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Host perception of jasmonates promotes infection by *Fusarium oxysporum* formae speciales that produce isoleucine- and leucine-conjugated jasmonates

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SUMMARY

Three pathogenic forms, or formae speciales (f. spp.), of *Fusarium oxysporum* infect the roots of *Arabidopsis thaliana* below ground, instigating symptoms of wilt disease in leaves above ground. In previous reports, *Arabidopsis* mutants that are deficient in the biosynthesis of abscisic acid or salicylic acid or insensitive to ethylene or jasmonates exhibited either more or less wilt disease, than the wild-type, implicating the involvement of hormones in the normal host response to *F. oxysporum*. Our analysis of hormone-related mutants finds no evidence that endogenous hormones contribute to infection in roots. Mutants that are deficient in abscisic acid and insensitive to ethylene show no less infection than the wild-type, although they exhibit less disease. Whether a mutant that is insensitive to jasmonates affects infection depends on which forma specialis (f. sp.) is infecting the roots. Insensitivity to jasmonates suppresses infection by *F. oxysporum* f. sp. *conglutinans* and *F. oxysporum* f. sp. *matthioli*, which produce isoleucine- and leucine-conjugated jasmonate (JA-Ile/Leu), respectively, in culture filtrates, whereas insensitivity to jasmonates has no effect on infection by *F. oxysporum* f. sp. *raphani*, which produces no detectable JA-Ile/Leu. Furthermore, insensitivity to jasmonates has no effect on wilt disease of tomato, and the tomato pathogen *F. oxysporum* f. sp. *lycopersici* produces no detectable jasmonates. Thus, some, but not all, *F. oxysporum* pathogens appear to utilize jasmonates as effectors, promoting infection in roots and/or the development of symptoms in shoots. Only when the infection of roots is promoted by jasmonates is wilt disease enhanced in a mutant deficient in salicylic acid biosynthesis.

Keywords: *Arabidopsis thaliana*, COI1, *Fusarium oxysporum*, Fusarium wilt, jasmonate.

INTRODUCTION

Fusarium wilt of *Arabidopsis thaliana* is an experimental pathosystem for the study of the genetics of host resistance to and pathogenesis of wilt fungi (Michielse and Rep, 2009). Wilt fungi are responsible for debilitating vascular infections in plant species that are important to agriculture, horticulture and silviculture (Mace *et al*., 1981). In *A. thaliana*, three pathogenic forms, or formae speciales (f. spp.), of the wilt fungus *Fusarium oxysporum* instigate disease (Diener and Ausubel, 2005). In the field, *F. oxysporum* f. sp. *conglutinans* (FOC), *F. oxysporum* f. sp. *raphani* (FOR) and *F. oxysporum* f. sp. *matthioli* (FOM) are isolated from diseased cabbage (*Brassica* species), radish (*Raphanus sativus*) and garden stock (*Matthiola incana*), respectively (Bosland and Williams, 1987). Foliar symptoms in field hosts, such as stunting, epinastic growth, perivascular yellowing and premature senescence of leaves, are reproduced in infected *A. thaliana* (Diener and Ausubel, 2005).

Interactions between wilt fungi and their hosts, including *F. oxysporum* and *A. thaliana*, are described by three phases (Diener, 2012; Talboys, 1972): In the primary determinative (or prevascular) phase, soil-borne *F. oxysporum* enters roots, usually at the tips, and colonizes extravascular tissue. In the secondary determinative (or vascular) phase, *F. oxysporum* invades the vascular cylinder and spreads via the water-conducting xylem vessels. In the expressive phase, symptoms appear in foliage above ground in advance of the infection of roots below ground.

More or less wilt disease is a characteristic of genetically dissimilar cultivars or wild accessions of a host species (Beckman and Roberts, 1995). For instance, the standard laboratory accession Columbia-0 (Col-0) of *A. thaliana* expresses complete resistance to FOM, whereas accession Taynuilt-0 (Ty-0) is highly susceptible (Diener and Ausubel, 2005). This natural variation in resistance is specific to the infecting f. sp., as Col-0 and Ty-0 exhibit similar resistance when they are instead infected with FOC or FOR. Inheritance of *RESISTANCE TO F. OXYSPORUM* (*RFO*) genes explains the difference between FOM-infected Col-0 and Ty-0, and suggests that variation in resistance is the result of expression of diversity in innate immunity (Cole and Diener, 2013; Diener, 2012; Shen and Diener, 2013).

Arabidopsis mutants with defects in hormone biosynthesis or signalling exhibit more or less wilt disease when infected with FOC. For example, genotypes that suppress pathogen-induced accumulation of salicylic acid (SA), such as *enhanced disease*

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susceptibility 5 (*eds5*), *salicylic acid induction-deficient 2* (*sid2*) and transgenic *nahG*, express more severe disease than the wild-type (Diener and Ausubel, 2005; Trusov *et al*., 2009). However, genotypes that are defective in the biosynthesis of abscisic acid [*abscisic acid-deficient 1* and *2* (*aba1* and *aba2*)], the perception of jasmonates [*coronatine-insensitive 1* (*coi1*) and *jasmonate-insensitive 1* (*jin1*)] or ethylene [*ethylene-insensitive 2* (*ein2*)] or the signalling (*auxin resistant 2*) or transport (*transport inhibitor resistant 3*) of auxin express less disease or no disease altogether (Anderson *et al*., 2004; Kidd *et al*., 2011; Thatcher *et al*., 2009; Trusov *et al*., 2009).

The effect of hormones on disease may be interpreted in terms of host resistance (Glazebrook, 2005). In general, signalling by jasmonate and ethylene is associated with less disease from infection by necrotrophic pathogens, and so jasmonate and ethylene are said to promote resistance to necrotrophs. SA accumulation often correlates with less disease from infection by biotrophic pathogens, and so SA is said to promote resistance to biotrophs. Furthermore, hormones sometimes have synergistic effects or antagonistic effects that work at cross-purposes and negate the contribution of either or both (Kunkel and Brooks, 2002).

The effects of hormones may be the consequence of interactions between hormone signalling and virulence, in which case pathogenesis depends on how hormone signalling interacts with the strategy of pathogens (Grant and Jones, 2009; Seilaniantz *et al*., 2011). Indeed, the pathogen's lifestyle, biotrophic or necrotrophic, rather than taxonomic grouping anticipates the effects of hormones. In addition, hormones usually have a more profound effect on infection by virulent pathogens than by non-adapted pathogens. From this perspective, jasmonate signalling is antithetical to the virulence expressed by necrotrophs and conducive to the virulence of biotrophs. Attributing the roles of hormones to virulence rather than host resistance may better explain the inconsistent roles played by hormones during infection by the same or similar microbes expressing different strategies.

For example, jasmonate signalling in *A. thaliana* either promotes or suppresses resistance to *F. oxysporum*, depending on the disease syndrome being observed (Aboul-Soud *et al*., 2004; Berrocal-Lobo and Molina, 2004; Epple *et al*., 1997; McGrath *et al*., 2005; Thatcher *et al*., 2009; Trusov *et al*., 2009). When conditions favour rotting by *F. oxysporum*, there is more necrosis of leaves or whole seedlings in the jasmonate-insensitive mutant *coi1* than in the wild-type. Thus, jasmonate signalling is critical for resistance or susceptibility depending on the pathogenesis elaborated by *F. oxysporum*.

In this study, we found that the host's perception of jasmonates is crucial for persistent root infection by *F. oxysporum* f. spp. that produce isoleucine- and leucine-conjugated jasmonate (JA-Ile/Leu) *in vitro*, that SA antagonizes the host's perception of jasmonates in roots and that the endogenous plant hormones abscisic acid, ethylene and jasmonates have no critical role in immunity. Instead, endogenous abscisic acid and ethylene probably contribute to the expression of foliar wilt symptoms.

RESULTS

COI1 promotes root infection by FOC

Previous studies have shown that the perception of jasmonates (by *COI1*), but not the synthesis of jasmonates (by *ALLENE OXIDE SYNTHASE*, *AOS*), is critical to the development of wilt disease in FOC-infected *A. thaliana* (Thatcher *et al*., 2009; Trusov *et al*., 2009). The effect and interaction of the synthesis and perception of jasmonates were scrutinized in the nine genotypes generated by the selfed dihybrid *AOS*/*aos COI1*/*coi1* (Fig. 1). In particular, we measured the radius of rosettes, which quantified the stunted expansion of rosette leaves, a conspicuous symptom of Fusarium wilt. *AOS* corresponds to the first step in jasmonate biosynthesis, and *aos* fails to accumulate jasmonates in response to stimuli, including biotic stress (Chehab *et al*., 2008). At 20 days post-infection (dpi), the rosette radii of FOC-infected plants that were capable (*AOS*/–) and incapable (*aos*) of accumulating jasmonates were similarly reduced (Fig. 1). *COI1* encodes the co-receptor of

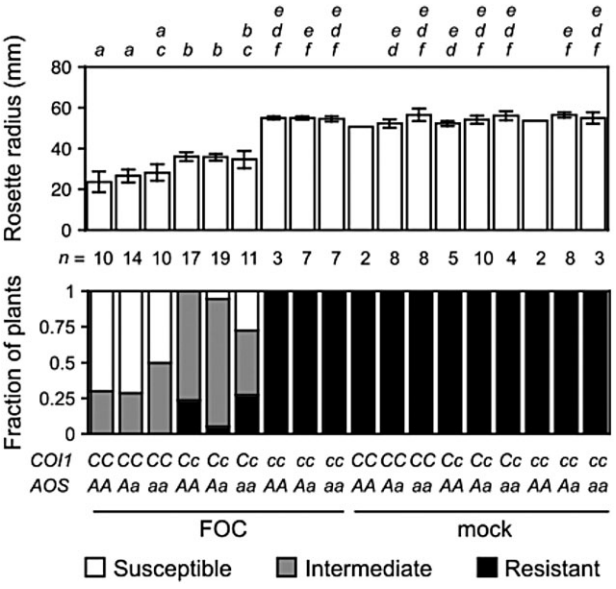

Fig. 1 *CORONATINE-INSENSITIVE 1* (*COI1*) promotes wilt disease from *Fusarium oxysporum* f. sp. *conglutinans* (FOC) infection. Genotypes of FOC-infected or mock-infected offspring of the dihybrid *COI1*/*coi1 ALLENE OXIDE SYNTHASE* (*AOX*)/*aos* were *COI1*/*COI1* (*CC*), *COI1*/*coi1* (*Cc*) or *coi1*/*coi1* (*cc*) and *AOS*/*AOS* (*AA*), *AOS*/*aos* (*Aa*) or *aos*/*aos* (*aa*). Wilt symptoms at 20 days post-infection (dpi). Top graph, the mean radius of rosettes of *n* plants in millimetres. Error bars are the confidence intervals of the means ($\alpha = 0.05$). Means with the same italicized letter (above) are not significantly different, according to Student's *t*-test ($P > 0.01$). Bottom graph: fractions of *n* plants resistant (health index, HI| ≥ 4) or susceptible (HI < 3) or with intermediate resistance (HI ≥ 3 and HI < 4).

the biologically active jasmonate (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), and *coi1* is insensitive to jasmonates, either produced by the plant or applied to the plant (Fonseca *et al*., 2009; Yan *et al*., 2009). In contrast, FOC-infected plants that were insensitive to jasmonates (*coi1*) developed no wilt symptoms, including no reduction in the rosette radius (Fig. 1). Furthermore, the double mutant *aos coi1* was unaffected, and so the absence of disease in *coi1* was not an effect of endogenous jasmonates in the absence of *COI1*.

Interestingly, symptom severity was sensitive to the gene dosage of *COI1*. The mean radius of rosettes of heterozygotes (*COI1*/*coi1*) was intermediate to that of *coi1* and the wild-type (*COI1*). The intermediate size of *COI1*/*coi1* rosettes was unperturbed by the presence or absence (*aos*) of *AOS*, which emphasized that *COI1*'s role is unrelated to endogenously produced jasmonates.

To examine whether *COI1* was critical for infection of roots in the primary and/or secondary determinative phases, infection in roots of *coi1* and the wild-type was compared in the initial week after soil was infested with FOC. *Fusarium oxysporum* infection in roots is stained blue when roots are treated with the indigogenic substrate of *Fusarium*-expressed arabinofuranosidase (ABF) 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-Ara) (Diener, 2012). In the first few days after soil infection, both root tips and lateral root primordia (LRP) were stained blue by X-Ara in the wild-type and *coi1*, indicating that *COI1* was not essential for FOC's initial invasion into roots. However, by 4–5 dpi, when wild-type rosettes still appeared asymptomatic, staining in roots of *coi1* and the wild-type was noticeably different. In particular, fine staining in the central vascular cylinder that extended from the colonized root apices of the wild-type (Fig. 2a,b) rarely extended from those of *coi1* (Fig. 2c,d).

To quantify the difference at X-Ara-stained root apices, four patterns of staining were recognized (Fig. 2e): (i) undifferentiated tissue at apices was incompletely stained; (ii) apices were stained throughout; (iii) stain extended into the vascular cylinder a short distance from the stained apices; and (iv) fine staining in the vascular cylinder extended for some distance from the stained apices.

At 5 dpi, X-Ara-stained roots were assigned to one of the four patterns, and patterns at the root tips and LRP were tallied separately (Fig. 2f). Comparable numbers of root tips from three whole-root systems of *coi1* (44, 48 and 71) and the wild-type (49, 55 and 63) were stained blue, whereas significantly fewer LRP from the

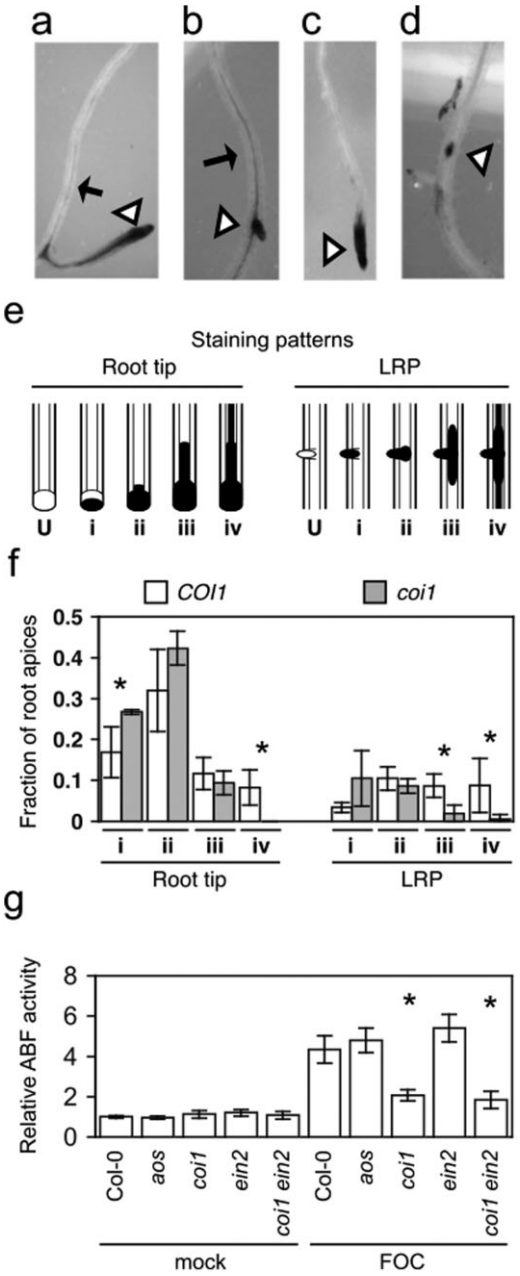

Fig. 2 *CORONATINE-INSENSITIVE 1* (*COI1*) promotes infection by *Fusarium oxysporum* f. sp. *conglutinans* (FOC). Representative FOC-infected apices (at triangles) of wild-type (a, b) and *coi1* (c, d), with 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-Ara) staining patterns (ii) and (iv), respectively. Arrows point to fine vascular staining. (e) Depictions of unstained root apices (U) and common patterns of X-Ara staining (filled) at root tips and lateral root primordia (LRP): (i) partial staining of undifferentiated apices; (ii) staining throughout undifferentiated apices; (iii) broad vascular staining extending for short distances from apices; and (iv) fine, extensive vascular staining away from apices. (f) Fractions of patterns in whole-root systems of three FOC-infected wild-type (*COI1*) and *coi1* at 4–5 days post-infection (dpi). Error bars are confidence intervals of the means ($\alpha = 0.05$). Asterisks indicate that the mean values of *COI1* and *coi1* are different, according to Student's *t*-test ($\alpha = 0.05$). (g) Relative arabinofuranosidase (ABF) activity was quantified in FOC-infected wild-type (Col-0, $n = 5$), *allene oxide synthase* (*aos*) ($n = 5$), *coi1* ($n = 4$), *ethylene-insensitive 2* (*ein2*) ($n = 5$) and *coi1 ein2* ($n = 5$) or mock-infected plants ($n = 3$) at 10 dpi, and values are relative to the mean of mock-infected Col-0 roots. Error bars are the confidence intervals of the means, and asterisks indicate that mean values of Col-0 and the mutant are different, according to Student's *t*-test ($\alpha = 0.05$).

root systems of *coi1* (13, 15 and 15) than the wild-type (23, 24 and 29) were stained blue. However, because the total number of LRP was not determined, the discrepancy could be explained by fewer LRP emerging from *coi1* rather than less infection in LRP of *coi1*. Showing that *COI1* was crucial for infection of the differentiated vascular cylinder, pattern (iv) [as well as pattern (iii) at LRP] was under-represented among the apices of *coi1* (Fig. 2f). Pattern (iv) was represented by only one LRP and no root tips of *coi1*, whereas 22 LRP and 20 root tips of the wild-type were assigned to pattern (iv). In addition to the deficit of pattern (iv), *coi1* had an excess of pattern (i) at the root tips, and probably at LRP, in comparison with the wild-type, suggesting that *COI1* also promoted the colonization of undifferentiated cells at apices after invasion.

In the second week after infection, when the wild-type exhibited visible symptoms, ABF activity indicated that FOC infection was profoundly suppressed in *coi1*, but was no different in *aos* and the wild-type. X-Ara staining in the vascular cylinder was extensive in the wild-type and sparse in *coi1*. This visible difference was quantified using the substrate nitrophenyl-α-L-arabinofuranoside (NP-Ara), and roots of the wild-type had 3.5-fold more *Fusarium*-derived ABF activity than *coi1* (Fig. 2g). In contrast, both visible staining and quantification of ABF activity in *aos* and the wild-type were similar and consistent with a similar appearance of foliar symptoms.

COI1 promotes symptoms in FOC-infected plants

Distinct roles for *COI1* expression in roots and shoots were observed in grafted plants infected with FOC. Grafting was used to make the four possible combinations of scions and rootstocks from wild-type (*COI1*) and *coi1* seedlings. As reported previously by Thatcher *et al*. (2009), all plants with *coi1* rootstocks exhibited the same absence of wilt disease as ungrafted *coi1* (Fig. 3a,b). However, wilt disease in plants with *COI1* rootstocks had an altered appearance depending on whether the scions were *coi1* or *COI1*. In plants with *coi1* scions, symptoms such as stunting of rosette leaves, accumulation of (anthocyanin) pigment in petioles and epinastic growth of leaf petioles were strongly suppressed, whereas other symptoms, such as perivascular yellowing and premature senescence, were delayed in comparison with plants with *COI1* scions (Fig. 3a,b). At 21 dpi, X-Ara staining was largely restricted to the root tips in *coi1* rootstocks, and so *COI1* in roots alone was sufficient to promote infection of the vascular cylinder (Fig. 3c). Interestingly, noticeably fewer roots in rootstocks of *COI1* were stained by X-Ara when grafted to *coi1* scions than *COI1* scions (Fig. 3c), suggesting that shoot-expressed *COI1* might also influence the infection of roots.

COI1 promotes wilt disease by FOM

In Diener and Ausubel (2005), Col-0 is resistant to wilt disease when soil is infested with FOM instead of FOC, and so sensitivity

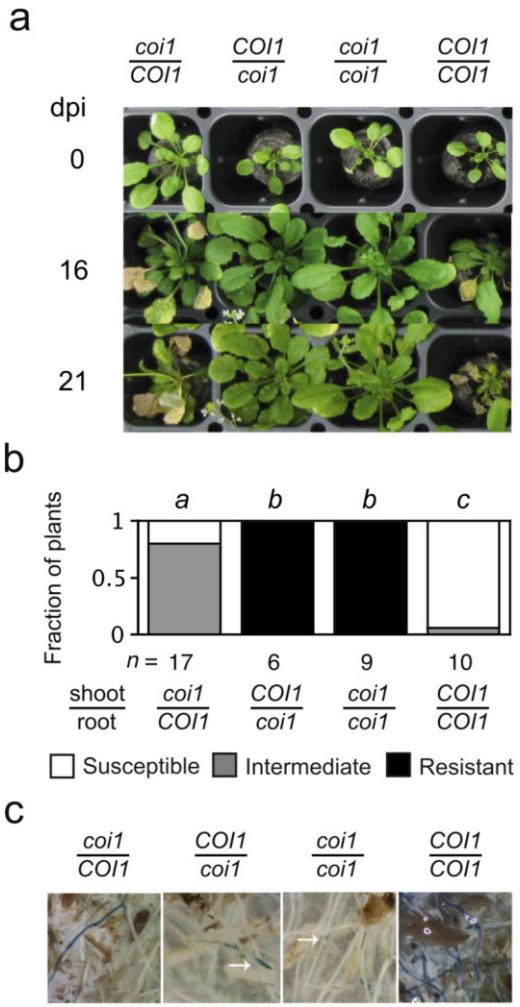

Fig. 3 *CORONATINE-INSENSITIVE 1* (*COI1*) promotes symptoms in *Fusarium oxysporum* f. sp. *conglutinans* (FOC)-infected plants. Grafted plants from scions (top) and rootstocks (bottom) that were wild-type (*COI1*) or *coi1* were infected with FOC. (a) Representative grafts are shown at 0, 16 and 21 days post-infection (dpi). (b) At 16 dpi, fractions of *n* grafted plants were susceptible ($0 \leq$ health index, HI < 2), had intermediate resistance ($2 \leq$ HI ≤ 3) or were resistant ($3 <$ HI ≤ 5). Grafts with the same italicized letter (above) had similar HI scores, according to Mann–Whitney *U*-test ($P > 0.01$). (c) At 21 days, 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-Ara) staining in roots of grafts is shown. Note that the staining of *coi1* rootstocks is restricted to the root tips (at arrows).

to jasmonate is not sufficient to make *A. thaliana* susceptible to Fusarium wilt disease. To investigate whether *COI1* nevertheless contributes to infection by FOM, wilt disease was evaluated in offspring of the selfed monohybrid *rfo1 COI1*/*coi1*, because *rfo1* compromises immunity to *F. oxysporum* (Diener, 2012). FOM-infected offspring that were *rfo1 COI1*/– exhibited wilt symptoms, including a reduced rosette radius, whereas *rfo1 coi1* offspring exhibited no disease (Fig. 4a). A wider rosette radius in *rfo1 COI1*/*coi1* heterozygotes and more severe symptoms in *rfo1 COI1*/*COI1*

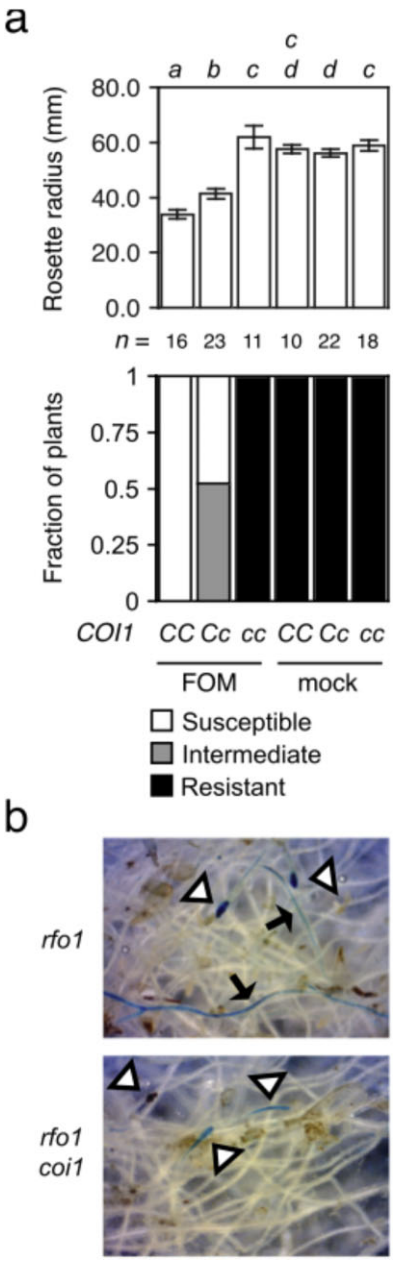

Fig. 4 *CORONATINE-INSENSITIVE 1* (*COI1*) promotes infection by *Fusarium oxysporum* f. sp. *matthioli* (FOM). Genotypes of FOM- or mock-infected offspring of the monohybrid double mutant *resistance to F. oxysporum 1* (*rfo1*) *COI1*/*coi* were *COI1*/*COI1* (*CC*), *COI1*/*coi1* (*Cc*) or *coi1*/*coi1* (*cc*). (a) Wilt symptoms at 20 days post-infection (dpi). Top graph: the mean radius of rosettes of *n* plants is in millimetres. Error bars are the confidence intervals of the means ($\alpha = 0.05$). Means with the same italicized letter (above) have no significant difference, according to Student's *t*-test ($P > 0.01$). Bottom graph: fractions of *n* plants that were resistant (health index, HI = 5), susceptible (HI = 3) or showed intermediate resistance (HI = 4). (b) At 20 dpi, 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-Ara) stains root apices (at triangles) of FOM-infected *rfo1* and *rfo1 coi1*, and arrows point to fine vascular staining in roots of *rfo1*.

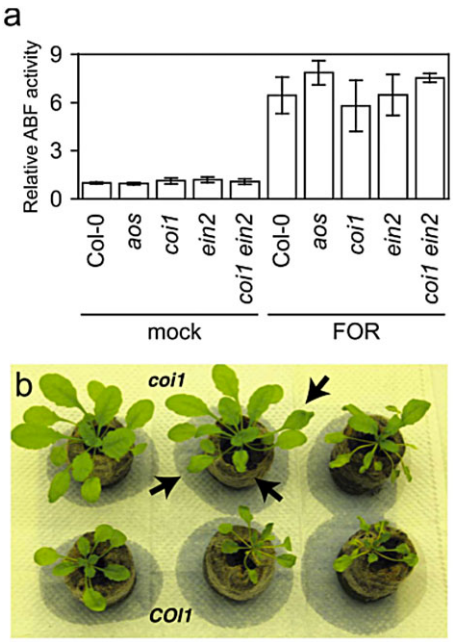

Fig. 5 *CORONATINE-INSENSITIVE 1* (*COI1*) promotes symptoms in *Fusarium oxysporum* f. sp. *raphani* (FOR)-infected plants. (a) Relative arabinofuranosidase (ABF) activity was quantified in FOR-infected wild-type (Col-0), *allene oxide synthase* (*aos*), *coi1*, *ethylene-insensitive 2* (*ein2*) and *coi1 ein2* or mock-infected plants ($n = 3$) at 10 days post-infection (dpi). Error bars are the confidence intervals of the means ($\alpha = 0.05$). (b) Foliar symptoms, such as stunting, epinasty and darkening (anthocyanin accumulation) of leaves, in FOR-infected wild-type (bottom row) were suppressed in infected *coi1* (top row). Note wilted leaves (at arrows) on *coi1* in watered soil.

homozygotes indicated that disease was sensitive to the gene dosage of *COI1*. Furthermore, X-Ara staining of the vascular cylinder was extensive in roots of *rfo1*, whereas staining at the root tips of *rfo1 coi1* rarely extended into the differentiated vascular cylinder (Fig. 4b).

COI1 promotes symptoms in FOR-infected plants.

Neither host synthesis nor perception of jasmonate was critical for infection by a third *F. oxysporum* pathogen, FOR. The root system and, specifically, the vascular cylinder in FOR-infected *aos*, *coi1* and the wild-type were similarly stained by X-Ara, and similar amounts of *Fusarium*-derived ABF activity were measured in the roots of *aos*, *coi1* and the wild-type at 10 dpi (Fig. 5a). Moreover, a dose of FOR that was lethal to the wild-type was also lethal to *aos* and *coi1*, although death of *coi1* could be delayed by a few days in comparison with *aos* and the wild-type.

Nevertheless, the appearances of FOR-infected *coi1* and the wild-type were strikingly different (Fig. 5b). Symptoms such as stunted growth of rosette leaves, epinastic growth of petioles and darker (anthocyanin) pigmentation, which were observed in the

wild-type, were largely suppressed in *coi1*, even in plants on the verge of death (see *coi1* plant in the centre of Fig. 5b). Often only wilting of leaves (at arrows in Fig. 5b) preceded the sudden decline and death of *coi1* plants (see *coi1* on the right of Fig. 5b). In contrast, wilt disease in FOR-infected *aos* and the wild-type was indistinguishable in all respects, and so the promotion of wilt symptoms by *COI1* could not be a response to endogenously produced jasmonates.

Fusarium oxysporum produces jasmonates

Because host biosynthesis of jasmonates was unnecessary for the contributions of *COI1*, the production of jasmonate activity by pathogenic *Fusarium* strains, including the wheat head blight *F. graminearum* and five *F. oxysporum* f. spp., FOC, FOM, FOR, *F. oxysporum* f. sp. *lycopersici* (FOL) and *F. oxysporum* f. sp. *tulipae* (FOT), was tested. Previous reports have shown that the culture filtrate of FOM contains jasmonate activity that induces the expression of the jasmonate-regulated gene *THI2.1* and the reporter gene *THI2.1*p:*uidA*, in which the *THI2.1* promoter sequence is fused to the *Escherichia coli β-glucuronidase* (*GUS*) gene (Epple *et al*., 1998; Hilpert *et al*., 2001; Vignutelli *et al*., 1998). The previously described jasmonate-induced expression of GUS activity by *THI2.1*p:*uidA* was confirmed using the indigogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-Gluc) (Fig. S1, see Supporting Information) (Bohlmann *et al*., 1998).

Culture filtrates of some, but not all, pathogenic *Fusarium* strains were capable of inducing *THI2.1*p:*uidA* expression. Transgenic seedlings that were wetted with filtrates from *Fusarium* cultures of FOC, FOM or FOT were stained blue by X-Gluc, whereas no staining was evident after treatment with filtrates of FOR, FOL and *F. graminearum* (Fig. 6). Because no GUS activity was evident in filtrate-treated wild-type or *aos* without the transgene (Fig. 6), the expression of *THI2.1*p:*uidA* and not the filtrates was the source of GUS activity. Because GUS activity was similarly induced in transgenic wild-type and *aos* (Fig. 6), filtrates and not endogenous biosynthesis were the source of the jasmonate hormone activity.

Jasmonates in *Fusarium* culture filtrates

The accumulation of specific jasmonates, jasmonic acid (JA) and JA-Ile/Leu, was measured in the culture filtrates of *Fusarium* strains. Jasmonates were extracted from filtrates of 3-week-old cultures, separated by high-performance liquid chromatography (HPLC) and detected by electrospray ionization coupled to multiple reaction monitoring (MRM). A single peak for JA that co-eluted with the JA standard and deuterated-JA was detected. JA was reproducibly present in cultures of FOC, FOM, FOR and FOT (in Table 1), but absent (<4.8 nM) in cultures of FOL and *F. graminearum*. When amounts of JA were quantified, much less JA was measured in the cultures of FOC and FOR than FOM and

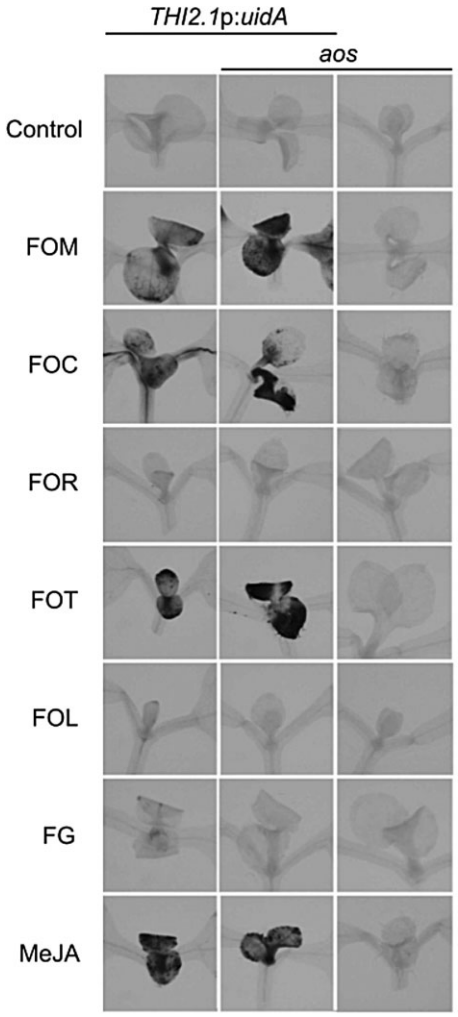

Fig. 6 *Fusarium* culture filtrates induce *THI2.1*p:*uidA*. *THI2.1p*:*uidA* is induced by jasmonates in meristems and the first true leaves of young seedlings and expresses β-glucuronidase (GUS), which hydrolyses the indigogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-Gluc) (Vignutelli *et al*., 1998). GUS activity in seedlings of *THI2.1*p:*uidA* (left), *allene oxide synthase* (*aos*) *THI2.1*p:*uidA* (middle) and *aos* (right) is stained blue by X-Gluc 1 day after treatment with culture filtrates of *Fusarium* strains, *F. oxysporum* f. sp. *matthioli* (FOM) race 2, *F. oxysporum* f. sp. *conglutinans* (FOC) race 1, *F. oxysporum* f. sp. *raphani* (FOR), *F. oxysporum* f. sp. *tulipae* (FOT), *F. oxysporum* f. sp. *lycopersici* (FOL) race 2 and *F. graminearum* (FG) or methyl jasmonate (MeJA). Results with FOL race 2 and race 3 (not shown) were similar.

FOT (in Table 1), and the most JA (200 nM) accumulated in cultures of FOT.

In the *m/z* 322 → 130 chromatograms, two closely eluting peaks corresponding to the natural stereoisomers of JA-Ile and JA-Leu were present in the same samples. JA-Ile and JA-Leu standards and ^{13}C-labelled JA-Ile internal control each eluted as two clearly separated peaks corresponding to alternative stereoisomers, one of which was the natural stereoisomer

Table 1 JA, JA-Ile and JA-Leu in axenic cultures of pathogenic *Fusarium*.

Fusarium pathogen	[JA]*	[JA-Ile]	[JA-Leu]
F. oxysporum f. sp. *matthioli* (FOM)	120 ± 29†	740 ± 160	410 ± 200
F. oxysporum f. sp. *conglutinans* (FOC)	10 ± 3.4	120 ± 27	74 ± 59
F. oxysporum f. sp. *raphani* (FOR)	8.7 ± 3.8	nd¶	nd
F. oxysporum f. sp. *tulipae* (FOT)	200 ± 77	6500 ± 1000	5800 ± 3400
F. oxysporum f. sp. *lycopersici* (FOL)‡	nd§	nd	nd
F. graminearum (FG)	nd	nd	nd

JA, jasmonic acid; JA-Ile, isoleucine-conjugated jasmonate; JA-Leu, leucine-conjugated jasmonate.
*Concentration in nanomoles (nM).
†The range (±) is the standard error of the mean ($n = 3$; $\alpha = 0.05$).
‡Similar results were obtained with FOL race 2 and race 3.
§Not detected, <4.8 nM.
¶Not detected, <6.2 nM.

(Fonseca *et al*., 2009). The early peak (at 40.8 min) in *Fusarium* extracts co-eluted with the later eluting peak in the JA-Ile standard and internal control, whereas the later peak (at 41.5 min) in *Fusarium* extracts co-eluted with the later eluting peak in the JA-Leu standard. The order of reverse phase separation of the stereoisomers of these compounds was the same as that reported by Fonseca *et al*. (2009). Both JA-Ile and JA-Leu were detected and measured in all strains that produced JA, with the exception of FOR, and neither amino acid conjugate was detected in FOR, FOL and *F. graminearum*. The molar amount of JA-Ile/Leu in extracts was consistently higher (from 3- to 32-fold more in Table 1) than JA, and the highest JA-Ile concentration was in FOT cultures (6.5 μM).

Infection of other hormone-related mutants

Other hormones may antagonize or reinforce the host's response to jasmonates. For example, wilt disease is more severe in *sid2*, which fails to accumulate SA (Diener and Ausubel, 2005; Nawrath and Métraux, 1999). The interaction of *sid2* and *coi1* in FOC-infected plants was scrutinized in the nine genotypes produced by the selfed dihybrid *COI1*/*coi1 SID2*/*sid2* (Fig. 7a). *COI1* again exhibited incomplete dominance, and the rosette radius of heterozygotes was intermediate to the radii of the wild-type and mutant. Among progeny that perceived jasmonates (*COI1*/–), *sid2* enhanced wilt disease, including a reduced radius. However, *sid2* failed to suppress the resistance of the *coi1 sid2* double mutant that was insensitive to jasmonates. In FOR-infected plants, in which *COI1* has no effect on root infection, *sid2* had no effect on wilt disease (Fig. 7b), and reduced expression of foliar symptoms in FOR-infected *coi1* was independent of *SID2*, as symptoms in *coi1 sid2* were less severe than those in *COI1*/– *sid2* (Fig. 7c).

Some responses to jasmonates also depend on ethylene perception (Lorenzo *et al*., 2003), and Trusov *et al*. (2009) reported that wilt disease is reduced in FOC-infected *ein2*, which is insensitive to ethylene (Alonso *et al*., 1999). We observed less yellowing and premature senescence of rosette leaves in FOC- and FOR-infected *ein2* in comparison with the wild-type; however, other symptoms and, ultimately, death were not appreciably affected by *ein2*. Infection by FOC and FOR produced similar X-Ara staining in roots of *ein2* and the wild-type at 10 dpi. Furthermore, *Fusarium*-derived ABF activity was comparable in FOC- and FOR-infected roots of the wild-type and *ein2*, or *coi1* and *ein2 coi1* (Figs 2g and 5a, respectively).

Similar to *coi1*, the abscisic acid-deficient *aba1* and *aba2*, which have abnormally small and dark green rosettes, exhibit less stunting, epinastic growth, yellowing and senescence of rosette leaves (Anderson *et al*., 2004; Trusov *et al*., 2009). However, X-Ara staining was not appreciably different in FOC-infected roots of *aba2* and the wild-type (Fig. 8a); indeed, the root system of *aba2* had more *Fusarium*-derived ABF activity than that of the wild-type (Fig. 8b). The smaller root mass of *aba2* presumably accounted for this modest difference, as *F. oxysporum* could colonize more of the smaller root system of *aba2*. In any case, *aba2* did not enhance immunity to root infection.

Jasmonate perception in Fusarium wilt of tomato

A role for the perception of jasmonates in Fusarium wilt of tomato was also tested. *JASMONIC ACID-INSENSITIVE 1* (*JAI1*) is the tomato orthologue of *Arabidopsis COI1* (Li *et al*., 2004). The gross appearance of mock-infected *jai1* and the wild-type was the same (Fig. 9a). In addition, FOL-infected *JAI1* and *jai1* plants expressed symptoms, such as epinastic growth of petioles and premature senescence of older leaves, with similar severity at 21 dpi (Fig. 9b,c), and yielded similar distributions of disease index scores at 35 dpi (Fig. 9d).

DISCUSSION

Our finding that *COI1* primarily promoted infection by FOC contradicts the observations of Thatcher *et al*. (2009), who concluded that there is 'no difference in the degree of fungal colonization of the wild-type and *coi1* plants until later stages of infection, when host necrosis [is] well developed'. Instead, Thatcher *et al*. (2009) reported that *COI1* primarily promotes the expression of foliar

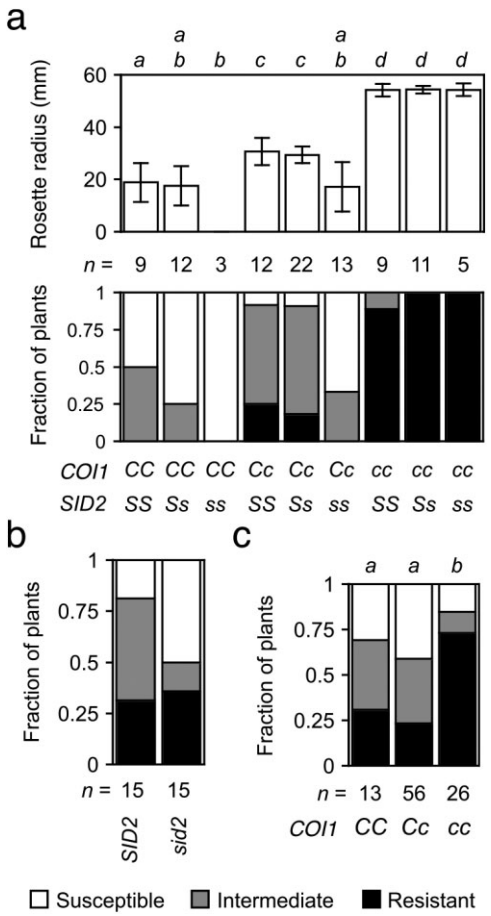

Fig. 7 *CORONATINE-INSENSITIVE 1* (*COI1*)-dependent susceptibility is independent of salicylic acid (SA). Wilt symptoms at 20 days post-infection (dpi). (a) *Fusarium oxysporum* f. sp. *conglutinans* (FOC)-infected offspring of the dihybrid *COI1*/*coi1 SALICYLIC ACID INDUCTION-DEFICIENT 2* (*SID2*)/*sid2* were *COI1*/*COI1* (*CC*), *COI1*/*coi1* (*Cc*) or *coi1*/*coi1* (*cc*) and *SID2*/*SID2* (*SS*), *SID2*/*sid2* (*Ss*) or *sid2*/*sid2* (*ss*). Top graph: mean radius of rosettes of *n* plants in millimetres. Error bars are the confidence intervals of the means (α = 0.05). Means with the same italicized letter (above) have no significant difference, according to Student's *t*-test ($P > 0.01$). Bottom graph of (a), and (b) and (c): fraction of *n* plants that were resistant (health index, HI > 3) or susceptible (HI < 2) or had intermediate resistance ($2 \leq HI \leq 3$). No symptoms were apparent in mock-infected plants (not shown). (b) Wild-type (*SID2*) and *sid2* were infected with *F. oxysporum* f. sp. *raphani* (FOR). (c) FOR-infected offspring of the monohybrid double mutant *sid2 COI1*/*coi1* were *COI1*/*COI1* (*CC*), *COI1*/*coi1* (*Cc*) or *coi1*/*coi1* (*cc*). Similar HI scores, according to Mann–Whitney *U*-test ($P > 0.01$), have the same italicized letter above.

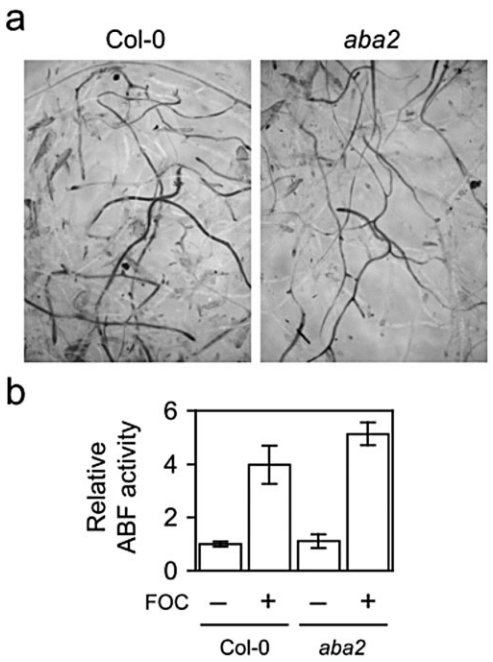

Fig. 8 *Fusarium oxysporum* f. sp. *conglutinans* (FOC) infection in *abscisic acid-deficient 2* (*aba2*). (a) At 7 days post-infection (dpi), 5-bromo-4-chloro-3-indoxyl-α-L-arabinofuranoside (X-Ara) staining was similar in roots of FOC-infected wild-type (Col-0) and *aba2*. (b) Relative arabinofuranosidase (ABF) activity in roots of FOC-infected (+; $n = 5$) or mock-infected (–; $n = 3$) plants was quantified using nitrophenyl-α-L-arabinofuranoside (NP-Ara). Fresh weights (± standard deviations) of whole-root systems of wild-type and *aba2* were 28 (± 8) and 15 (± 3) mg, respectively. Comparison of ABF activity in infected wild-type and *aba2* using the Student's *t*-test gives two-tailed $P = 0.026$.

symptoms and, specifically, senescence. Their conclusion rests on the detection of similar amounts of *F. oxysporum* DNA in *coi1* and the wild-type early in infection. However, Thatcher *et al*. (2009) quantified *F. oxysporum* DNA in shoots where little if any *F. oxysporum* would be present until late in the infection cycle. Indeed, when infected plants were stained with X-Ara, *F. oxysporum* infection was detected early in roots, late in stems and never in leaves (S. J. Cole, personal observation). The absence of *F. oxysporum* in shoots would explain why Thatcher *et al*. (2009) detected no early difference in fungal DNA in shoots of *coi1* and the wild-type.

Our finding that endogenous jasmonates were inconsequential to host resistance is remarkable because the accumulation of jasmonates is a common response to wounding and infection in plants (Koo and Howe, 2009), and previous investigations have clearly shown the induction of jasmonate-regulated, defence-related transcription in FOC-infected *A. thaliana* (Anderson *et al*., 2004; Kidd *et al*., 2011). Considering such a precedent, Thatcher *et al*. (2009) posited that *COI1* also mediates defence responses that restrict FOC growth. However, the absence of endogenous jasmonates (in *aos*) had no discernible effect on infection or wilt disease in general.

The effect of SA was dependent on *COI1*. When infected with FOR, *COI1* was unnecessary for root infection, and a deficiency in SA had no effect on wilt disease. When infected with FOC, which was dependent on *COI1* for root infection, *sid2* exhibited more severe disease; however, this enhanced susceptibility was dependent on *COI1* because the *sid2 coi1* double mutant appeared as resistant as *SID2*/– *coi1*. Previous reports have shown that the

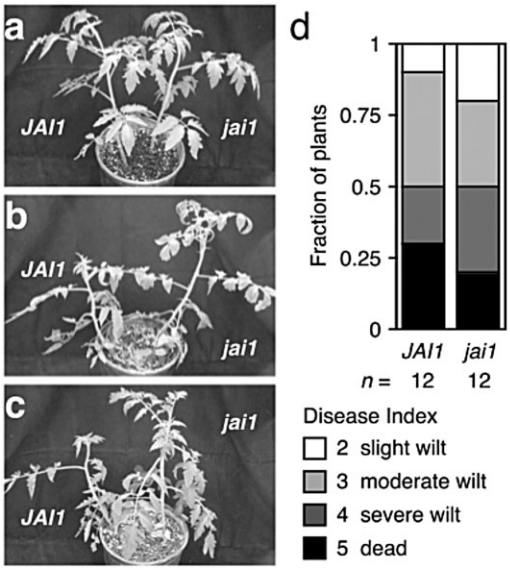

Fig. 9 Fusarium wilt in tomato *jasmonic acid-insensitive 1* (*jai1*). Representative wild-type (*JAI1*) and jasmonate-insensitive *jai1* plants that were mock infected (a) or infected with *Fusarium oxysporum* f. sp. *lycopersici* (FOL) race 3 (b, c) are shown at 21 days post-infection (dpi). Lower leaves of FOL-infected plants exhibit epinasty and premature senescence when compared with uninfected plants. (d) At 35 dpi, symptoms of FOL-infected *JAI1* ($n = 12$) and *jai1* ($n = 12$) plants were scored using a disease index: healthy (0), slightly wilted (1), moderately wilted (2), severely wilted (4) and dead (5). Mock-infected *JAI1* ($n = 6$) and *jai1* ($n = 6$) plants exhibited no wilt symptoms.

enhanced susceptibility of SA-deficient genotypes *nahG* or *eds5* is also suppressed by *coi1* (Thatcher *et al*., 2009; Trusov *et al*., 2009). Because JA signalling may be exaggerated in SA-deficient mutants (Kunkel and Brooks, 2002), it is likely that the enhanced susceptibility of *sid2* is an abnormal response to *Fusarium*-derived jasmonates, and that normal SA signalling is inconsequential to wilt disease in the wild-type.

Jasmonates, which were detected by Miersch *et al*. (1999a) in culture filtrates of FOM, have not been identified previously as products of other f. spp. Miersch *et al*. (1999a) detected 22 jasmonate-related compounds, including JA and JA-Ile, in filtrates of a FOM isolate. However, in contrast with Miersch *et al*. (1999a), who reported the accumulation of (20-fold) more JA than JA-Ile, we detected (six-fold) less JA than JA-Ile. Because more JA-Ile than JA accumulated in cultures of isolates from three f. spp., the difference in the major products in the two studies is probably a consequence of growth conditions rather than differences in the FOM strains used here and in Miersch *et al*. (1999a).

JA-Ile is the only reported amino acid conjugate of JA in fungal cultures, and so the detection of comparable amounts of JA-Ile and JA-Leu was unanticipated (Cross and Webster, 1970; Miersch *et al*., 1999a). JA-Leu also normally accumulates in plants (Kramell *et al*., 1995); however, JA-Leu has equivalent or weaker hormone activity than JA-Ile when the two are compared in physiological tests, and so the interactions of JA-Ile and JA-Leu with COI1 might yield distinct hormone responses (Katsir *et al*., 2008; Miersch *et al*., 1999b; Tamogami *et al*., 1997).

A role for *COI1*/*JAI1* correlated with the *in vitro* production of JA and JA-Ile/Leu. Similarly, *COI1* is critical to the pathogenesis of *Pseudomonas syringae* pathovars that are capable of producing the polyketide effector coronatine, which mimics JA-Ile (Bender *et al*., 1999; Feys *et al*., 1994), and this suggests that jasmonates are effectors for some, but not all, *F. oxysporum* pathogens. For instance, FOL, which produced no detectable JA or JA-Ile/Leu, epitomized those f. spp. for which jasmonates are not critical effectors. However, f. spp. that produced JA, including FOR, FOC and FOM, relied on *COI1* for the expression of foliar symptoms. Although the accumulation of JA in filtrates of FOR was too low to induce *THI2.1*p:*uidA*, FOR-derived jasmonate *in planta* was crucial for the expression of foliar symptoms, but inconsequential for root infection. Furthermore, f. spp. that produced substantial quantities of JA-Ile/Leu, namely FOC and FOM, were further dependent on *COI1* for root infection and, ultimately, the death of the host. The separate associations of JA-Ile/Leu with root infection and JA with foliar symptoms are intriguing, and suggest that different jasmonates might function as distinct effectors and target different host processes through the same COI1 receptor.

Fungal-derived jasmonates are also likely effectors in hosts other than *A. thaliana* and commonplace in fungal pathogenesis. The three jasmonate-producing f. spp., FOC, FOM and FOR, represent three distinct phylogenetic lineages in the *F. oxysporum* species complex and are virulent to related dicotyledons in the mustard family (O'Donnell *et al*., 2009); in contrast, the jasmonate-producing f. sp. FOT is virulent to tulip (*Tulipa gesneriana*), a monocotyledon. A characteristic of FOT-infected tulip bulbs is gum exudation, or gummosis, and methyl jasmonate (MeJA) treatment of tulip bulbs phenocopies the gummosis associated with Fusarium bulb rot (Saniewski *et al*., 2004, 2006). JA was first identified in culture filtrates of *Lasiodiplodia theobromae* (Miersch *et al*., 1987), which is responsible for dieback diseases in peach and other fruit trees (Saniewski *et al*., 1998, 2006). A common symptom of *Lasiodiplodia*-instigated diseases is gummosis, which is copied by MeJA treatment of peach stems (Saniewski *et al*., 1998).

How *Fusarium*-derived jasmonates promote infection in roots remains unclear. Possibly, jasmonates promote gummosis (as cited above) and the blockage of xylem vessels. Histological analysis has long associated infection by wilt fungi with pectin-like gum deposition in xylem vessels (Beckman, 1987; Mace *et al*., 1981). Deposition of polysaccharide in nutrient-poor xylem sap may be beneficial to the growth of *F. oxysporum*. Alternatively, jasmonate signalling could be suppressing the host's defence response or the cell wall damage response (Denness *et al*., 2011; Millet *et al*., 2010).

Differences in the susceptibility of cultivated varieties usually correlate with the extent of infection in roots, presumably because the differences reflect variations in innate immunity (Beckman and Roberts, 1995; Smith and Walker, 1930). However, when the relative susceptibilities of hormone-related mutants are compared, the severity of symptoms may not correlate with the extent of infection, because hormones may mediate the development of symptoms as well as the response to infection (Grant and Jones, 2009; Seilaniantz *et al*., 2011). Loss of abscisic acid biosynthesis (*aba2*) and insensitivity to ethylene (*ein2*) compromised the expression of foliar symptoms without curtailing infection, just as the auxin-related mutant *tir3* suppresses the development of foliar symptoms without suppressing the infection of roots (Diener, 2012; Kidd *et al*., 2011).

EXPERIMENTAL PROCEDURES

Standards

(±) JA (Research Products International Corp., Mount Prospect, IL, USA) and (±) $^{2}H_4$ JA (C/D/N Isotopes Inc., Pointe-Claire, QC, Canada) were purchased. JA-Leu, JA-Ile and $^{13}C_6$-JA-Ile were generously provided by Paul Staswick (University of Nebraska, Lincoln, NE, USA).

Plant and *Fusarium* stocks

Published sources or the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA) provided seeds: *sid2-2* (Wildermuth *et al*., 2001), *rfo1* (Diener and Ausubel, 2005), *coi1-1* (Feys *et al*., 1994), *aos* (SALK_017756C), *ein2-1* (CS3071), *aba2-1* (CS156), *THI2.1*p:*uidA* (Vignutelli *et al*., 1998) and *JAI1*/*jai1* in the cultivar Castlemart (Li *et al*., 2004). Seeds were grown on Jiffy 7 peat pellets (Growers Solution, Cookeville, TN, USA) under cool white fluorescent lighting with moderate intensity for a 12-h day length at 22 °C before infection and 28 °C after infection. Seeds were axenically grown on Plant Nutrient (PN) agar plates (Diener and Ausubel, 2005) with or without 0.5% sucrose. FOC race 1 (strain 777), FOR (strain 815) and FOM race 2 (strain 726) originate from P. H. Williams through H. C. Kistler (Bosland and Williams, 1987; Kistler *et al*., 1991). Kerry O'Donnell (USDA/ARS, Peoria, IL, USA) provided FOL race 2 (strain 4287) and FOT (NRRL 26954), and H. C. Kistler provided FOL race 3 (MN-25) and *F. graminearum* (Gz3639).

Infection assays

Fusarium strains were grown on Czapek–Dox medium (Oxoid Ltd., Basingstoke, Hampshire, UK), and conidial suspensions were harvested from 5-day-old shaken cultures and washed three times with water. Two- to three-week-old *Arabidopsis* plants were irrigated with 10^6–10^8 conidia/mL or mock-infected with water. Disease severity was scored using a health index (HI), described as a disease index in Diener and Ausubel (2005). Briefly, HI scores ranged from 0 to 5 in increments of 0.5, and indicated the appearance of foliar symptoms: HI = 0 for dead plants and HI = 5 for unaffected plants. Rosettes of plants with intermediate scores exhibited stunting (HI ≤ 4), perivascular yellowing (HI ≤ 3), senescence (HI ≤ 2) and necrosis (HI ≤ 1). ABF activity in infected roots was visualized and quantified as described by Diener (2012). The rosette radius was the mean length of the midrib of three leaves emerging from the stem at roughly 0°, 120° and 240°. Two-week-old tomato seedlings in peat pellets were irrigated with 10^6 FOL conidia/mL (or water for mock infection) and transplanted to soil pots. In each pot, *jai1*/*jai1* and *JAI1*/*JAI1* plants were paired: 12 pots were FOL-infected plants and six were mock-infected plants. Tomato plants were scored using a disease index described in Fig. 9.

Genotyping

Plants were genotyped as described in Diener and Ausubel (2005). Marker DNA of *aos* or *AOS* was PCR amplified using primers 5'-CTAACCGG AGGCTACCGTATC-3' and either 5'-GCGTGGACCGCTTGCTGCAACT-3' (LBb1) or 5'-AACAACAAAATCCTTACCGGC-3', respectively. For *coi1-1*, a 612-bp product was PCR amplified using primers 5'-TCGACCGGGAAGAA AGGATTA-3' and 5'-ACACAGTTTGTGGAAACCCCA-3', and *Xcm*I digested wild-type but not *coi1-1* DNA. Primer sequences for genotyping *jai1*, *rfo1* and *sid2-2* are described in Li *et al*. (2004), Diener and Ausubel (2005) and Wildermuth *et al*. (2001), respectively.

Quantification of jasmonates

Still cultures in 100 mL of Czapek–Dox medium were kept in darkness at room temperature for 3 weeks, centrifuged, filtered and stored at −80 °C. Internal standards $^{2}H_4$ JA (934 pmol) and $^{13}C_6$ JA-Ile (978 pmol) were added to 1-mL aliquots of thawed culture filtrates, acidified to pH 2 with 0.1 M HCl and extracted three times with 1 mL of ethyl acetate. Pooled organic phases were dried under vacuum. Extracts were resuspended in 10 μL methanol/110 μL water, and 50-μL aliquots were separated by reverse-phase HPLC (C18 Asentis Express, 15 cm × 2.1 mm × 2.7 μm, Supelco Analytics, Sigma-Aldrich, St. Louis, MO, USA), equilibrated in 80% buffer A (water containing 10 mM HCO_2NH_4 and 0.1 mM cetyltrimethylammonium bromide)/20% buffer B (CH_3CN/aqueous 10 mM HCO_2NH_4, 90/10, v/v), and eluted (200 μL/min) with an increasing concentration of buffer B (min/% B; 0/20, 2/20, 52/80, 55/20, 60/20). The effluent from the column was passed to a flow splitter, and a proportion of the flow (about 20%) was passed to an Ionspray™ source connected to a triple quadrupole mass spectrometer (Perkin-Elmer Sciex, ABI III^{+}, Thornhill, Ontario, Canada) operating in the negative ion tandem mass spectrometric MRM mode, in which the intensities of specific parent → fragment ion transitions (JA *m/z* 209 → 59, $^{2}H_4$ JA *m/z* 213 → 61, JA-Ile/JA-Leu *m/z* 322 → 130, $^{13}C_6$ JA-Ile *m/z* 328 → 136) were monitored under previously optimized conditions (orifice −55 V; argon collision gas at instrumental collision gas thickness setting of 180) using instrument manufacturer-supplied software (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) for data acquisition (Tune version 2.5 and RAD version 2.6) and analysis (MacSpec version 3.3 and BioMultiView version 1.3.1).

Two closely eluting peaks in the sample chromatograms with the same transition as JA-Ile/Leu (*m/z* 322 → 130) were tentatively identified using JA-Ile and JA-Leu standards and co-chromatography experiments. Under the prescribed chromatographic conditions, the stereoisomers in the JA-Ile standard eluted as two equally intense peaks at 39.2 and 40.8 min, whereas the stereoisomers in the JA-Leu standard eluted as two equally intense peaks at 40.0 and 41.5 min. The four peaks of JA-Ile/Leu exhibited

near-baseline separation when equal amounts of JA-Ile and JA-Leu standards were injected. The identity of JA-Ile/Leu peaks in sample extracts was confirmed by co-chromatography experiments by the addition of authentic JA-Leu or JA-Ile to FOT fungal extract. In these experiments, the peaks from the sample co-chromatographed with the early (40.8 min) or later (41.5 min) peak, respectively.

*THI2.1*p:*uidA* bioassay

Seedlings grown for 7 days on PN agar were vacuum infiltrated with culture filtrates, 100 μM MeJA in 0.02% acetone, 100 μM $AgNO_3$ or mock treated with corresponding solvent. Wounded seedlings were cut with a scalpel. GUS activity was detected by staining overnight with 0.2 mg/mL X-Gluc (Gold Biotechnologies, St. Louis, MO, USA) in 0.1 M sodium phosphate buffer, pH 7.0, at 37 °C. Seedlings were destained in ethanol–water, rehydrated and photographed.

Grafting

Grafts were essentially performed as described in Turnbull *et al*. (2002). MeJA-resistant *coi1* progeny of *COI1*/*coi1* were transferred to PN agar plates after selection on plates with 30 μM MeJA. After joining scion and rootstocks, grafts were left on PN agar plates for 10 days before transplanting to peat pellets. Four-week-old grafted plants were infected 2 weeks after transplanting.

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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 Induction of *THI2.1p*:*uidA* by methyl jasmonate, silver nitrate and wounding.