C). It was also determined that 12-oxo-phytodienoic acid (OPDA), a precursor of JA, was accumulated in tomato plants in response to *B. cinerea* inoculation, and that hexanoic acid treatment raised the OPDA levels 72 h after fungal infection (Fig. 7D). These OPDA fluctuations suggest that Hx-IR could be mediated by the accumulation of this oxylipin, which plays a role in defense signaling and which presents antimicrobial properties (Stintzi et al. 2001; Ribot et al. 2008).

Hexanoic acid is effective against *P. syringae*.

It was studied whether hexanoic acid treatment was able to protect tomato plants against a pathogen with a different infection strategy such as *P. syringae*. It was observed that hexanoic

Fig. 5. Influence of jasmonic acid (JA) signaling pathway on hexanoic acid-induced resistance against *Botrytis cinerea*. **A**, Lesion diameter from wild-type tomato plant cv. Castlemart and the JA-impaired mutants *defenseless1* (*def1*) and *jasmonic insensitive1* (*jai1*) was measured at 72 h after inoculation. Data show the lesion diameter (mm) $\pm$ standard error (SE) ($n = 20$). **B**, Callose deposition in response to *B. cinerea* in Castlemart and mutant *def1* and *jai1*. Quantification was performed by determining the number of yellow pixels per million corresponding to pathogen-induced callose on digital photographs of infected leaves. Data show average values $\pm$ SE ($n = 10$). **C**, Lesion diameter from wild-type tomato plants cv. Ailsa Craig and the JA-impaired mutant *def1* was measured at 72 h after inoculation. Data show the lesion diameter (mm) $\pm$ SE ($n = 20$). **D**, Callose deposition in response to *B. cinerea* in Ailsa Craig and mutant *def1*. Data show average values $\pm$ SE ($n = 10$). Different letters represent statistical significant differences (A, B, C, and D) ($P < 0.05$; least significant difference test).

acid treatment (0.6 mM) of tomato plants (cv. Ailsa Craig) significantly reduced the number of bacterial colonies and the disease symptoms 72 h after inoculation (Fig. 8A and B).

DISCUSSION

In this study, we have analyzed the effectiveness and mode of action of Hx-IR against *B. cinerea*. We recently reported that this compound protected tomato plants against this necrotroph, which is difficult to control (Leyva et al. 2008).

In this work, we found that treatment of tomato plants with 0.6 mM hexanoic acid for 48 h under hydroponic conditions clearly reduced the incidence of the disease and led to smaller lesions 72 h after inoculation in all the tomato backgrounds tested (Ailsa Craig, Castlemart, Moneymaker, and Rheinlands Ruhm). This concentration had no antimicrobial effect on *B. cinerea* (Leyva et al. 2008), and shorter conditioning times were not sufficient to protect the plant against this pathogen, which strongly supports the inducer effect of this treatment. The fact that hexanoic acid did not significantly accumulate in root-treated plants suggests that its effects might result from specific interactions with plant defense systems.

We have demonstrated that hexanoic acid treatment resulted in an enhanced callose deposition in most of the tomato backgrounds studied, although this effect seems to be cultivar dependent because Castlemart plants displayed Hx-IR with no accumulation of callose. In this work, we also showed that treatment with BABA, a potent inducer of a broad spectrum of disease resistance in different plant species (Jakab et al. 2001; Cohen 2002), primed for callose accumulation upon *B. cinerea* in tomato. Under these experimental conditions, SA also protected tomato plants against *B. cinerea* but was less effective than hexanoic acid and BABA. Accordingly, SA slightly induced callose upon fungal infection. Inhibition of callose accumulation by treatment with the callose inhibitor 2-DDG slightly reduced protection against *B. cinerea* in hexanoic acid-treated plants (cv. Ailsa Craig). This suggests that primed callose deposition can contribute to the IR but hexanoic acid has effects other than induction of callose. The involvement of callose in the basal defense against *B. cinerea* seems to be cultivar dependent in tomato because Ailsa Craig plants did not accumulate callose, which was a basal response to this necrotroph for the rest of the cultivars analyzed. Interestingly, hexanoic acid treatments also induced callose accumulation in the Ailsa Craig plants, thus demonstrating that the mechanisms for IR do not always coincide with basal defense mechanisms. The levels of caffeic acid were also increased after hexanoic acid treatment, which further supports the role of the reinforcement of the cell wall in this inducer activity.

We also investigated the effect of hexanoic acid on the expression of defense-related genes. Although the basal level of expression of *PR1* and *ACCox* was reduced after hexanoic acid treatment in the absence of infection, no increase for both genes was observed in hexanoic acid-treated plants with respect to water-treated plants upon infection. However, hexanoic acid treatment increased the accumulation of the *LoxD* transcript in comparison with control plants 72 h after *B. cinerea* infection. Interestingly, BABA treatment primed the induction of *PR1* upon *B. cinerea* infection but reduced the *LoxD* expression, probably as a result of the antagonistic interaction between SA and JA pathways. These results show differences in the mechanism of action of hexanoic acid and BABA as inducers in tomato plants.

The analysis of hormones and metabolites related to plant-pathogen interactions confirmed that the JA-signaling pathway play a role in the defense against this necrotroph in tomato. The basal levels of JA, OPDA, and JA-Ile increased after *B.*

cinerea inoculation in tomato plants. Hexanoic acid treatment induced a faster and stronger accumulation of JA-Ile at 48 h after inoculation and of OPDA at 72 h after inoculation compared with water-treated plants. Interestingly, the increase of JA observed in water-treated plants upon infection was not observed in hexanoic acid-treated plants, which may be explained by a recirculation of this hormone into its conjugated forms. Hence, Hx-IR is mediated by the accumulation of the bioactive signal JA-Ile (Chico et al. 2008) and of the oxylipin OPDA, which is a regulator of plant defenses and is also active against microorganisms, including *B. cinerea* (Prost et al. 2005; Vellosillo et al. 2007). The fact that hexanoic acid treatment increased the accumulation of the *LoxD* transcript compared with control plants 72 h after *B. cinerea* infection supports the induction of oxidation and the further transformation of fatty acids. In addition, the analysis of the *coil* homolog mutant in tomato *jail* confirmed that the JA-signaling pathway is important for both basal IR and Hx-IR against *B. cinerea*. On the other hand, *def1* plants deficient in JA biosynthesis still displayed protection by hexanoic acid but perceived better the priming for callose than wild-type plants, which suggests a JA link between hexanoic acid perception and callose priming that requires further studies.

In this work, we also analyzed the role of ABA in Hx-IR against *B. cinerea*. The ABA-deficient mutant *flacca* was unable to express Hx-IR in all the tomato backgrounds tested (Ailsa Craig, Moneymaker, and Rheinlands Ruhm). Hence, ABA signaling could play a role in the protection against *B. cinerea* mediated by hexanoic acid. The basal ABA levels did not increase upon fungal infection in tomato plants (cv. Ailsa Craig) and hexanoic acid did not alter ABA accumulation, sup-

Fig. 6. Influence of abscisic acid (ABA) signaling on hexanoic acid-induced resistance against *Botrytis cinerea*. **A**, Lesion diameter from wild-type tomato plants of cvs. Rheinlands Ruhm, Moneymaker, and Ailsa Craig and their respective ABA-impaired mutant *flacca* was measured at 72 h after inoculation. Data show the lesion diameter (mm) $\pm$ standard error (SE) ($n = 15$). **B**, Callose was quantified 72 h after infection as described in Figure 2. Data show average values $\pm$ SE ($n = 10$). Different letters represent statistical significant differences (A and B) ($P < 0.05$; least significant difference test).

porting the idea that basal level of ABA is necessary and sufficient to mediate Hx-IR.

In our experimental conditions, none of the *flacca* mutants showed enhanced basal resistance relative to their respective wild-type plants as was previously reported by Audenaert and associates (2002a) in cv. Moneymaker. This discrepancy might be due to the different experimental system employed. Although we used whole plants incubated at 100% relative humidity (RH), in cv. Moneymaker the experiments were performed in detached leaves. Therefore, in our system, ABA might not be relevant as a susceptibility factor but is important for hexanoic acid-mediated callose priming. The mutant plants accumulated reduced amounts of callose with respect to the wild-type plants upon infection, and hexanoic-acid treatment did not boost callose deposition upon fungal infection. This indicates that the disruption of the ABA signaling results in the loss of both basal and primed callose deposition, resulting in a loss of Hx-IR against *B. cinerea*. Hence, ABA can act as a positive regulator of Hx-IR by enhancing callose deposition, as already reported for BABA-IR in *Arabidopsis* (Ton and Mauch-Mani 2004; Flors et al. 2008). Further studies will be carried out to establish whether hexanoic acid responses are directly affected by ABA or by a more complex signaling crosstalk.

In conclusion, the resistance induced by hexanoic acid in tomato plants against *B. cinerea* is based in part on a boosted callose accumulation in papillae that stops fungal progression and which requires intact ABA signaling. Hexanoic acid also enhances the oxylipins pathway by increasing OPDA accumu-

lation and the JA conjugate JA-Ile that could participate in the defense against the pathogen. A proposed model for Hx-IR against *B. cinerea* is included in Figure 9, where it is shown that callose and JA-signaling priming are mechanisms involved in this IR.

Hexanoic acid treatment also protected tomato plants against the hemibiotrophic bacterium *P. syringae*, which reflects the fact that it induces a wide-spectrum disease resistance in tomato.

Although further research is required to assess any additional factors involved in hexanoic acid-mediated protection, the presented data show that this new inducer could act on different targets than the other previously described inducers, which contributes to the knowledge of IR in tomato plants.

MATERIALS AND METHODS

Microbial strains and growth conditions.

The necrotrophic fungus used in this study was *B. cinerea* CECT2100 (Spanish collection of type cultures, Universidad de Valencia, 46100 Burjassot, Spain). It was routinely cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit) at 24°C. *B. cinerea* spores were collected from 10- to 15-day-old cultures with sterile water containing 0.01% (vol/vol) Tween-20, which was then filtered, quantified with a hemacytometer, and adjusted to an appropriate concentration (*B. cinerea* spores at 1×10^6 ml⁻¹).

P. syringae DC3000 was grown in a King's B medium (KB) (King et al. 1954) at 28°C. Rifampicin was added to KB at 50 µg ml⁻¹. Bacterial suspensions were adjusted to CFU at 10⁵

Fig. 7. Hormone levels in water- and hexanoic acid-treated tomato plants (cv. Ailsa Craig) upon *Botrytis cinerea* infection. Leaves were collected at various time points and **A**, abscisic acid (ABA); **B**, jasmonic acid (JA); **C**, JA-Ile; and **D**, 12-oxo-phytodienoic acid (OPDA) levels were determined in freeze-dried material by high-performance liquid chromatography mass spectrometry. Data show a representative experiment that has been repeated five times; each point is the average of a pool of 10 plants $\pm$ standard error.

(460) ml⁻¹ in sterile distilled water containing 0.015% surfactant Silwet L-77 (Osi Specialties, Danbury, CT, U.S.A.) as previously described (Katagiri et al. 2002).

Plant material.

Different tomato (*Solanum lycopersicum* Mill) genotypes were used in our studies: wild-type Ailsa Craig, Rheinlands Ruhm, Moneymaker, and Castlemart. The JA pathway mutants used were *def1* and *jail-1* mutants in background Castlemart (G. Howe, Michigan state University) and *def1* mutant in background Ailsa Craig (LA 3749), provided by the Tomato Genetics Resource Center (TGRC). The ABA pathway mutants used were ABA-deficient mutant *flacca* in backgrounds Ailsa (LA3613) and Rheinlands Ruhm (LA0673), both provided by TGRC, and *flacca* mutant in the background Moneymaker (J. A. López, Estación Experimental del Zaidín). Seed were germinated in vermiculite. After 4 weeks, seedlings were transferred to hydroponic conditions in tanks containing Hoagland solution. Plants were grown at day and night temperatures of 24 and 18°C, respectively, with 16 h of light and 8 h of darkness and RH = 60%. After 4 days, plants were inoculated with the pathogen and maintained at day and night temperatures of 24 and 18°C, respectively, with 16 h of light and 8 h of darkness and RH = 100%.

The *jail-1* homozygous plants were selected from the F2 population using a PCR-based method with the primers forward = GTG GAG ACG ATA TGT TGA GAC TAA, reverse 1 = CCA TGG AGT CCA TCA CCT AAC AGT, and reverse 2 =

GTG GTC AGA TCA GAG CCC TCT ATT, as described by Li and associates (2004).

B. cinerea bioassays.

Conidia collected from 10- to 15-day-old PDA plates supplemented with tomato leaves at 40 mg ml⁻¹ were maintained in Gambor's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 10 mM sucrose and 10 mM KH₂PO₄ for 2 h in the dark with no shaking.

Plants were treated with water, hexanoic acid (0.6 mM) at pH 6 (Leyva et al. 2008), and BABA (0.5 mM) or SA (0.5 mM) in the nutrient solution 48 h before inoculation.

Plant inoculation was done on intact plants at 100% RH as described by Flors and associates (2007). Untreated or hexanoic acid-treated tomato plants were challenged by applying 5-μl droplets of a suspension of *B. cinerea* spores at 1 × 10⁶ ml⁻¹, previously incubated in Gambor's B5 medium supplemented with sucrose (0.1 mM) and phosphate (0.1 mM) for 2 h.

Fungal hyphae grew concentrically from the inoculation site, resulting in visible necrosis at 48 h after inoculation. Disease symptoms were assessed 72 h after inoculation by determining the average lesion diameter in 20 plants per genotype.

P. syringae bioassays.

Plants were treated with water and hexanoic acid (0.6 mM) at pH 6 in the nutrient solution 48 h before inoculation (Leyva et al. 2008). Four week-old plants were grown and treated under the same conditions as those described by Flors and associates (2007). In addition, the disease rate was also scored by counting the percentage of leaves showing dark-brown spots. 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.

Quantification of callose deposition.

Callose deposition and effect of the callose inhibitor 2-DDG on resistance against *B. cinerea* was determined as described

Fig. 8. Hexanoic acid-induced resistance against *Pseudomonas syringae*. After 72 of inoculation, disease rating was scored by **A**, plating in agar-King's B medium bacterial extracts from infected leaves and **B**, measuring the percentage of diseased leaves. Data show average values ± standard error ($n = 20$). Different letters represent statistical significant differences (A and B) ($P < 0.05$; least significant difference test).

Fig. 9. Model for hexanoic acid-induced resistance in tomato plants against *Botrytis cinerea*. Infection by *B. cinerea* leads to activation of salicylic acid (SA)-dependent defense pathway while abscisic acid (ABA) could act as susceptibility factor depending on the experimental conditions. According to our results, the jasmonic acid (JA) pathway appears to play a role in the basal defense. Treatment with hexanoic acid does not depend on the SA pathway. Hexanoic acid treatment induces ABA-mediated callose deposition. On the other hand, the contribution of the JA-dependent defense pathway is observed, by priming JA-Ile and 12-oxo-phytodienoic acid (OPDA) after hexanoic acid treatment. Thus, callose accumulation and the JA signaling pathway are relevant for hexanoic acid-induced resistance against *B. cinerea*.

by Flors and associates (2007). Disease symptoms were assessed 72 h after inoculation and 10 leaves were collected for callose staining.

Analysis of gene expression by RT-qPCR.

Ribonucleic acid was extracted from tomato leaves with the Total quick RNA cells and tissues kit (Talent, Trieste, Italy) at 72 h after inoculation. Leaf tissue was collected from five treated and untreated plants. Conditions of RT-qPCR experiments were described by Flors and associates (2007). Differences in cycle numbers during the linear amplification phase between samples containing cDNA from infected and noninfected plants were used to determine the differential gene expression. The expression detected from the tomato *actin* and *tubulin* genes was used as an internal standard. The primers using for *actin*, *PR1*, and *LoxD* were described by Flors and associates (2007). In addition, in this work, we used the primers *Letub* forward primer 5'-CCAGGTTTGCCACTCACTTG-3' and *Letub* reverse primer 5'-GGAAACGAAGAATGTGAGCAT-3', and *LeACCOx* forward primer 5'-CCATGTCCTAAGCCCGATTGTAT-3' and *LeACCOx* reverse primer, 5'-ACTCACTTTGTCATCTTGGAACAGA-3'. Triplicate analyses were performed on all occasions using the cDNA samples derived from three independent experiments.

Chromatographic analysis.

For hormone analysis, fresh material was frozen in liquid nitrogen. Dry tissue (0.05 g) was immediately homogenized in 2.5 ml of ultrapure water. For hexanoic acid uptake assays, ¹³C-hexanoic acid (Sigma, Poole, U.K.) 0.6 mM was supplied to the hydroponic solution. Plants were allowed to uptake it for 48 h and leaves were analyzed at different time points. Dry tissue (0.05 g) was homogenized in 2.5 ml of ultrapure water. For hormone analysis, a mixture of internal standards was added before the extraction (100 ng of [³H₆]-ABA, 100 ng of prostaglandin B1 (Pinfield-Wells et al. 2005), dihydrojasmonic acid (Flors et al. 2008), and propylparaben. For hormone and ¹³C-hexanoic acid analysis, extractions and experimental procedures were performed as described by Flors and associates (2008). After extraction, a 20-μl aliquot was directly injected into the HPLC system. Analyses were carried out using a Waters Alliance 2690 HPLC system (Milford, MA, U.S.A.) with nucleosil ODS reversed-phase column (100 by 2 mm i.d.; 5 μm) (Scharlab, Barcelona, Spain). The chromatographic system was interfaced to a Quatro LC (quadrupole-hexapolequadrupole) mass spectrometer (Micromass, Manchester, U.K.).

Statistical analysis.

Statistical analysis was carried out by one-way analysis of variance using the Statgraphycs-plus software for Windows V.5 (Statistical Graphycs Corp., MD, U.S.A.). Means were expressed by a standard error. They were compared using Fisher's least significant difference at 95%. All the experiments were repeated at least three times.

ACKNOWLEDGMENTS

This work was supported by grants from the National R&D Plan AGL2003-08481; Plan 2004 of Universitat Jaume I (UJI) for research promotion (P1 1B2004-35); Generalitat Valenciana GRUPOS04/029; and the National R&D Plan AGL2006-12711-C02-01, AGL2006-12711-C02-02, AGL2007-66282-C02-01/ALI, and AGL2007-66282-C02-02/ALI. M. de la O. Leyva is the recipient of a Ph.D. fellowship from grant AGL2003-08481 and now a Ph.D. fellowship from Plan 2004 of UJI for research promotion. I. Finiti is the recipient of a long-term predoctoral fellowship from Generalitat Valenciana. We thank the SCIC of the UJI and Ted Farmer of the University of Lausanne, and B. Mauch-Mani of the University of Neuchâtel for the JA-Ile standard.

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