Running Title: Vascular disease-promoting function of COI1

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The vascular pathogen *Verticillium longisporum* requires a jasmonic acid-independent COI1 function in roots to elicit disease symptoms in *Arabidopsis thaliana* shoots

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ABSTRACT

Verticillium longisporum is a soil-borne vascular pathogen that causes reduced shoot growth and early senescence in Arabidopsis thaliana. Here we report that these disease symptoms are less pronounced in plants, which lack the receptor of the plant defense hormone jasmonic acid (JA), CORONATINE INSENSITIVE 1 (COI1). Initial colonization of the roots was comparable in wild-type and coi1 plants and fungal DNA accumulated to almost similar levels in petioles of wild-type and coi1 plants at 10 days post infection. Completion of the fungal life cycle was impaired in coi1 as indicated by the reduced number of plants with microsclerotia, which are detected on dead plant material at late stages of the disease. Contrary to the expectation that the hormone receptor mutant coi1 should display the same phenotype as the corresponding hormone biosynthesis mutant dde2, dde2 plants developed wild-type-like disease symptoms. Marker genes of the JA and the JA/ethylene defense pathway were induced in petioles of wild-type but not in petioles of dde2 plants indicating that fungal compounds that would activate the known COI1-dependent signal transduction chain were absent. Grafting experiments revealed that the susceptibility-enhancing COI1 function acts in the roots. Moreover, we show that the coi1-mediated tolerance is not due to the hyper-activation of the salicylic acid pathway. Together, our results have unravelled a novel COI1 function in the roots, which acts independently from JA-Ile or any JA-Ile mimic. This COI1 activity is required for a yet unknown root-to-shoot signaling process which enables V. longisporum to elicit disease symptoms in Arabidopsis.
INTRODUCTION

Microbial plant pathogens have evolved different colonization mechanisms to gain access to plant fixed carbon sources: Necrotrophic pathogens kill plant cells and feed on the remains, whereas biotrophic pathogens establish specific structures to retrieve nutrients from living cells. Hemibiotrophic pathogens first colonize their hosts as biotrophs before killing them during the subsequent necrotrophic phase. Vascular pathogens like *Fusarium oxysporum* or *Verticillium spec.*, which persist during the first part of their life cycle in the xylem before causing severe tissue damage, can thus be regarded as hemibiotrophs (Thatcher et al., 2009; Klosterman et al., 2011).

Upon recognition of pathogen or damage associated molecular patterns (PAMPs/DAMPs) by membrane bound plant receptor kinases, plant defense hormones are synthesized (Nurnberger et al., 2004; Chisholm et al., 2006; Pieterse et al., 2009). These orchestrate massive transcriptional reprogramming, finally restricting pathogen proliferation and disease development. Though being a simplified concept, it is generally recognized that defense responses mediated by the phytohormone salicylic acid (SA) are effective against biotrophic pathogens, whereas jasmonates (JAs) and ethylene (ET) act as crucial signaling molecules that activate responses counteracting necrotrophs (Glazebrook, 2005).

Analyses of these defense pathways with respect to the hemibiotrophic vascular pathogen *F. oxysporum* had unraveled that exogenous SA and ectopic activation of the JA pathway provides increased resistance in the model plant *Arabidopsis thaliana* (Edgar et al., 2006). The latter result has been obtained by overexpression of transcription factors ERF1 (ETHYLENE RESPONSE FACTOR 1) or AtERF2 (Berrocal-Lobo and Molina, 2004; McGrath et al., 2005) which are normally induced at the transcriptional level after recognition of increased JA-isoleucine (JA-Ile) levels by the JA receptor CORONATINE INSENSITIVE 1 (COI1 (Berrocal-Lobo and Molina, 2004)).
However, coi1 mutant plants were more tolerant (Thatcher et al., 2009), which contradicts the concept that activation of COI1 by JA leads to activation of ERF1 and other transcription factors finally resulting in resistance. It was concluded that COI1 can be “hi-jacked” by the fungus to induce senescence which in turn facilitates the disease. 

Since the JA-Ile biosynthesis mutant dde2-2 was as susceptible as wild-type plants, it was hypothesized that fungal oxylipins activate COI1 leading on the one hand to defense responses but also to enhanced susceptibility as a consequence of pre-mature senescence.

In order to further explore the exceptional role of COI1 for vascular diseases, we analyzed the function of COI1 and JAs in the interaction between Arabidopsis and Verticillium longisporum. Verticillium longisporum is a vascular pathogen that enters its hosts through the roots (Fradin and Thomma, 2006; Eynck et al., 2007). After having reached the xylem vessels it spreads systemically by either hyphal growth or through the formation of conidia that are transported to the shoot with the transpiration stream. In contrast to Fusarium wilt diseases, V. longisporum does not affect the water status thus causing no wilt symptoms (Floerl et al., 2008; Floerl et al., 2010). The life cycle is completed after formation of thick-walled melanized microsclerotia which can survive for more than a decade in the soil. V. longisporum infects predominantly crucifers and belongs to the most important diseases of Brassicaceae, in particular of oilseed rape (Zeise and von Tiedemann, 2002). Due to the growing demand for oil crops as sources for nutritional oils and bio-fuels, novel strategies to limit the spread of V. longisporum are needed. Since these can be based on the molecular mechanisms of defense responses, several groups have started to investigate the interaction between V. longisporum and the model plant Arabidopsis thaliana (Steventon et al., 2001; Veronese et al., 2003; Johansson et al., 2006; Floerl et al., 2010; Floerl et al., 2012).
Here we show that JAs and metabolites of the SA pathway are synthesized after infection with *V. longisporum* and that respective marker genes are activated. However, the corresponding hormone biosynthesis mutants did not show major differences in disease susceptibility. Importantly, *V. longisporum* requires COI1 in the roots but not any JAs or JA mimics to enhance susceptibility in the shoot.

**RESULTS**

*Verticillium longisporum* Completes its Life Cycle Less Efficiently on *coi1* than on JA Biosynthesis Mutants

The importance of the plant defense hormone JA and its receptor COI1 for the response of *Arabidopsis thaliana* to *Verticillium longisporum* was assessed by infection of the JA biosynthesis mutant *dde2-2* (Park *et al.*, 2002) and the receptor mutant *coi1-t* (Mosblech *et al.*, 2011). Three-week old plants of each genotype were up-rooted and the roots were incubated in a solution containing $10^6$ *V. longisporum* spores per ml. Plants were subsequently transferred back to soil. At 15 days post inoculation (dpi), leaf area was reduced down to 50 to 60% in wild-type and the JA biosynthesis mutant *dde2-2* but not in the JA receptor mutant *coi1-t* (Figs. 1A and B). Since it is unusual that a hormone receptor mutant shows a different phenotype than the corresponding biosynthesis mutant, the well characterized *coi1-1* mutant (Xie *et al.*, 1998) and the two independent JA biosynthesis mutants *fad3-2 fad7-2 fad8* (McConn and Browse, 1996) and *acx1/5* (Schilmiller *et al.*, 2007) were analyzed. Again, shoot growth of the receptor mutant was less severely affected than shoot growth of the biosynthesis mutants (Fig. S1). At 22 dpi, senescence-like symptoms became apparent in infected wild-type and *dde2-2* plants. In contrast to the natural senescence, which starts at leaf tips, yellowing started at the petioles (Fig. 1C). These symptoms were much less pronounced in *coi1-*
At 35 dpi, most of the wild-type and dde2-2 plants were dead while most of the coi1-t plants had remained green (Fig. 1D). Appearance of microsclerotia correlated with this disease phenotype resulting in 73% wild-type, 79% dde2-2 and 27% infected coi1-t plants carrying microsclerotia primarily around the petioles (Figs 1E and 1F).

Impaired disease progression in coi1-t was also detected by histological analysis of petioles (Fig. 2). Leaf vascular bundles of uninfected Arabidopsis plants display dorsoventral polarity with xylem cells in the adaxial position above the paired procambium-derived cells and the abaxial phloem. At 10 dpi, the cytosol of cells at the abaxial side became denser in infected wild-type vascular bundles. At 15 dpi, the layer of cells with dense cytosol was interspersed with cells displaying a xylem vessel-like appearance (large lumen, lignified cell walls). Islands of small cells that might be phloem sieve elements and/or companion cells were now found towards the middle of vascular bundle. In addition, the pro-cambium-derived paired cells were not visible any more. Consistent with the less severe disease phenotype, these changes were less pronounced in coi1-t: At 10 dpi, the layer of cells with a denser cytosol was thinner than in the wild-type and at 15 dpi, cells with lignified cell walls were not yet formed at the abaxial side (for pictures of mock-infected coi1-t, see Fig. S2A). Again, the dde2-2 mutant responded like the wild-type in this assay (Fig. S2B).

The coi1-t Mutant is Colonized by *Verticillium longisporum*

Next, we tested how the apparent impaired or delayed disease progression in coi1-t related with fungal biomass. Petioles were harvested for quantification of fungal DNA since the localization of microsclerotia indicated preferential colonization of this tissue (Fig. 1F). At 10 dpi, no significant differences in the amounts of fungal DNA were detected in coi1-t as compared to the wild-type (Fig. 3A). At later time points, fungal proliferation was less efficient in coi1-t as compared to wild-type and dde2-2 plants.
Though not being quantitative in nature, laser scan microscopy indicated entry of GFP-tagged *V. longisporum* (Eynck *et al.*, 2007) into the xylem vessels of *coi1-t* roots (Fig. 3B). Although the differences were not significant, higher levels of fungal DNA were consistently observed in *dde2-2* plants than in wild-type plants supporting the idea that the JA-Ile-mediated defense pathway can restrict fungal growth at later stages of infection whereas a yet unknown COI1-dependent pathway supports fungal proliferation.

**Verticillium longisporum** does not Induce COI1-Dependent Defense Genes in the JA Biosynthesis Mutant *dde2-2*

Since disease development depends on COI1 but not on plant-derived JAs, jasmonate levels were determined in all three genotypes (Fig. 4A). JA increased significantly in infected wild-type plants and was absent in *dde2-2*. In *coi1-t*, JA levels were slightly elevated in mock-infected plants and did not show a significant increase after infection. Like JA, the active hormone JA-Ile was more abundant in wild-type petioles after infection and was absent in the *dde2-2* mutant. The *coi1-t* mutant had increased JA-Ile levels already after mock infection and reacted to the fungus with a further increase. In order to get an estimate on the magnitude of these responses in relation to known JA biosynthesis-inducing treatments, JA and JA-Ile levels were measured in petioles two hours after wounding. Although the increase in JA was clearly higher than after *V. longisporum* infection, the levels of JA-Ile were comparable.

The lack of any biochemically detectable JA or JA-Ile in the infected *dde2-2* mutant suggested that *V. longisporum* cannot synthesize JA. In order to investigate whether *V. longisporum* might produce a yet unknown JA mimic to activate COI1, we determined transcript levels of two marker genes of the JA-Ile-dependent COI1 response in petioles at 15 dpi, namely *VSP2* and *PDF1.2*. Both genes were only induced in wild-
type plants (Fig. 4B) indicating that no fungal-derived JAs or JA mimics that would activate the established COI1-dependent defense genes are effective in *V. longisporum*-infected *dde2-2* plants.

Consistent with the result that similar amounts of JA-Ile were found in wild-type plants after *V. longisporum* infection and wounding, *VSP2* transcript levels were induced to comparable levels under both conditions. In contrast, the JA/ET marker gene *PDF1.2*, which is highly expressed after infection with the foliar pathogen *Botrytis cinerea*, is not efficiently induced in *V. longisporum*-colonized plant tissue, at least at this early time point. The observed increase in ABA (Fig. 4C), which is known to inhibit the JA/ET pathway, might explain the low *PDF1.2* transcript levels (Anderson et al., 2004).

**COI1 in the Roots Determines the Disease Phenotype of the Shoot**

Our findings that JA signaling but not JA biosynthesis is required for full *V. longisporum* disease development is reminiscent of previously published data on the *F. oxysporum*/Arabidopsis interaction (Thatcher et al., 2009). Grafting experiments had revealed that the genotype of the root determines disease symptoms in the shoot. In order to further analyze whether *V. longisporum* and *F. oxysporum* exploit similar mechanism to manipulate their hosts, we generated chimeric plants with either a wild-type shoot grafted on a *coi1-16* root or a *coi1-16* shoot grafted on a wild-type root. In accordance to what has been described for the *F. oxysporum*/Arabidopsis system, a wild-type shoot developed lesser disease symptoms when the root stock was from the *coi1-16* genotype whereas a *coi1-16* shoot showed disease symptoms when the root stock was from wild-type plants (Fig. 5).
The coi1-Mediated Tolerance is not due to Hyper-activation of the SA Pathway

Increased resistance of the coi1 mutant has been detected before in a screen for resistance against the hemibiotrophic pathogen *Pseudomonas syringae* (Kloek et al., 2001). In this interaction, the bacterial JA-Ile mimic coronatine activates COI1 to suppress the SA pathway (Kloek et al., 2001; Laurie-Berry et al., 2006). To analyze, whether a similar scenario would explain the coi1-mediated tolerance towards *V. longisporum*, SA synthesis and SA signaling were analyzed in infected wild-type, dde2-2 and coi1-t plants (Fig. 6). Free SA levels did not increase after infection in wild-type plants and reached similar levels in all three infected genotypes. Lower basal SA levels were detected in dde2-2. In contrast, the SA glucoside (SAG) and the SA-derived metabolite dihydroxybenzoic acid (DHBA) were elevated in all three genotypes after infection (Fig. 6A). Like the relative levels of SAG and DHBA, which showed the highest values in the wild-type followed by intermediate levels in the dde2-2 mutant and even lower levels in coi1-t, PR-1 expression followed the same pattern (Fig. 6B). These results indicate that the tolerant coi1 phenotype is not due to hyper-induction of the SA pathway.

The correlation between the levels of the SA-derived metabolites SAG and DHBA and PR-1 transcripts suggested that one of these SA metabolites might be important for PR-1 transcription. Taking into account that free SA levels were not increased after infection, we aimed to substantiate the result that PR-1 expression was due to the activation of the SA biosynthesis pathway. Indeed, induction of PR-1 was abolished in the biosynthesis mutant sid2-2 (Dewdney et al., 2000; Wildermuth et al., 2001). Consistently, transgenic plants expressing the SA-hydrolyzing bacterial enzyme NahG show reduced levels of PR-1 (Fig. S3A; (Lawton et al., 1995)). These findings correlated with lower amounts SAG and DHBA in sid2-2 and nahG plants (Fig. S3B). The global regulator of the SA-dependent defense response systemic acquired
resistance, NPR1 (Cao et al., 1997), was important for induction of the majority of PR-1 transcript levels. Though the SA pathway was induced to 20% of the levels obtained after the induction of a strong resistance response by *Pseudomonas syringae* pv. *maculicola* ES4326/avrRps4 (Fig. 6B), the leaf area was reduced to similar extents in wild-type, *sid2-2*, *nahG* and *npr1-1* plants (Fig. 6C).

**DISCUSSION**

Many necrotrophic pathogens that cause foliar diseases colonize the leaf tissue either by damaging the epidermis or by entering through stomatal openings or wound sites. In contrast, soil-borne pathogens like e.g. *Fusarium oxysporum f. sp.* and *Verticillium spec.* penetrate the roots and reach the aerial parts through the vascular system. Later, they break out of the vessels and form resting structures like spores, conidia or microsclerotia. Here we show that *V. longisporum* requires COI1 in the root for efficient completion of its life cycle in the shoot. The disease-promoting COI1 activity can operate in the absence of plant-derived oxylipins or fungal-derived JA-Ile mimics.

**COI1 Aggravates the *V. longisporum*-Induced Disease Phenotype**

The JA-Ile receptor mutant *coi1-t* showed less severe disease progression (reduced shoot growth, premature senescence, alterations of the anatomy of the vascular bundle and reduced microsclerotia formation (Figs. 1 and 2)) towards *V. longisporum* than wild-type plants. Reduced symptom development of *coi1-1* has also been described for the *F. oxysporum*/Arabidopsis interaction. In this system, wild-type-like initial colonization of *coi1-1* by *F. oxysporum* was followed by compromised fungal propagation at later stages of the infection. Our results point at a similar scenario (Fig. 3). Colonization of the xylem was observed in *coi1-t* roots and almost the same levels
of fungal DNA were detected in coi1-t petioles at 10 dpi. Differences in fungal biomass between coi1-t and wild-type increased over time leading finally to a higher percentage of wild-type plants with microsclerotia as compared to coi1-t.

In addition to growth inhibition and premature senescence, V. longisporum-induced alterations of the vascular bundles were different in wild-type and coi1-t plants. As previously observed for V. albo-astrum-infected hop (Talboys, 1958), Arabidopsis forms additional xylem-like cells. At 15 dpi, these cells appeared at the abaxial side in the wild-type, where the phloem is normally localized. In contrast, the coi1-t mutant contained several layers of cells with denser cytosol in this region (Fig. 2). In order to analyze whether these cells might be in a transition state, we investigated wild-type and coi1-t plants at 10 dpi and found that these characteristically stained cells were also observed in the wild-type at this earlier stage of the disease. The appearance of cells with dense cytosol in the vascular bundle has been described before when petioles were treated with 1 mg/L 2,4-dichlorophenoxyacetic and 0.1 mg/L Kinetin (Li et al., 2012). The expression of WUSCHEL in these cells was taken as evidence that this process is related to de-differentiation. In coi1-t plants, the de-differentiation process is already visible at 10 dpi, but less intense than in the wild-type. At 15 dpi, these cells have not yet re-differentiated into xylem-like cells. Whether the delayed restructuring of the vascular system in coi1-t limits proliferation of the fungus or whether slight reductions in fungal biomass already at 10 dpi are responsible for the observed slower restructuring is unclear. As both processes might influence each other, this question is difficult to resolve.
COI1 Influences the Disease Phenotype in the Absence of JA-Ile or Fungal-Derived JA-Ile Mimics

The JA biosynthesis mutant dde2-2 showed stronger disease symptoms than the JA receptor mutant coi1-t (Fig. 1). Although initial colonization is similar, more fungal DNA tends to accumulate in dde2-2 than in coi1-t at later stages of the infection (Fig. 3). This phenomenon is noteworthy, since both mutants should show the same phenotype. One plausible explanation is that the fungus synthesizes JA-Ile or a JA-Ile mimic that activates COI1. However, this does not seem to be the case since known JA-Ile-induced COI1-dependent responses like activation of the marker genes VSP2 or PDF1.2 are not induced in V. longisporum-infected dde2-2 mutant plants (Fig. 4). Thus, V. longisporum infections require COI1 through a mechanism that is different from that evolved by virulent Pseudomonas strains (Laurie-Berry et al., 2006). This hemibiotrophic pathogen produces the JA-Ile mimic coronatine to suppress SA-dependent defense responses in a COI1-dependent manner. A higher susceptibility of dde2-2 as compared to coi1-1 has also been described for the F. oxysporum/Arabidopsis interaction (Thatcher et al., 2009). It had been speculated that F. oxysporum-derived oxylipins might induce a senescence-promoting COI1 activity that would facilitate disease. For V. longisporum, we can rule out the existence of such a JA-Ile-like compound as deduced from the lack of VSP2 and PDF1.2 expression in infected dde2-2 plants (Fig. 4B).

Another example for a non-canonical COI1 function was described in the root knot nematode (Meloidogyne spp.)/tomato interaction. Root knot nematodes produce less number of eggs per g root on the tomato JA-receptor mutant jai1 than on the tomato JA biosynthesis mutant def1 (Bhattarai et al., 2008). This might be due to a nematode-derived effector triggering COI1 to promote egg production. Analysis of JA-Ile-dependent responses in the infected def1 mutant would reveal whether this effector is
a JA-Ile mimic or a different signal. A JA-Ile-independent COI1 function in roots was recently described for ET-mediated root-growth inhibition in Arabidopsis (Adams and Turner, 2010). The two JA biosynthesis mutants dde2-2 and opr3 showed a wild-type root growth inhibition response on 4 µM of the ET precursor ACC, whereas root growth of the coi1-16 mutant was less sensitive.

COI1 in the Roots Influences the Disease Phenotype of the Shoot

Grafting studies revealed that impaired shoot growth and early senescence depended on a functional COI1 allele in roots, whereas COI1 in shoots was not necessary for a visible disease phenotype (Fig. 5). The finding that the disease-promoting COI1 function operates in the roots was reported before for the F. oxysporum/Arabidopsis interaction (Thatcher et al., 2009). In this system, the same amount of fungal DNA was detected in wild-type and coi1 shoots before the onset of necrosis indicating that fungal entry and initial fungal growth was not restricted. Only later, when senescence processes were initiated in a COI1-dependent manner, fungal growth was restricted in coi1-1. The situation is similar in the V. longisporum/Arabidopsis interaction. As described above, initial penetration into the roots and colonization of the petioles at 10 dpi did not unravel major differences between wild-type and coi1-t plants and fungal growth became more restricted in coi1-t plants as the disease progressed (Fig. 3).

Having evidence that coi1 roots do not restrict fungal entry, the question emerges, why the wild-type shoot grafted on coi1 roots is less susceptible. Since V. longisporum does not change the water status (Floerl et al., 2008; Floerl et al., 2010), we do not think that clogging of the vessels in the root is responsible for the induction of disease symptoms of the shoot. We rather favor the idea that susceptibility of the shoot is caused by a root-borne mobile signal. One option is that a mobile signal released from coi1 roots induces a yet unknown antifungal resistance program in the shoot. A second
explanation is that the mobile signal is synthesized in a COI1-dependent manner and favors premature senescence. This developmental program initiates the mobilization of nutrients from the mesophyll (Quirino et al., 2000). The observed distortion of the vascular system (Fig. 2) might lead to leakage of the organic compounds to the otherwise nutrient-poor xylem to support fungal growth. The mobile signal might either be sufficient to induce premature senescence, or alternatively, it might alter the responsiveness of the above-ground-tissue to the infection. In the latter case, a feed-forward loop would be generated, with initial small manipulations of the senescence program facilitating fungal growth which in turn leads to an acceleration of these disease-promoting processes. The observation that shoots of uninfected coi1-1 plants are less sensitive to F. oxysporum extracts supports this scenario (Thatcher et al., 2009).

Since disease symptoms were similar in wild-type, dde2-2 and sid2-2, we question plant-derived jasmonates or salicylates as potential candidates for the postulated mobile signal. Since ET influences senescence and growth, the disease phenotype might be related to this hormone or its precursor 1-aminocyclopropane-1-carboxylic acid (ACC). Experimental evidence for the role of ET as a root-borne susceptibility factor has been reported before: expression of an ACC-deaminase in roots of tomato plants generated tolerance (e.g. reduced symptoms albeit wild-type-like colonization) towards V. dahliae (Robinson et al., 2001). Moreover, the Arabidopsis ET receptor mutant etr1-1 showed reduced symptoms and reduced V. dahliae biomass from 5 dpi on. However, no significant alterations in disease resistance/susceptibility towards F. oxysporum were found in ET-signaling mutants ein2 and etr1-1 (Thatcher et al., 2009). Still, as these alleles might not affect all ET responses, further studies with transgenic or mutant Arabidopsis plants with reduced production of ACC in the roots are required.
However, other hormones which may be discussed with respect to root-to-shoot signaling like e.g. cytokinins have to be taken into account (Dodd, 2005).

**Verticillium longisporum Infections Cause Increased Biosynthesis of JA-Ile, SA Metabolites and ABA**

Phytohormone measurements revealed that *V. longisporum* infections lead to increases in the amount of JA-Ile, SA metabolites and ABA (Figs 4 and 6). Increased JA-Ile levels are associated with two competing defense programs: the JA pathway, which elicits responses against herbivores and the JA/ET pathway, which is effective against fungi. Expression analysis of the corresponding marker genes (*VSP2* for the JA pathway and *PDF1.2* for the JA/ET pathway) revealed that the JA pathway is as efficiently induced as after wounding, whereas the JA/ET pathway is not as highly activated as by necrotrophic pathogens at least at 15 dpi. This might be an indication of low ET levels. Alternatively enhanced ABA levels, which are known to suppress the JA/ET pathway, might be responsible for relatively low *PDF1.2* expression levels. The weak activation of the JA/ET signal transduction pathway explains that the disease phenotype was not altered in the *dde2-2* mutant. Slightly higher levels of fungal biomass were detected at 15 and 19 dpi pointing at a minor protective role of the JA/ET pathway.

When comparing the relative amounts of *V. longisporum*-induced JA and JA-Ile levels with the relative amounts of these two metabolites after wounding, we found that JA-Ile levels reached similar amounts under both condition. JA levels showed a stronger increase after wounding pointing at a more efficient flux through this pathway in the continuous presence of the fungus. Elevated JA-Ile levels were observed in mock- and *V. longisporum*-infected *coi1-t* plants which is consistent with previously published data that COI1 is required for the induction of JA-Ile-metabolizing enzymes (Koo et al.,
These elevated levels of JA-Ile are unlikely to interfere with fungal growth since coi1-16 shoots grafted on wild-type roots are as susceptible as shoots of wild-type plants (Fig. 5). If increased JA-Ile levels would be able to induce a resistance mechanism in the absence of COI1, this process would be localized in the roots and should lead to the synthesis of a mobile signal that induces resistance in the shoot. Since preliminary microarray data have not unraveled hyper-activation of a specific set of genes in petioles of coi1-t plants (data not shown), we consider this scenario as unlikely.

In addition to JA-Ile and ABA, metabolites of the SA pathway increased after V. longisporum infection, leading to the induction of the SA marker gene PR-1. Interestingly, free SA levels did not accumulate suggesting that SA derivatives are responsible for the activation of PR-1. Candidates are SAG and DHBA which are derived from the activated isochorismate synthesis pathway after infection (Fig. S3). SAG might serve as a storage form of SA, whereas DHBA, which has a weak PR-1-inducing activity (Bartsch et al., 2010), is likely to be the active metabolite. Expression of PR-1 was partially reduced in dde2-2 and even further compromised in coi1-t (Fig. 6) which corresponds well with the relative amounts of SAG and DHBA. This suggests that activation of SA biosynthesis is partially dependent on JA in V. longisporum-colonized petioles. Importantly, PR-1 is not hyper-induced in coi1-t as observed after infections with Pseudomonas syringae (Kloek et al., 2001) or Plectosphaerella cucumeria (Hernandez-Blanco et al., 2007) to which coi1 is more resistant. In the Pseudomonas syringae/Arabidopsis interaction, the increased resistance of coi1 is due to suppression of the SA pathway by the JA-Ile mimic coronatine and was reverted to susceptibility after transformation with the NahG gene (Brooks et al., 2004; Laurie-Berry et al., 2006). Since a JA-Ile mimic influencing the V. longisporum/Arabidopsis interaction is unlikely (Fig. 4), it is consistent that the SA pathway is not hyper-induced.
in coi1-t. Moreover, the coi1-mediated tolerance towards F. oxysporum was not affected in coi1 nahG plants (Thatcher et al., 2009). Given the similar phenotypes of coi1 in the interactions to V. longisporum and F. oxysporum, and the observation that the SA pathway is not up-regulated in V. longisporum-infected coi1 plants, we conclude that the coi1-mediated tolerance is independent of SA.

In summary, we have demonstrated that V. longisporum requires a COI1-dependent but JA-Ile-independent mechanism in the roots to efficiently complete its life cycle in the Arabidopsis shoot. These observations support previous results obtained by the analysis of the interaction between Arabidopsis and F. oxysporum (Thatcher et al., 2009). Our work extends this study by experimental evidence demonstrating that COI1 acts independently of any JA-Ile or JA-Ile mimic. In view of these data, the interpretations of the results obtained for the Fusarium system may have to be reconsidered. It might well be that no fungus-derived oxylipins have to be postulated that would induce the COI1-mediated susceptibility.

Both fungi belong to the subclass Hypocreomycetidae of ascomycete fungi, but are in different phylogenetic lineages (Klosterman et al., 2011). Thus, the ability to colonize the xylem might have arisen independently. Comparison of the genome sequences of V. dahliae, V. albo-atrum and F. oxysporum pointed at a homolog of a bacterial glucosyltransferase as a common virulence factor (Klosterman et al., 2011), but did not reveal any other conspicuous similarities. V. longisporum and F. oxysporum both require COI1-dependent plant root-to-shoot signaling processes to adjust the anatomy and the physiology of the shoot for their own benefit. Manipulation of this mechanism in crop plants might lead to a strategy to combat these devastating vascular diseases.
MATERIALS AND METHODS

Plant Genotypes

The Columbia ecotype (Col-0 or Col-gl) of *Arabidopsis thaliana* were used as wild types. Mutants were obtained from the following sources: *dde2-2* (Park et al., 2002) from B. von Malek and B. Keller, University of Zurich, Zurich, Switzerland; *coi1-t* (SALK 035548) from I. Heilmann, Martin-Luther-University, Halle, Germany; *coi1-1* and *coi1-16* (Xie et al., 1998) from J. Turner, University of East Anglia, Norwich, UK; *acx1/5* (Schilmiller et al., 2007) from G. Howe, Michigan State University, East Lansing, USA; *fad3-2 fad7-2 fad8* (McConn and Browse, 1996) from J. Browse, Washington State University, Pullman, USA; *npr1-1* (Cao et al., 1994) from Nottingham *Arabidopsis* Stock Center, Nottingham, UK; *nahG* (Gaffney et al., 1993; Lawton et al., 1995) from L. Friedrich (Syngenta Biotechnology, RTP, NC, USA); *sid2-2* (Nawrath and Metraux, 1999; Wildermuth et al., 2001) from F.M. Ausubel, Harvard University, Cambridge, USA.

Fungal Culture

The *V. longisporum* isolate *Vl43* (Zeise and von Tiedemann, 2002) was obtained from A. von Tiedemann, Georg-August-University Göttingen (Germany). Long term storage was performed as conidial suspensions in a concentration of 1-3 x 10^9 conidia ml⁻¹ in Czapek-Dox medium (SIGMA, Steinheim, Germany) supplemented with 25% glycerol at −80°C. For propagation, droplets of these suspensions were plated onto potato dextrose agar (PDA, SIGMA, Steinheim, Germany) and incubated for 14 days at 21°C in the dark. Spores were obtained by transferring blocks of agar with mycelium to 120 ml potato dextrose broth (PDB, SIGMA, Steinheim, Germany) supplemented with 0.5 mg l⁻¹ Cefotaxim. The cultures were subsequently incubated for 2 to 4 weeks on a
rotary shaker at 21°C in the dark. To start sporulation, PDB was replaced by Czapek-Dox broth (SIGMA, Steinheim, Germany). After 4 to 7 days, spores were harvested by filtering through a fluted filter (Macherey-Nagel, Düren, Germany). The conidia were washed once with sterile tap water. Spore concentration was determined with a haemocytometer and diluted to 1 x 10⁶ spores ml⁻¹.

**Plant Inoculation**

For experiments including *coi1*, seeds from all genotypes were surface sterilized and sown on agar plates containing Murashige and Skoog (MS) medium. To identify homozygous *coi1* plants, 50 µM MeJA was added to the plates. Plates were incubated for two (*coi1-t and coi1-1*) or three days (*wild-type and dde2-2*) at 4°C in the dark to promote germination. Plants were grown subsequently under controlled environmental conditions (22 °C, ~ 140 µmol m⁻² sec⁻¹ PAR; 8-h-light/16-h-dark photoperiod). After 12 days, plants were transferred to pots containing a 1:1 mixture of silica grit (Vitakraft, Nr 12262, Bremen, Germany) and soil (Archut, Fruhstorfer Erde, T25, Str1 fein) on a layer of seramis (Masterfoods GmbH, Verden/Aller, Germany) in a growth chamber. Initial watering of this setup was done with 0.1% of the fertilizer Wuxal (Manna, Düsseldorf, Germany). After 7 to 12 days, plants were uprooted. Roots were rinsed with tap water to get rid of residual substrate material and incubated for 45 min in a conidial suspension (10⁶ conidia ml⁻¹ H₂O). For mock inoculations, roots of were incubated in tap water. Plants were transplanted to soil and kept under a transparent cover for two days to ensure high humidity. Plants were subsequently grown under short day conditions as described above. Reciprocal grafts between wild-type and *coi1-16* were generated using 5-day-old seedlings by the micrografting technique (Turnbull *et al.*, 2002). Grafts were kept under continuous light on solidified mineral MS medium for one week (17 °C, ~ 50 µmol m⁻² sec⁻¹ PAR) before transferring them to short-day conditions.
as described above. Successful grafts were transferred to the silica grit/soil mixture as described above.

**Quantification of *Verticillium longisporum* DNA**

Fungal biomass was quantified by determination of fungal DNA in infected plant extracts with real-time PCR. DNA extraction from infected petioles was conducted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The iCycler System (BioRad, Hercules, CA, USA) was used for the amplification and quantification of *V. longisporum* DNA using primers OLG70 (5’-CAGCGAAACGCGATATGTAG-3’) and OLG71 (5’-GGCTTGTAGGGGGTTAGA-3’) spanning internal transcribed sequences of ribosomal RNA genes (Eynck *et al.*, 2007). The Advantage 2 Polymerase (Clontech, Mountain View, CA, USA) and 20-30 ng of template DNA were used. For normalization, the Arabidopsis *Actin8* gene (At1g49240) was amplified with primers 5’-GGTTTTCCCCAGTTGTGTGTTG-3’ and 5’-CTCCATGTCATCCAGTTG-3’).

**Leaf Surface Measurement**

Photographs were taken with a digital camera and a custom-made software (Bildanalyseprogramm, Datinf GmbH Tübingen, Germany) was used to quantify the projected leaf area.

**Quantitative Real-Time RT-PCR Analysis**

RNA extraction and quantitative RT PCR analysis was performed as described (Fode *et al.*, 2008). Calculations were done according to the 2^-ΔCT method (Livak and Schmittgen, 2001). *UBQ5* served as a reference (Kesarwani *et al.*, 2007). Primers used
to amplify and quantify the cDNA are indicated in Table S1 (*PDF1.2* (At5g44420), *PR-1* (At2g14610), *VSP2* (At5g24770), *UBQ5* (At3g62250)).

**Anatomical Studies**

Petioles (2 to 4 mm) were stored in a mixture of 37 % formaldehyde, 100% acetic acid and 70 % ethanol (FAE, 5:5:90, v/v/v). Samples were successively infiltrated with the following solutions: 70% ethanol for 24 h, 80% ethanol for 2 h, 90 % ethanol for 2 h, 100 ethanol for 2 h, 100 % ethanol for 12 h, 100 % ethanol : 100% acetone (1:1) for 2 h, 100 % acetone for 2 h (2 times), acetone : plastic (1:1) for 4 h, acetone : plastic (1:3) for 12 h, 100 % plastic for 12 h (2 times). Plastic was a mixture of styrene (Merck, Darmstadt, Germany) and butyl methacrylate (Sigma, Steinheim, Germany) (1:1) containing 2 % dibenzoylperoxide with 50 % phthalate (Peroxid Chemie GmbH, Pullach, Germany). The samples were transferred into gelatine capsules (Plano GmbH, Wetzlar, Germany), mounted with fresh plastic solution which was then polymerized at 60°C for 3 days and at 37°C for 10 additional days. Transverse cross-sections (1 µm) of the embedded samples were obtained with a microtome (Autocut, Reichert-Jung, Heidelberg, Germany) using a diamond knife (Chisto Diatome, Drukker International, Cuijk, Netherlands). The sections were placed on glass slides which were covered with 0.5 % (w/v) gelatine containing 1.77 mM KCr(SO₄)₂ in distilled H₂O. For histochemical analyses, cross sections were stained with 0.05 % toluidine blue in 1% boric acid for 10 min at 60°C, mounted in DePex (Serva, Heidelberg, Germany) and photographed under a bright field microscope (Axioskop, Zeiss, Oberkochen, Germany).

**Determination of JA, JA-Ile, SA, DHBA, SAG and ABA levels by HPLC-MS/MS**

Extraction was performed as previously described for lipids (Matyash *et al.*, 2008) with modifications as described in Document S1.
Supplemental Data

The following materials can be found in the online version of this article.

**Supplemental Figure S1.** Disease phenotype of independent JA biosynthesis mutants and *coi1-1* after *Verticillium longisporum* infection

**Supplemental Figure S2.** Structure of the vascular bundle in petioles of *Verticillium longisporum*-infected plants

**Supplemental Figure S3.** *PR-1* gene expression and SA, SAG and DHBA levels in SA biosynthesis and signaling mutants

**Supplemental Table S1.** Sequence of primers used for quantitative RT-PCR

**Supplemental Document S1:** Determination of JA, JA-Ile, SA, DHBA, SAG and ABA levels by HPLC-MS/MS

**ACKNOWLEDGEMENTS**

We thank Ronald Scholz, Sabine Freitag and Merle Fastenrath for excellent technical assistance and Dr. Alexander Christmann for help with the grafting experiments. We are particularly grateful to the Prof. Lipka and co-workers (Georg-August-University Göttingen) for help with the Laser Scan Microscopy and to Prof. Dr. A. Tiedemann for providing *V.l.* 43 and the corresponding GFP-tagged line. This work was supported by grants of the Deutsche Forschungsgemeinschaft (DFG GA330/14-3, DFG FE 446/2-3, and DFG PO 362/15-1/2 within the Research Group FOR546).
REFERENCES


FIGURE LEGENDS

Figure 1. Disease Phenotype of Verticillium longisporum-Infected Wild-type, dde2-2 and coi1-t Plants

(A) Typical V. longisporum (V.l.) disease symptoms of wild-type (upper row), dde2-2 (middle row) and coi1-t (lower row) plants at 15 days post inoculation (dpi). One representative mock-treated plant of each genotype (left row) and 4 representative infected plants of each genotype are shown.

(B) Projected leaf area of mock-infected and V. longisporum-infected wild-type, dde2-2 and coi1-t plants. Data are means (+/- SEM) of 43-48 replicates from three independent experiments. Stars indicate significant differences at P < 0.0001 (two-way ANOVA followed by Bonferroni multiple comparison test; ns, not significant) between V. longisporum (V.l.)- and mock-infected samples.

(C) Single leaves of mock-infected and V. longisporum-infected wild-type, dde2-2 and coi1-t plants at 22 dpi. Leaves from corresponding positions (mock- and V. longisporum-infected) are shown.

(D) Representative disease symptoms of wild-type (upper row), dde2-2 (middle row) and coi1-t (lower row) plants at 35 dpi. One representative mock-treated plant and one infected plant are shown.
(E) Percentage of wild-type, *dde2-2* and *coi1-t* plants with microsclerotia after 35 dpi. Numbers are from three independent experiments with 16 mock- and 16 *V. longisporum*-infected plants/experiment. Microsclerotia were only observed on plants showing the severe phenotype as shown in (D). Different letters indicate significant differences at $P < 0.01$ (one-way ANOVA followed by Tukey-Kramer multiple comparison test).

(F) Photograph of a typical *V. longisporum*-infected Col-0 leaf at 35 dpi showing microsclerotia primarily around the petiole.

**Figure 2.** Structure of the Vascular Bundle in Petioles of *Verticillium longisporum*-Infected Plants

(A) Cross-sections of petioles from mock-inoculated and *V. longisporum*-infected Col-0 plants at 10 and 15 dpi. Sections were stained with toluidine blue to detect lignification of secondary cell walls. Xy, xylem vessel; Ph, phloem; Pc, procambium-derived paired cells. Red arrows exemplarily denote two cells which look like xylem vessels at the abaxial side, the green arrow exemplarily marks putative phloem cells in the middle of the vascular bundle (bar = 20 µm).

(B) Cross-sections of petioles from *V. longisporum*-infected *coi1-t* plants at 10 and 15 dpi (bar = 20 µm).

**Figure 3.** Fungal Biomass of *Verticillium longisporum*-Infected Wild-type, *dde2-2* and *coi1-t* Plants and Root Colonization of *coi1-t*

(A) Relative quantification of fungal biomass by real-time PCR on DNA extracted from petioles of *V. longisporum*-infected wild-type, *dde2-2* and *coi1-t* plants at 10, 15 and 19 dpi. Amplification values for fungal internal ribosomal spacer regions were normalized
to the abundance of Arabidopsis Actin8 sequences. Relative amounts of fungal DNA were set to 100% for the wild-type. 10 and 15 dpi: Bars indicate means (+/- SEM) of 11 biological replicates from three independent experiments. Each replicate is a pool of four plants. 19 dpi: Bars indicate means (+/- SEM) of three independent experiments, with three biological replicates. Each replicate represents a pool of four plants. Stars indicate significant differences at $P < 0.0001$ (unpaired t-test) between Col-0 and coi1-t.

(B) Confocal image of a coi1-t root infected with a GFP-tagged V. longisporum strain at 7 dpi. The black and the white arrows indicate fungal hyphae inside and outside of the vascular cylinder, respectively.

**Figure 4.** Activation of JA Biosynthesis and Signaling Pathways in V. longisporum-Infected Wild-type, dde2-2 and coi1-t Plants

(A,C) HPLC-MS/MS analysis for detection of JA-, JA-Ile and ABA levels in petioles from wild-type, dde2-2 and coi1-t plants at 15 days after mock and V. longisporum infection. Data are the means (+/- SEM) of eight replicates from two independent experiments. Each replicate is a pool of four plants. As positive controls, petioles were wounded with forceps and harvested after 2 hours. Data are from three biological replicates.

(B) Quantitative RT-PCR analysis of relative VSP2 and PDF1.2 transcript levels in petioles from wild-type, dde2-2 and coi1-t plants at 15 days after mock and V. longisporum infection. Data indicate means (+/- SEM) of three independent experiments with 16 individual plants/experiment. Wounded petioles were harvested for RNA extraction after 2 hours (three biological replicates), Botrytis cinerea-infected samples were harvested after 3 days (four biological replicates). Relative transcript levels of the infected wild-type were set to 100%.
Different letters denote significant differences between samples (one-way ANOVA followed by Tukey-Kramer multiple comparison test; $P < 0.05$ for (A) and (B) PDF1.2; $P < 0.001$ for (b) VSP2 and (C)). FW, fresh weight.

**Figure 5.** Disease Phenotype of Reciprocal Grafts Between Wild-type and *coi1-16* Plants

Reciprocal grafts between wild-type (Col-0) and *coi1-16* plants were inoculated with *V. longisporum* (*V.l.*) or mock-treated and disease symptoms were recorded after 3 weeks. Scions and rootstocks are indicated by the labels above and below the lines, respectively.

**Figure 6.** Activation of SA Biosynthesis and Signaling Pathways in *V. longisporum*-Infected Wild-type, *dde2-2* and *coi1-t* Plants and Disease Phenotype in SA Biosynthesis and Signaling Mutants

(A) HPLC-MS/MS analysis for detection of SA-, SAG and DHBA levels in petioles from wild-type, *dde2-2* and *coi1-t* plants at 15 days after mock and *V. longisporum* infection. Data are the means (+/- SEM) of eight replicates from two independent experiments. Each replicate is a pool of four plants (same material as in Fig. 4). FW, fresh weight.

(B) Quantitative RT-PCR analysis of relative *PR-1* transcript levels in petioles from wild-type, *dde2-2* and *coi1-t* plants at 15 days after mock and *V. longisporum* infection. Data indicate means (+/- SEM) of three independent experiments with 16 individual plants/experiment (same material as in Fig. 4). *Pseudomonas syringae* pv. *maculicola* ES4326/avrRps4-infected leaf samples were harvested after 3 days (three biological replicates). Relative transcript levels of the *V.l.*-infected wild-type were set to 100%.
Projected leaf area of mock-infected and *V. longisporum*-infected wild-type, *sid2-2*, *NahG* and *npr1-1* plants. Data indicate means (+/- SEM) of 29-34 replicates from two independent experiments.

Different letters denote significant differences between samples (one-way ANOVA followed by Tukey-Kramer multiple comparison test; P < 0.01 for (A), SA and SAG; P < 0.05 for (A), DHBA and (B)). In (C), stars indicate significant differences at P < 0.0001 (two-way ANOVA followed by Bonferroni multiple comparison test) between *V. longisporum*- and mock-infected samples.
Fig. 1

A. Mock 15 dpi V.l. Col-0, dde2-2, coi1-t

B. 15 dpi

<table>
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<th>Col-0</th>
<th>dde2-2</th>
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<tr>
<td>−</td>
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<td>relative leaf area</td>
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C. Mock 22 dpi V.l. Col-0, dde2-2, coi1-t

D. Mock 35 dpi V.l.

E. 35 dpi

% of plants with microsclerotia

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<tr>
<td>a</td>
<td>a</td>
<td>b</td>
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F. Col-0, 35 dpi V.l.
Fig. 2

A

Col-0, mock

Col-0, V.l.

10 dpi

B

col1-t, V.l.

10 dpi

15 dpi

15 dpi

Xy

Pc

Ph

Ralhan et al.
Fig. 3

A

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B

Vascular cylinder

![Image](image)
Fig. 4

A. Relative expression of PDF1.2 and VAHD1 in Col-0 (wild type) and dde2-2 coi1-t mutants under control and wounded conditions. The VSP2 gene was also examined as a control. Significant differences were determined using a Tukey’s test (p ≤ 0.05).

B. JA and JA-Ile levels in Col-0 (wild type) and dde2-2 coi1-t mutants under control and wounded conditions. A Tukey’s test (p ≤ 0.05) was used to determine significant differences.

C. ABA levels in Col-0 (wild type) and dde2-2 coi1-t mutants under control and Botrytis-infected conditions. A Tukey’s test (p ≤ 0.05) was used to determine significant differences.
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Fig. 6

A

SA

SAG

DHBA

nmol per g FW

V.I. Col-0 dde2-2 coi1-t

B

PR-1

relative expression

V.I. Col-0 dde2-2 coi1-t

C

relative leaf area

V.I. Col-0 sid2-2 nahG

Col-0 npr1-1