Degradation of aromatic compounds through the β -ketoadipate pathway is required for pathogenicity of the tomato wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici*

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SUMMARY

Plant roots react to pathogen attack by the activation of general and systemic resistance, including the lignification of cell walls and increased release of phenolic compounds in root exudate. Some fungi have the capacity to degrade lignin using ligninolytic extracellular peroxidases and laccases. Aromatic lignin breakdown products are further catabolized via the β -ketoadipate pathway. In this study, we investigated the role of 3-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE), an enzyme of the β -ketoadipate pathway, in the pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici* towards its host, tomato. As expected, the *cml*e deletion mutant cannot catabolize phenolic compounds known to be degraded via the β -ketoadipate pathway. In addition, the mutant is impaired in root invasion and is nonpathogenic, even though it shows normal superficial root colonization. We hypothesize that the β -ketoadipate pathway in plant-pathogenic, soil-borne fungi is necessary to degrade phenolic compounds in root exudate and/or inside roots in order to establish disease.

INTRODUCTION

The rhizosphere, defined as that part of the soil which is adjacent to the root system of a plant, is a highly dynamic place with numerous interactions between plant roots, microbes and soil fauna. Plant roots not only serve as an anchor for the plant in the soil or as a feeding structure to obtain water and nutrients for growth, but also release compounds, known as the root exudate. The root exudate contains amino acids, organic acids, sugars, enzymes, polysaccharides, vitamins, phenolics and other secondary metabolites (Bais *et al.*, 2006; Singh and Mukerji, 2006). These

compounds can change the chemical and physical properties of the soil, affect the composition of the soil microbial community and inhibit the growth of competing plant species (Nardi *et al.*, 2000). In addition, root exudates can act as attractants or repellents for beneficial or detrimental microbes. Positive plant-microbe interactions include, for instance, the symbiosis between leguminous plants and *Rhizobia*, triggered by specific flavonoids, or between arbuscular mycorrhizal fungi and plant roots. Rhizobacteria attracted by carbohydrates and amino acids can stimulate plant growth or suppress plant disease caused by soil fungi and bacteria through competition for nutrients, niche exclusion or by the production of antimicrobial metabolites (Bais *et al.*, 2006 and references cited therein).

On pathogen attack, plant roots strengthen their cell walls by lignification and can produce a broad spectrum of antimicrobial compounds, some of which are species specific (van Loon, 2000; Okubara and Paulitz, 2005). For example, Bais *et al.* (2002) showed that, in response to *Pythium ultimum* attack, hairy roots of *Ocimum basilicum* secrete rosmarinic acid, a root exudate with a repellent function against an array of soil-borne pathogens. Comparison of susceptible and resistant crop varieties also revealed that increased lignification and higher levels of phenolics can be correlated with disease resistance (de Ascensao and Dubery, 2000; Eynck *et al.*, 2009; de Farias Viegas Aquije *et al.*, 2010; Taddei *et al.*, 2002). In addition, several studies have reported reduced plant disease after pretreatment of the plants with various (fungal) elicitors, associated with increased lignification or increased synthesis of phenolics, such as ferulic acid (Benhamou *et al.*, 1994; Bhaskara Reddy *et al.*, 1999; He *et al.*, 2002; Takenaka *et al.*, 2003). However, many pathogens are still able to overcome this type of plant defence and cause disease, such as *Fusarium* wilt of tomato caused by the fungus *Fusarium oxysporum* f. sp. *lycopersici*.

Fusarium oxysporum is an abundant saprophyte in soil and organic matter and an inhabitant of the rhizosphere. Many isolates found in the soil are nonpathogenic; however, strains causing wilt or rot disease in important agricultural and ornamental plant species have also been found (Gordon and Martyn, 1997; Recorbet

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et al., 2003). These pathogenic isolates are grouped into *formae specialis* depending on their host range (Armstrong and Armstrong, 1981; Gordon and Martyn, 1997). *Fusarium oxysporum* is capable of overcoming plant defence responses during root infection, including changes in root cell wall and root exudate composition (reviewed by Di Pietro *et al.*, 2003; Michielse and Rep, 2009). Several studies have reported cell wall strengthening through the deposition of lignin, the accumulation of cell wall-bound phenolics, as well as an effect of root exudates on conidia germination and growth of *Fusarium* on elicitation (de Ascensao and Dubery, 2000, 2003; van den Berg *et al.*, 2007; Cvikrova *et al.*, 1993; Mandal and Mitra, 2007, 2008; Panina *et al.*, 2007; Steinkeller *et al.*, 2005, 2008). de Ascensao and Dubery (2000, 2003) reported banana root cell wall reinforcement on elicitation with cell wall fractions of *F. oxysporum* f. sp. *cubense* race 4, and the accumulation of soluble and cell wall-bound phenolics, such as vanillic acid, sinapic acid, *p*-coumaric acid and ferulic acid. Treatment of tomato roots or hairy root tomato cultures with an elicitor derived from *F. oxysporum* f. sp. *lycopersici* resulted in increased lignin deposition and increased concentrations of the phenolic compounds ferulic acid, 4-hydroxybenzoic acid and *p*-coumaric acid (Mandal and Mitra, 2007, 2008). Steinkeller *et al.* (2005) showed that tomato root exudates contain phenolic compounds that inhibit microconidial germination. How certain *F. oxysporum* strains overcome the increased root cell wall lignification and phenolic compounds in root exudates and cause disease is not fully understood.

Fungi, including *F. oxysporum*, can degrade lignin using ligninolytic extracellular peroxidases and laccases (Bugg *et al.*, 2011; Martinez *et al.*, 2005). The aromatic breakdown products are further degraded through the β -ketoadipate pathway (Harwood and Parales, 1996). In *F. oxysporum* f. sp. *lycopersici*, six laccase genes have been analysed and, although three single deletion mutants (*lcc1*, *lcc3* and *lcc5*) showed reduced laccase activity, they were not impaired in virulence (Canero and Roncero, 2008a). A decrease in laccase activity and virulence was observed, however, for a mutant disrupted in a gene encoding a chloride channel (*CLC1*) (Canero and Roncero, 2008b). Therefore, it may be that full laccase activity is required for pathogenicity.

In this article, we report the characterization of a nonpathogenic *F. oxysporum* f. sp. *lycopersici* mutant, previously identified in an insertional mutagenesis screen, carrying a T-DNA insertion in a gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme (*CMLE*) (Michielse *et al.*, 2009a), an enzyme in the β -ketoadipate pathway. We show that the inactivation of *CMLE* does not affect superficial tomato root colonization, but does inhibit *in planta* growth, as well as growth and conidial germination in the presence of certain phenolic compounds. A possible link between pathogenicity, the β -ketoadipate pathway and cell wall lignification and alterations in phenolic compounds in root exudates is discussed.

RESULTS

Isolation and structural analysis of *CMLE*

In an insertional mutagenesis screen of *Fusarium oxysporum*, two mutants, 72C7 and 75H3, were identified with strongly reduced pathogenicity (Michielse *et al.*, 2009a). Both mutants carry a single T-DNA insertion in the open reading frame of *FOXG_17757* (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). Database searches using the BLASTX algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>) revealed that this gene has homology to 3-carboxy-*cis,cis*-muconate lactonizing enzyme (*CMLE*), an enzyme that functions in aromatic compound degradation through the β -ketoadipate pathway. In this pathway, protocatechuate, derived from phenolic compounds, is converted into β -ketoadipate, which, in two subsequent steps, is converted into the tricarboxylic acid intermediates succinyl-CoA and acetyl-CoA. *CMLE* catalyses the reversible γ -lactonization of 3-carboxy-*cis,cis*-muconate in this reaction (Harwood and Parales, 1996).

The annotated *FOXG_17757* gene lacks a 5' and 3' end. In this article, we suggest a new gene model based on FGENESH and AUGUSTUS gene prediction software (<http://www.softberry.com>, <http://augustus.gobics.de/>). This model was verified by reverse transcriptase-polymerase chain reaction (RT-PCR) (data not shown). The *CMLE* gene consists of an open reading frame of 1190 nucleotides containing two introns of 49 and 46 nucleotides, which results in a protein of 364 amino acids. The nucleotide sequence has been deposited in the National Center for Biotechnology Information (NCBI) database (Accession Number JQ234921).

CMLE is required for pathogenicity

To investigate whether *CMLE* is indeed required for pathogenicity, the *CMLE* gene was replaced by a hygromycin resistance cassette in the wild-type strain by homologous recombination (Fig. S1, see Supporting Information), using *Agrobacterium*-mediated transformation. Numerous transformants were obtained and correct homologous recombination was verified by PCR (data not shown) and Southern analysis (Fig. S2, see Supporting Information). Five independent *cmle* mutants were tested for pathogenicity in a root-dip bioassay. In this assay, none of the *cmle* mutants was able to cause any disease symptoms on tomato plants (Fig. 1A). To confirm that the nonpathogenic phenotype was caused by a loss of *CMLE*, the gene was re-introduced by homologous recombination into one *cmle* deletion strain (*CMLEKO6*) (Fig. S1). Correct homologous recombination was again verified by PCR (data not shown) and by Southern analysis (Fig. S2). Five independent complemented strains were used in a root-dip bioassay and re-introduction of the *CMLE* gene led to restoration of pathogenicity (Fig. 1B). Thus, *CMLE* is required for pathogenicity in *F. oxysporum* f. sp. *lycopersici*.

Fig. 1 3-Carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) is required for pathogenicity. Nine- to eleven-day-old tomato seedlings were inoculated with fungal spore suspensions using root-dip inoculation, and the disease index (0, healthy plant; 4, severely diseased plant or dead plant) was scored after 3 weeks. Error bars indicate the standard deviation and capitals define statistically significantly different groups [analysis of variance (ANOVA), $P = 0.95$]. (A) Average disease index of 20 plants 3 weeks after mock inoculation (H₂O) or inoculation with five independent *cmle* deletion mutants (*CMLEKO4*, 5, 6, 7 and 9), insertional mutants 72C7 and 75H3 or wild-type (4287). (B) Average disease index of 20 plants 3 weeks after mock inoculation (H₂O) or inoculation with *cmle* deletion mutants (*CMLEKO6* and 9), five independent complemented strains (*CMLEcom9*, 10, 19, 20 and 42) or wild-type (4287).

CMLE is required for growth on various aromatic compounds

All insertional mutagenesis pathogenicity mutants identified previously were analysed for growth on various carbon sources, including the *cmle* mutants 72C7 and 75H3. In this analysis, the two *cmle* mutants did not show any major aberrant growth phenotype (Michielse *et al.*, 2009a). To characterize the growth phenotype of the *cmle* mutants in more detail, the mutants and complemented strains were subjected to several growth assays.

First, carbon source utilization ability on 95 different sources was tested in a BIOLOG assay. On the majority of the carbon sources tested, including various mono-, di- and polysaccharides, carboxylic acids, sugar alcohols and amino acids, no difference was observed between the *cmle* mutant (KO6) and the wild-type strain (Table S1, see Supporting Information). However, on amygdalin, D-galacturonic acid and quinic acid, the *cmle* mutant was reduced in growth (Fig. S3, see Supporting Information). This phenotype was verified in a plate assay for amygdalin and quinic acid (Fig. 2).

Secondly, growth was analysed on minimal and rich medium supplemented with the aromatic compounds ferulic acid, coumaric acid, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, cinnamic acid, catechol or lignin. As no other carbon source was added to the minimal medium, growth on these media indicates the capacity of the strains to assimilate the respective aromatic com-

pounds. The *cmle* deletion mutants (KO6 and KO9) and insertional mutants 72C7 and 75H3 displayed significantly reduced growth on minimal medium supplemented with ferulic acid, coumaric acid, vanillic acid, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde and cinnamic acid (Table 1). These are all compounds that are converted to protocatechuate and are subsequently degraded through the β-ketoadipate pathway (Cain *et al.*, 1968; Harwood and Parales, 1996; Middelhoven, 1993; Sparnins *et al.*, 1979). Growth was restored to the wild-type level in the complemented strains (Table 1). In addition, on hairy root extract and on lignin, growth of the *cmle* deletion mutants and the insertional mutants 72C7 and 75H3 was reduced significantly, except for *CMLEKO6* on lignin (Table 1). This indicates that *CMLE* is also required for vigorous growth on complex plant components which, on degradation, generate aromatic compounds. None of the mutants displayed reduced growth on catechol, which is also degraded through the β-ketoadipate pathway, but does not require the enzymatic activity of *CMLE* (Harwood and Parales, 1996). On rich medium, i.e. in the presence of a carbon source, sucrose, no growth reduction was observed for the *cmle* mutants on catechol. Indeed, in some cases, growth was even enhanced (Table 2). However, as observed on minimal medium, growth of the *cmle* mutants was reduced significantly on five of the six aromatic compounds tested, except for mutant 75H3 on ferulic and coumaric acids, which appeared to be reduced in growth, but the difference from the wild-type was not significant at 95% (Table 2). Thus, *CMLE* is required for the

Fig. 2 Deletion of *CMLE* (gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme) leads to reduced growth on amygdalin and quinic acid. Mycelial plugs were used to inoculate buffered minimal medium (pH 5.3) containing sucrose (1%), amygdalin (500 mg/L) or quinic acid (500 mg/L), followed by incubation at 25 °C. Photographs were taken after 5 days.

Table 1 Average colony diameter (cm) after 5 days of growth on minimal medium containing various aromatic compounds.

Compound	Wild-type	<i>CMLE</i> KO6	<i>CMLE</i> KO9	<i>CMLE</i> com19	<i>CMLE</i> com42	72C7	75H3
Potato dextrose agar	4.4 ± 0.3	4.3 ± 0.1	4.0 ± 0.2	4.2 ± 0.3	4.2 ± 0.4	4.1 ± 0.2	4.0 ± 0.1
Sucrose	5.4 ± 0.1	5.5 ± 0.2	5.3 ± 0.1	5.4 ± 0.1	5.4 ± 0.1	5.2 ± 0.3	5.4 ± 0.1
Ferulic acid	4.8 ± 0.1	1.9 ± 0.1	1.6 ± 0.2	5.1 ± 0.2	5.0 ± 0.1	1.4 ± 0.2	1.6 ± 0.2
Coumaric acid	5.1 ± 0.2	1.8 ± 0.2	2.4 ± 0.4	5.1 ± 0.3	5.3 ± 0.3	2.1 ± 0.1	2.2 ± 0.2
Vanillic acid	5.3 ± 0.1	3.4 ± 0.2	2.2 ± 0.2	5.7 ± 0.2	5.2 ± 0.2	3.2 ± 0.2	2.2 ± 0.3
4-Hydroxybenzoic acid	5.4 ± 0.1	2.8 ± 0.1	2.8 ± 0.3	5.1 ± 0.2	5.2 ± 0.6	3.1 ± 0.1	2.8 ± 0.2
4-Hydroxybenzaldehyde	5.1 ± 0.2	2.1 ± 0.2	1.5 ± 0.3	5.4 ± 0.1	5.2 ± 0.3	2.3 ± 0.3	1.9 ± 0.2
Cinnamic acid	5.2 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	5.2 ± 0.2	5.4 ± 0.4	1.9 ± 0.2	1.5 ± 0.3
Catechol	4.8 ± 0.2	4.8 ± 0.2	4.7 ± 0.1	5.1 ± 0.1	4.9 ± 0.2	5.1 ± 0.1	4.7 ± 0.1
Lignin	5.4 ± 0.2	5.4 ± 0.2	4.3 ± 0.1	5.4 ± 0.1	5.2 ± 0.1	5.0 ± 0.2	4.6 ± 0.1
Hairy roots	7.9 ± 0.1	6.8 ± 0.1	6.6 ± 0.3	8.1 ± 0.1	8.0 ± 0.1	7.2 ± 0.1	6.9 ± 0.2

CMLE, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme.

Bold type and shaded grey, significantly different from wild-type at a 95% confidence interval.

Table 2 Average colony diameter (cm) after 5 days of growth on rich medium containing various aromatic compounds.

Compound	Wild type	<i>CMLE</i> KO6	<i>CMLE</i> KO9	<i>CMLE</i> com19	<i>CMLE</i> com42	72C7	75H3
Potato dextrose agar	5.4 ± 0.2	5.5 ± 0.4	5.1 ± 0.1	5.4 ± 0.2	5.9 ± 0.3	4.8 ± 0.2	5.5 ± 0.2
Sucrose	6.8 ± 0.3	6.9 ± 0.2	7.1 ± 0.1	6.7 ± 0.1	6.6 ± 0.3	6.7 ± 0.1	7.3 ± 0.2
Ferulic acid	5.4 ± 0.1	4.2 ± 0.2	4.1 ± 0.4	5.3 ± 0.4	5.4 ± 0.2	4.2 ± 0.3	5.0 ± 0.2
Coumaric acid	6.1 ± 0.1	5.4 ± 0.1	5.2 ± 0.2	6.6 ± 0.3	6.3 ± 0.3	5.3 ± 0.2	5.8 ± 0.2
Vanillic acid	6.1 ± 0.2	5.1 ± 0.2	5.6 ± 0.2	6.3 ± 0.2	6.1 ± 0.3	5.0 ± 0.2	5.3 ± 0.1
4-Hydroxybenzoic acid	6.7 ± 0.3	3.8 ± 0.5	3.3 ± 0.7	6.3 ± 0.2	6.3 ± 0.2	3.9 ± 0.2	5.1 ± 0.6
4-Hydroxybenzaldehyde	6.0 ± 0.4	4.0 ± 0.2	4.3 ± 0.2	6.2 ± 0.1	6.2 ± 0.2	4.7 ± 0.2	4.7 ± 0.2
Cinnamic acid	5.8 ± 0.1	6.0 ± 0.1	6.3 ± 0.4	5.9 ± 0.1	6.0 ± 0.2	6.1 ± 0.4	6.4 ± 0.2
Catechol	6.1 ± 0.2	5.9 ± 0.1	7.0 ± 0.1	6.7 ± 0.2	6.3 ± 0.3	6.5 ± 0.1	6.8 ± 0.1
Lignin	6.1 ± 0.1	5.1 ± 0.2	6.2 ± 0.1	6.4 ± 0.2	6.4 ± 0.1	5.9 ± 0.2	6.2 ± 0.1

CMLE, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme.

Bold type and shaded grey, significantly different from wild-type at a 95% confidence interval.

metabolism of aromatic compounds, most probably through the protocatechuate branch of the β -ketoadipate pathway; in the absence of a functional *CMLE* protein, these aromatic compounds appear to be toxic to *F. oxysporum*.

The *cmle* mutant is affected in conidial germination in the presence of some aromatic compounds

Aromatic compounds have been reported to stimulate conidial germination of *F. oxysporum* f. sp. *niveum* at low concentrations and/or to inhibit germination at higher concentrations (Wu *et al.*, 2008a, b, c, 2009a, b, c, d, 2010). Therefore, germination rates of the wild-type and *cmle* deletion mutant KO6 were assessed in the presence of various aromatic compounds. In the presence of low concentrations of coumaric or ferulic acid (10 or 50 mg/L), germination rates did not differ significantly between the wild-type and the *cmle* mutant (Fig. 3A,B). However, at a higher concentration (100 mg/L), a significant reduction in the germination rate of the *cmle* mutant was observed (Fig. 3A,B). In the presence of 5, 10 or 50 mg/L vanillic acid, the *cmle* mutant germinated more efficiently than the wild-type. At the highest concentration (50 mg/L), the germination rates of both the wild-type and *cmle* mutant decreased to a similar extent (Fig. 3C). No difference in germination rate between the wild-type and the *cmle* mutant was observed in the presence of 4-hydroxybenzaldehyde (Fig. 3D).

Higher concentrations of 4-hydroxybenzaldehyde led to aberrant growth of the wild-type strain and were therefore not tested in the germination assay. In summary, no enhanced germination rate of the wild-type in the presence of the aromatic compounds tested could be observed, and deletion of *CMLE* led to a decrease in germination rate in the presence of high concentrations of coumaric and ferulic acid, and to an increased germination rate in the presence of vanillic acid.

CMLE is required for *in planta* growth, but not for superficial root colonization

To assess whether the inactivation of *CMLE* affects the colonization behaviour of *F. oxysporum*, tomato roots were infected with wild-type and mutant strains and colonization was analysed using confocal fluorescence microscopy. The insertional mutagenesis mutants 72C7 and 75H3 were used to assess superficial colonization. These mutants contain, on their T-DNA, a translational fusion of the hygromycin B resistance gene and a cytoplasmic green fluorescence protein (GFP) gene driven by the *gpd* promoter (Michielse *et al.*, 2009a). Ectopic complementation of both insertional mutagenesis mutants with the *CMLE* gene restored pathogenicity to the wild-type level (Fig. S4, see Supporting Information), indicating that only the mutation in the *CMLE* gene is responsible for the observed reduced pathogenicity of the mutants

Fig. 3 Conidial germination of the *cmle* mutant is reduced on coumaric and ferulic acid, but increased on vanillic acid. Germination rates of the wild-type strain and a *cmle* mutant on various concentrations of coumaric acid, ferulic acid, vanillic acid and 4-hydroxybenzaldehyde, as indicated, were determined by inoculating 600 spores per well in a 24-well plate containing 250 μ L of 2% water agar. After an overnight incubation at 4 °C and a subsequent incubation of 6 h at 25 °C, the germinated conidia were counted. Filled squares, wild-type; open squares, *cmle* deletion mutant. *CMLE*, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme.

Fig. 4 The *cmle* mutant is not impaired in root colonization. Nine- to eleven-day-old seedlings were inoculated with fungal spore suspensions of a *cmle* mutant (72C7) or a control strain without pathogenicity defect (38D9), both expressing cytoplasmic green fluorescence protein (GFP). Images show confocal micrographs taken 2 and 3 days after inoculation. *CMLE*, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme.

and that these mutants can therefore be used to analyse the colonization behaviour of *F. oxysporum* in the absence of a functional *CMLE* gene. As control, a pathogenic GFP-tagged strain (38D9) was used. The insertional mutants were both still able to germinate and colonize tomato roots and no difference could be observed between the mutants and the control strain 3 days after inoculation (Fig. 4).

In planta growth of these mutants was assessed using the *psixGFP* reporter system (van der Does *et al.*, 2008). In this system, *GFP* expression will only be visible in hyphae that have penetrated the root cortex (van der Does *et al.*, 2008). To this end, the *psixGFP* reporter construct was introduced into the *cmle* deletion mutant (*CMLEKO6*). Introduction of this construct did not affect the non-pathogenicity phenotype of the mutant (Fig. S5, see Supporting Information) and, on incubation of the *cmle-psixGFP* strain together with MSK8 tomato cells, a clear *GFP* signal was visible (Fig. S6, see Supporting Information), indicating the functionality of this expression system in a *CMLE* deletion background. *In planta* growth of the *cmle* mutant expressing *GFP* driven by the *six1* promoter was monitored over time. As a control, the colonization behaviour of the original strain expressing *GFP* driven by the *SIX1* promoter (van der Does *et al.*, 2008) was analysed. As observed earlier, for the latter strain, an increase in hyphae expressing *GFP* could be observed, ranging from just penetrated hyphae at the earliest time point to intracellular growth and xylem colonization at later time points (Fig. 5, right panels). In contrast, no *GFP*-expressing hyphae could be observed in roots infected with the *cmle* mutant (Fig. 5, left panels). On the basis of these experiments, we conclude that *CMLE* is not required for superficial root colonization on tomato roots, but is required for *in planta* growth.

The *cmle* mutant displays biocontrol activity

To further investigate whether the *cmle* mutant is capable of normal superficial root colonization, its biocontrol activity, which

is dependent on the colonization rate, was determined. It has been shown that *F. oxysporum* f. sp. *lycopersici* exerts biocontrol activity on flax when added in a 100-fold excess relative to an *F. oxysporum* strain pathogenic towards flax (Alabouvette *et al.*, 2009). Therefore, flax cv. Viking was inoculated with a flax pathogenic strain (Foln3) alone or in a 1:100 combination with the *F. oxysporum* f. sp. *lycopersici* wild-type strain or the *cmle* mutant. As controls, the plants were mock inoculated or inoculated with the flax pathogenic strain in a 1:100 combination with the biocontrol strain Fo47. The addition of the biocontrol strain Fo47, as well as the addition of the *F. oxysporum* f. sp. *lycopersici* wild-type strain or the *cmle* deletion mutant, delayed the development of the first disease symptoms by 3 days (Fig. 6). After 56 days, the plants treated with the pathogenic strain all developed disease symptoms. However, significant decreases in wilting symptoms (at a 95% confidence interval) of 67% and 77% were observed in plants inoculated with the pathogenic flax strain in combination with the *F. oxysporum* f. sp. *lycopersici* wild-type or *cmle* mutant, respectively. Thus, both the wild-type and *cmle* mutant display a similar degree of biocontrol activity, and it is thus likely that both strains display a similar root colonization behaviour.

DISCUSSION

Plant root cell wall lignification and the release of root exudates with antimicrobial properties have been commonly accepted as defence mechanisms against soil pathogens (Bais *et al.*, 2006; Mandal *et al.*, 2010; Okubara and Paulitz, 2005). Fungi are able to degrade lignin with the aid of extracellular oxidative enzymes, such as laccases, lignin peroxidases, manganese peroxidases and mycelium-associated dehydrogenases (Bugg *et al.*, 2011; Martinez *et al.*, 2005). The products of lignin degradation, such as aromatic carboxylic acids and aromatic acids, are further degraded through the β -ketoadipate pathway and eventually incorporated into intracellular catabolic routes (Harwood and Parales, 1996). In this

Fig. 5 The *cmle* mutant is impaired in *in planta* growth. Nine- to eleven-day-old seedlings were inoculated with fungal spore suspensions of the *CMLE* KO6 or *six1* deletion mutant, both expressing green fluorescence protein (GFP) driven by the *six1* promoter. Images of tomato roots were taken with a fluorescence microscope at the indicated times after inoculation. The *SIX1* promoter is only active inside plant tissue; therefore, only fungal structures inside roots are fluorescent. *CMLE*, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme.

study, we have characterized an *F. oxysporum* f. sp. *lycopersici* mutant carrying a deletion in *CMLE*, coding for an enzyme of the β -ketoadipate pathway, and provide evidence for a role of this enzyme in root invasion.

Deletion of the *CMLE* gene in *F. oxysporum* resulted in a non-pathogenic phenotype towards tomato plants. Microscopic analysis revealed that the nonpathogenic phenotype was not a result of reduced root attachment or superficial root colonization, as the *cmle* mutant was equally efficient as the wild-type in colonizing tomato roots. This was further confirmed by biocontrol experiments; the *cmle* mutant was capable of exerting biocontrol activity to the same extent as the wild-type, indicative of a similar root colonization behaviour. However, the *cmle* mutant appeared not to be able to grow *in planta*. Although the wild-type strain is capable of growing intercellularly and extensively colonized the xylem

within 4 days after infection, the *cmle* mutant did not display any *in planta* growth. In root-dip pathogenicity assays, tomato plants with minor disease symptoms (disease index 1 or 2) on infection with a *cmle* deletion background were occasionally observed (four of 414 plants). This indicates that, on very rare occasions, the *cmle* mutant is capable of causing slight browning of the xylem vessel in the hypocotyl, possibly as a result of some *in planta* growth. What is clear, however, is that the *cmle* mutant is at least severely impaired in *in planta* growth.

Tomato roots respond to *F. oxysporum* infection by lignification of the root cell wall and increased concentrations of phenolic compounds in root exudates (Mandal and Mitra, 2007, 2008; Panina *et al.*, 2007). It could be that the nonpathogenic phenotype of the *cmle* mutant is a result of its inability to degrade these phenolic compounds or, alternatively, to penetrate lignified cell

Fig. 6 The *cmle* mutant and its parental strain exhibit biocontrol activity on flax. The protective capacity of the *cmle* mutant and its parental strain was determined on flax. Flax cv. Viking was inoculated with the pathogenic isolate *Fusarium oxysporum* f. sp. *lini* (10^4 conidia/mL) alone or in combination with the biocontrol strain (Fo47), *cmle* mutant or the wild-type strain (10^6 conidia/mL). There were three replicates of 16 individual plants per treatment. *Fusarium* wilt incidence is expressed as the percentage of wilted plants. Filled diamonds, uninoculated control; filled squares, *F. oxysporum* f. sp. *lini*; open squares, *F. oxysporum* f. sp. *lini* and biocontrol strain Fo47; open circles, *F. oxysporum* f. sp. *lini* and *cmle* mutant; filled circles, *F. oxysporum* f. sp. *lini* and the wild-type strain 4287. CMLE, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme.

walls. Analysis of the growth phenotype of the *cmle* mutant in the presence of various carbon and nitrogen sources using the BIOLOG assay and in the presence of various phenolic compounds revealed that the general fitness of the *cmle* mutant is not impaired. The mutant can grow on all mono-, di- and polysaccharides, carboxylic acids, sugar alcohols and amino acids present in the BIOLOG plate. However, in the same assay, the growth of the *cmle* mutant on amygdalin and quinic acid was severely reduced. Amygdalin, a cyanogenic glycoside widely present in plant species, is metabolized into benzaldehyde, hydrogen cyanide and glucose in fungi (Brimer *et al.*, 1996; Davis, 1991). As *F. oxysporum* is capable of detoxifying hydrogen cyanide (Periera *et al.*, 1997), it can be speculated that the reduced growth of the *cmle* mutant on amygdalin is a result of its inability to metabolize benzaldehyde. This compound and quinic acid are both metabolized in eukaryotes through the β -ketoadipate pathway (Harwood and Parales, 1996). Reduced growth of the mutant was also observed on the phenolic compounds ferulic acid, coumaric acid, vanillic acid, cinnamic acid, 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde, all compounds which are degraded via the protocatechuate branch of the β -ketoadipate pathway in fungi (Cain *et al.*, 1968; Harwood and Parales, 1996; Middelhoven, 1993; Sparrins *et al.*, 1979). As expected, growth of the *cmle* mutant was not impaired on catechol, which is degraded via the catechol branch of the β -ketoadipate pathway and does not require CMLE (Harwood and

Parales, 1996). In addition, growth of the *cmle* mutant was significantly reduced on lignin. It could be that the *cmle* mutant is impaired in lignin degradation or that the intermediate phenolic aromatic breakdown products accumulate, potentially reaching toxic levels. The reduced growth on ferulic acid, coumaric acid, vanillic acid, 4-hydroxybenzoic acid and 4-hydroxybenzaldehyde, observed in the presence of sucrose, suggests that these compounds, even though they can serve as a carbon source, can inhibit the growth of *F. oxysporum*. Wu *et al.* (2009b, d) described a concentration-dependent effect of sinapic acid and benzoic acid on conidial germination of *F. oxysporum* f. sp. *niveum*. In our study, no positive effect on germination on addition of coumaric acid, ferulic acid, vanillic acid or 4-hydroxybenzaldehyde was observed for the wild-type strain or the *cmle* mutant. In arbuscular mycorrhizal symbiosis, low concentrations of phenolics can have a stimulating effect on fungal growth and a positive effect on colonization, but, at higher concentrations, the same phenolics can have inhibitory effects on fungal pathogens (Mandal *et al.*, 2010). In line with this, we observed that the germination rates of the *cmle* mutant decreased with increased concentrations of coumaric and ferulic acid. This implies that the inability of the *cmle* mutant to degrade these phenolics and/or their toxic effect could contribute to the nonpathogenic phenotype of the mutant. However, as the mutant is unaffected in its ability to colonize roots superficially, the level of phenolic compounds in root exudate alone cannot explain its nonpathogenic phenotype.

The *cmle* mutant is equally efficient as the wild-type in breaking down polygalacturonide, a carbohydrate component of the plant cell wall, and in the penetration of cellophane (data not shown), an assay used as an indication of plant cell wall penetration (Prados Rosales and Di Pietro, 2008). This suggests that the breaching of the cell walls *per se* is not severely affected in the mutant. Possibly, local release of phenolic compounds from lignified cell walls and secreted by cells at sites of attempted penetration are of a level that prevent further ingrowth of the mutant. Local release of high concentrations of phenolic compounds could involve phenolic-storing cells that have been shown to be present in root tissue of several plant species, including tomato. On vascular infection by *F. oxysporum*, these phenolic-storing cells release their content (Beckman and Halmos, 1962; Mace *et al.*, 1972; Waggoner and Diamond, 1956).

In conclusion, we have shown that the β -ketoadipate pathway in *F. oxysporum* is required for root invasion and for the degradation of several phenolic compounds. Such compounds are known to increase in concentration in root exudates on pathogen attack and some are intermediates of lignin breakdown. As the β -ketoadipate pathway is well conserved in the fungal kingdom and the up-regulations of phenolic compounds and cell wall lignification are common defence responses of plants, it is possible that other plant pathogens also rely on the β -ketoadipate pathway for successful infection.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Fusarium oxysporum f. sp. *lycopersici* strain 4287 (race 2; FGSC9935) was used in this study. It was stored as a monoclinal culture at -80°C and revitalized on potato dextrose agar (PDA, Difco, Le Pont de Claix, France) at 25°C . For *Agrobacterium*-mediated transformation of *F. oxysporum*, *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993) was used and was grown in 2x yeast extract tryptone medium (Sambrook and Russel, 2001) containing 20 mg/mL rifampicin at 28°C . Introduction of the plasmids into the *Agrobacterium* strain was performed by electroporation, as described by Mattanovich *et al.* (1989). *Escherichia coli* DH5 α (Invitrogen, Bleiswijk, the Netherlands) was used for the construction, propagation and amplification of the plasmids, and was grown in Luria–Bertani (LB) medium at 37°C containing either 100 mg/mL ampicillin or 50 mg/mL kanamycin, depending on the resistance marker of the plasmid used.

Construction of deletion and complementation constructs

The *CMLE* gene deletion construct was generated by amplification of 933-bp upstream and 722-bp downstream fragments from genomic DNA by PCR with the primer pairs FP1825 (CCCAAGCTTCAAGGATCGCCATGACTGC)–FP1783 (AGGGCGCGCGAGTGCAGGTGCGATACAAG) and FP1782 (GGGGTACCCTGCTGTCATCAATGCTGCG)–FP1781 (CCTTAATTAAGATGAGTATTACTGACCACAC), respectively. The PCR products were cloned into pGEM-T easy (Promega, Leiden, the Netherlands), and *HindIII*/*Ascl* upstream and *KpnI*/*PacI* downstream fragments were sequentially cloned into pRW2h (Houterman *et al.*, 2008), resulting in pCMLEKO.

The *CMLE* complementation construct, pCMLEcom, was generated by amplification of a 3032-bp fragment containing the *CMLE* open reading frame, including 970-bp upstream and 872-bp downstream sequences, with the primer pair FP1989 (TTGGCGCGCCCAAGGATCGCCATGACTGC)–FP1990 (AAACTGCAGGATGAGTATTACTGACCACA). The PCR fragment was cloned into pGEM-T easy and an *Ascl*/*PstI* *CMLE* complementation fragment was subsequently cloned into pRW1p (Houterman *et al.*, 2008).

Fungal transformation, plant infections and Southern analysis

Agrobacterium-mediated transformation of *F. oxysporum* f. sp. *lycopersici* was performed as described by Mullins *et al.* (2001) with minor adjustments (Takken *et al.*, 2004). *CMLE* gene deletion transformants were selected on Czapek Dox agar (CDA, Oxoid, Basingstoke, Hampshire, UK) containing 100 mg/mL hygromycin (Duchefa, Haarlem, the Netherlands), and *CMLE* complementation transformants and transformants harbouring pPZpsixGFP (van der Does *et al.*, 2008) were selected on CDA containing 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8, and 100 mg/mL Zeocin (InvivoGen, Toulouse, France).

Pathogenicity assays were performed on plant line MoneyMaker ss590 (Gebr. Eveleens b.v., the Netherlands). Nine- to eleven-day-old seedlings were infected following the root-dip inoculation method (Wellman, 1939). The disease index was scored and statistical analysis was performed as described earlier (Michiels *et al.*, 2009a). Biocontrol assays were per-

formed on flax (*Linum usitatissimum*) cv. Viking using *F. oxysporum* f. sp. *lini* (Foln3, MYA-1201), as described earlier (Michiels *et al.*, 2009b).

Genomic DNA isolation and Southern blotting was performed as described earlier (Michiels *et al.*, 2009b). Genomic DNA was digested with *Bgl*II and, as a probe, a 440-bp PCR fragment obtained with primers FP2090 (CCAATTCAACGATCAATGAAG) and FP2091 (GAGAGCCATGCTATCATGAC), corresponding to the *CMLE* upstream region, was used.

Growth and germination assays

To assess the growth of the wild-type and the various mutants on different carbon sources and aromatic compounds, microconidia were isolated from liquid minimal medium (3% sucrose, 10 mM KNO₃ and 0.17% yeast nitrogen base without amino acids and ammonia) grown for 5 days on a rotary shaker at 25°C . The conidia were harvested through miracloth (CalBiochem, Darmstadt, Germany) and washed twice with sterile water. The conidial concentration was determined with a Bürker-Türk haemocytometer.

A BIOLOG FF MicroPlate (BIOLOG, Hayward, CA, USA) was used to test carbon source utilization. A conidial suspension (150 μL) was inoculated in each well of the plate (10^4 conidia per well), followed by incubation at 25°C . The absorbance of each well was measured with a microtitre plate reader (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL, USA) after 4 days of incubation. Verification of the BIOLOG results was performed on buffered minimal medium [10 mM KNO₃, 0.17% yeast nitrogen base without amino acids and ammonia, 10 mM 2-(*N*-morpholino)ethanesulphonic acid (Mes), pH 5.3, and 1.5% agar] supplemented with 1% sucrose, amygdalin (500 mg/L) or quinic acid (500 mg/L).

Radial growth of the wild-type and various mutants was tested in quintuplet on buffered minimal medium and on buffered PDA (10 mM Mes, pH 5.3) supplemented with one of the following compounds: ferulic acid (100 mg/L), 4-hydroxybenzoic acid (50 mg/L), catechol (100 mg/L), coumaric acid (100 mg/L), cinnamic acid (10 mg/L), vanillic acid (10 mg/L), 4-hydroxybenzaldehyde (50 mg/L), lignin (50 mg/L) or hairy roots (1%). Minimal medium supplemented with 1% sucrose was used as a positive control. A mycelial plug from a 5-day-old PDA plate was used to inoculate the various plates and, after an incubation of 5 days at 25°C , the colony diameter was measured.

Germination rates in the presence of coumaric acid (10, 50, 100 mg/L), ferulic acid (10, 50, 100 mg/L), vanillic acid (1, 5, 10, 50 mg/L) and 4-hydroxybenzaldehyde (1, 10, 50 mg/L) were analyzed in sextuplet. To this end, 50 μL of a 1.2×10^4 conidial suspension were added to a 24-well plate, with each well containing 250 μL of 2% water agar, and incubated overnight at 4°C . After an incubation of 6 h at 25°C , the germinated conidia were counted. Statistical analysis [analysis of variance (ANOVA) and Fisher's *post hoc* test] was performed using StatView™SE+ v1.03.

Microscopic analysis

A Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Sliedrecht, the Netherlands) was used to monitor root colonization by the wild-type and *cmle* deletion mutant. Excitation was provided with argon (488 nm) and helium–neon (543 nm) lasers; dichroic beam splitters were used to divide the fluorescence emission into a GFP channel and a channel in which the autofluorescence of the plant cells was detected. Images were

scanned using a water objective C-apochromat 40×/1.2. Pictures were analysed with Zeiss LSM510 and ImageJ software. For preparation of the slides, 9-day-old tomato seedlings were carefully removed from the potting soil, rinsed with water, and intact roots were inoculated with 20 mL of tap water containing 10⁷ conidia/mL and incubated for 2–3 days at room temperature in a Petri dish. The roots were rinsed with water, cut from the hypocotyl, placed in a drop of water on a glass slide and covered with a cover glass. A bridge mounted on the glass slide prevented squashing of the root material.

Root invasion by *F. oxysporum* strains expressing GFP under the control of the *SIX1* promoter was monitored using an EVOS digital inverted microscope with a GFP light cube (AMG—Advanced Microscopy Group, Bothell, WA, USA). For preparation of the slides, 10-day-old tomato seedlings were carefully removed from the potting soil, rinsed with water, and intact roots were inoculated with 20 mL of tap water containing 10⁷ conidia/mL and incubated for 1–4 days at room temperature in a Petri dish. The roots were rinsed with water, cut from the hypocotyl, placed in a drop of water on a glass slide and covered with a cover glass. Each day, entire root systems of seedlings were monitored for fungal invasion.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Gene deletion and complementation strategy. A gene deletion and complementation construct was introduced into wild-type and various *cmle* mutants, respectively. (A) Schematic representation of the *CMLE* gene (gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme) deletion strategy drawn to scale. The arrow heads indicate the original T-DNA insertion sites in the mutants 72C7 and 75H3. The T-DNA of the *CMLE* gene deletion construct harbours the hygromycin resistance cassette (*hph*) flanked by 933-bp *CMLE* upstream and 722-bp *CMLE* downstream sequences. (B) Schematic representation of the *cmle* complementation strategy drawn to scale. T-DNA of the *cmle* complementation construct harbours the phleomycin resistance cassette (*BLE*) and *CMLE* open reading frame flanked by 970-bp upstream and 872-bp downstream sequences. The striped box indicates the probe used in Southern analysis. B, *Bgl*II restriction site.

Fig. S2 Southern analysis of the insertional mutagenesis mutants 72C7 and 75H3 and the *CMLE* deletion and complementation mutants. Genomic DNA was digested with *Bgl*II, blotted and hybridized with a 440-bp probe corresponding to the *CMLE* (gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme) upstream region. The *CMLE* locus in the wild-type strain is visible as a 1.5-kb fragment. As expected, this 1.5-kb band shifted to 11.3 kb in the *cmle* deletion mutants because of homologous recombination at the *CMLE* locus. Restoration of the locus by homologous recombination in the complemented strains resulted again in the 1.5-kb fragment. In the insertional mutagenesis mutants 72C7 and 75H3, the wild-type 1.5-kb fragment is replaced by ~8-kb and ~6.5-kb fragments, respectively, because of the presence of the T-DNA in the *CMLE* locus. Ectopic complementation of these insertional mutagenesis mutants led to an additional band of 1.5 kb corresponding to the *CMLE* gene. The 1-kb ladder of Fermentas (St. Leon-Rot, Germany) is used as a marker.

Fig. S3 Deletion of *CMLE* (gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme) leads to reduced growth on amygdalin, D-galacturonic acid and quinic acid in the BIOLOG plate assay. BIOLOG FF MicroPlates were inoculated with conidial spore suspensions of wild-type, *cmle* mutant or complemented strain (10⁴ spores per well). After 4 days of incubation at 25 °C, the optical density was measured at 600 nm.

Fig. S4 Complementation of the *cmle* (*CMLE*, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme) insertional mutagenesis mutants restores pathogenicity. Nine- to eleven-day-old tomato seedlings were inoculated with fungal spore suspensions

using root-dip inoculation, and the disease index (0, healthy plant; 4, severely diseased plant or dead plant) was scored after 3 weeks. Error bars indicate the standard deviation and capitals define statistically significantly different groups [analysis of variance (ANOVA), $P = 0.95$]. Average disease index of 20 plants, 3 weeks after mock inoculation (H_2O), inoculation with five independent *cmle* 72C7 complemented strains (72C7com12, 15, 20, 42 and 47), five independent *cmle* 75H3 complemented strains (75H3com2, 5, 7, 12 and 15), insertional mutants 72C7 and 75H3 or wild-type (4287).

Fig. S5 Green fluorescence protein (GFP) driven by the *six1* promoter does not affect the pathogenicity defect of the *cmle* mutants (*CMLE*, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme). Nine- to eleven-day-old tomato seedlings were inoculated with fungal spore suspensions using root-dip inoculation, and the disease index (0, healthy plant; 4, severely diseased plant or dead plant) was scored after 3 weeks. Error bars indicate the standard deviation and capitals define statistically significantly different groups [analysis of variance (ANOVA), $P = 0.95$]. Average disease index of 20 plants, 3 weeks after mock inocula-

tion (H_2O), inoculation with four independent *cmle sixGFP* strains (*CMLE* KO6-*sixGFP*4, 7, 12 and 29), insertional mutants 72C7 and 75H3 or wild-type (4287).

Fig. S6 The *cmle* mutant (*CMLE*, gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme) expresses green fluorescence protein (GFP) driven by the *six1* promoter in plant cell cultures. Spore suspensions of $\Delta six1$ -*psix1::GFP* or $\Delta cmle$ -*psix1::GFP* were incubated with tomato MSK8 plant cells. Images were taken 24 h post-inoculation with a BX50 fluorescence microscope with the appropriate excitation and emission filters for GFP (Olympus, Hamburg, Germany).

Table S1 Growth ratio of *CMLE* (gene encoding 3-carboxy-*cis,cis*-muconate lactonizing enzyme) knockout and complementation mutants in the BIOLOG assay.

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