Fusaric acid contributes to virulence of *Fusarium oxysporum* on plant and mammalian hosts

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SUMMARY

Fusaric acid (FA) is among the oldest identified secondary metabolites produced by *Fusarium* species, known for a long time to display strong phytotoxicity and moderate toxicity to animal cells; however, the cellular targets of FA as well as its function in fungal pathogenicity remain unknown. Here, we investigated the role of FA in *Fusarium oxysporum*, a soil-borne cross-kingdom pathogen that causes vascular wilt on more than a hundred plant species and opportunistic infections in humans. Targeted deletion of *fub1*, encoding a predicted orthologue of the polyketide synthase involved in FA biosynthesis in *F. verticillioides* and *F. fujikuroi*, abolished production of FA and its derivatives in *F. oxysporum*. We further show that expression of *fub1* is positively controlled by the master regulator of secondary metabolism LaeA and the alkaline pH regulator PacC through modulation of chromatin accessibility at the *fub1* locus. FA exhibited strong phytotoxicity on tomato plants, which was rescued by exogenous supply of copper, iron or zinc, suggesting a possible function of FA as a chelating agent of these metal ions. Importantly, severity of vascular wilt symptoms on tomato plants and mortality of immunosuppressed mice were significantly reduced in the *fub1*Δ mutants and fully restored in the complemented strains. Collectively, these results provide new insights on the regulation and mode of action of FA as well as on the function of this phytotoxin during the infection process of *F. oxysporum*.

INTRODUCTION

Fungi produce major plant diseases and destroy or contaminate each year a significant part of the global agricultural production, making them by far the most damaging class of plant pathogens (Strange & Scott, 2005, Fisher *et al.*, 2012). Moreover, opportunistic fungal pathogens of humans can provoke life-threatening systemic infections, particularly on immunocompromised patients (Fridkin, 2005). The soil-inhabiting fungus *Fusarium oxysporum* has been ranked in the Top 10 fungal pathogens in molecular plant pathology based on scientific/economic importance and causes vascular wilt disease in more than 100 different crops (Armstrong & Armstrong, 1981, Dean *et al.*, 2012). In addition, *F. oxysporum* isolates can cause opportunistic infections in humans ranging from superficial or locally invasive to disseminated, depending on the immune status of the host (Nucci & Anaissie, 2007). *F. oxysporum* f. sp. *lycopersici* FGSC 9935 (FOL 4287) is a fully sequenced isolate (Ma *et al.*, 2010) able to kill both tomato plants and immunosuppressed mice (Ortoneda *et al.*, 2004). Therefore, this isolate represents

an excellent model for studying the genetic basis of cross-kingdom pathogenicity in fungi.

Many fungi produce secondary metabolites that are toxic to plants or animals (Berthiller *et al.*, 2013). Fusaric acid (FA), a picolinic acid derivative originally isolated from *Fusarium heterosporium* (Yabuta *et al.*, 1937), was the first fungal phytotoxin isolated from infected host plants (Gäumann, 1957) and is known for its high phytotoxicity (Niehaus *et al.*, 2014, Stipanovic *et al.*, 2011). FA also exhibits toxicity towards animals including notochord malformation in zebrafish (Yin *et al.*, 2015) or neurotoxicity in mammals (Porter *et al.*, 1995), and towards bacteria (Bacon *et al.*, 2006, Ruiz *et al.*, 2015). Although several studies on the mode of action of FA have been conducted, the cellular basis for its toxicity remains poorly understood. Suggested mechanisms include the modification of cell membrane potential, inhibition of ATP synthesis, chelation of metal ions or electrolyte leakage (D'Alton & Etherton, 1984, Pavlovkin, 1998, Ruiz et al., 2015, Marrè *et al.*, 1993). Recently, chromatin condensation, cytochrome *c* release, DNA fragmentation and hydrogen peroxide accumulation were reported in FA treated plant cell cultures suggesting a possible involvement of programmed cell death in FA toxicity (Jiao *et al.*, 2013, Samadi & Shahsavan Behboodi, 2006).

The polyketide synthase (PKS) Fub1 was recently identified as the first enzyme of the FA biosynthetic pathway in *F. verticillioides* (Brown *et al.*, 2012). The *fub1* gene is part of the FA gene cluster, and its inactivation is sufficient to completely block FA production (Brown et al., 2012, Niehaus et al., 2014). In the present work we studied the role of Fub1 in *F. oxysporum*. We found that *fub1* is essential for production of FA and its derivatives in this fungus, and that its transcription is positively regulated by LaeA, a master regulator of secondary metabolism, and the alkaline pH regulator PacC. We further demonstrate that loss of Fub1 and FA in *F. oxysporum* leads to reduced virulence in tomato plants and immunodepressed mice. Finally, we show that phytotoxicity of FA can be reduced by supplying copper, iron or zinc to the plants. Our results establish a functional role for FA in fungal virulence on plants and mammals.

RESULTS

Inactivation of the polyketide synthase Fub1 abolishes FA production in *F. oxysporum*

A BlastP search in the *Fusarium* Comparative Database (Broad Institute) using Fub1 from *F. fujikuroi* (FFUJ_02105) as a bait identified a single predicted Fub1 orthologue

(FOXG_15248) displaying 89% overall identity with the query protein. Manual inspection of the *F. oxysporum fub1* locus identified all other members of the FA gene cluster previously described in *F. verticillioides* (Brown et al., 2012) and *F. fujikuroi* (Niehaus et al., 2014) (Fig. S1A). Interestingly, two additional putative genes were present between *fub3* and *fub4* in different *F. oxysporum* isolates, including the reference strain FOL 4287 (Fig. S1A) (Brown *et al.*, 2015). Both genes are neighbors in other *Fusarium* species, but not located in the FA gene cluster. For example, in *F. fujikuroi* the orthologues of these two genes, *FFUJ_11046* and *FFUJ_11047*, are located on chromosome 10 while the FA gene cluster is located on chromosome 3 (Fig. S1A). It is currently unknown whether the insertion of these two additional genes has any effect on the FA gene cluster.

Recently, additional components of the cluster, including two $Zn(II)_2Cys_6$ transcription factors, have been identified in different *Fusarium* species (Studt *et al.*, 2016, Brown et al., 2015). To study the role of Fub1 in FA production by *F. oxysporum*, we replaced the entire *FOXG_15248* coding sequence with the hygromycin B resistance gene (*hph*^{r}), generating several *fub1*Δ strains (Fig. S1B and S1C). To determine whether FOXG_15248 was responsible for FA production in *F. oxysporum*, extracts from cultures of the different strains grown on potato dextrose agar (PDA) or czapek-dox agar (CDA) were analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS/MS). This approach allows reliable and sensitive quantification of several hundred of fungal analytes, including almost all mycotoxins for which standards are commercially available (Malachova *et al.*, 2014). FA and its derivative fusarinolic acid (FnA) were detected in wild-type extracts but not in those of the *fub1*Δ mutant (Fig. 1A). Interestingly, the total amount of mycotoxin (FA + FnA) was approximately 2.5 times higher in CDA than in PDA cultures, with a FA:FnA ratio of 29.5:1 in the former and of 1:2.75 in the latter (Fig. 1A). These data are consistent with those reported in *F. fujikuroi* (Niehaus et al., 2014). In addition to FA and FnA, beauvericin (Bea) and bikaverin (Bik) were detected in all samples. Interestingly, both compounds were more abundant in the *fub1*Δ cultures compared to the wild-type, especially in CDA (Fig. 1B). Reintroduction of the intact *fub1* allele into *fub1*Δ, yielding the complemented *fub1*Δ^C strain (Fig. S2), fully restored the wild-type FA levels (Fig. 1). Thus, Fub1 is responsible for production of FA and its derivatives in *F. oxysporum*.

Next, we tested the potential toxicity of FA on *F. oxysporum*. When the wild-type strain or *fub1*Δ were cultured on PDA supplemented with 0.25 or 0.5 mg/ml FA, both showed a significant and comparable reduction in radial growth, while no growth was detectable at 0.75 mg/ml FA (Fig. S3).

Effect of pH and nutrients on *fub1* transcript levels and FA production

In *F. fujikuroi*, *fub1* transcription is positively regulated by the pH response factor PacC at pH 8, but not at pH 4 (Niehaus et al., 2014). We noted that CDA has an initial pH of 6.8 ± 0.2 while PDA has about 5.6 ± 0.2. Moreover, pH in CDA, where $NaNO_3$ is the sole nitrogen source, tended to increase during fungal growth (data not shown). To discriminate between the effects of media composition and pH on FA biosynthesis, we germinated conidia of the wild-type strain in potato dextrose broth (PDB) and transferred the germlings to czapek-dox liquid (CDL) or fresh PDB buffered either to pH 5 or 7 (see experimental procedures for details). Unexpectedly, in both media *fub1* transcription and FA production were much higher under moderate acidic conditions, although as expected, CDL induced more FA (Fig. 2A and 2B). The effect of pH on FA production was stronger than that of the medium composition, as reflected by the finding that FA production was higher in PDB at pH 5 than in CDL at pH 7 (Fig. 2A and 2B). Our data indicate that both pH and nutrients are important factors in the regulation of FA biosynthesis, and that this regulation may differ between *F. oxysporum* and *F. fujikuroi*. We next tested the role of PacC in *fub1* regulation using a *pacC* loss-of-function mutant (Caracuel *et al.*, 2003). When germlings of the different strains were grown in Glutamine minimal medium (GMM) buffered either to pH 5 or 7, *fub1* transcript levels were 10 times lower in *pacC*Δ compared to the wild-type at both pH values (Fig. 2C). Thus, PacC functions as a positive regulator of *fub1* within this pH range.

Chromatin structure at the *fub1* locus is controlled by the global regulator of secondary metabolism LaeA and the pH response factor PacC

While the *fub1*Δ mutants did not show a detectable growth defect on PDA or CDA, we noted that their growth in the presence of hygromycin B was markedly reduced (Fig. 3A and S4). Interestingly, the complemented *fub1*Δ^C strains showed a similar growth defect on hygromycin, whereas the transformants carrying an ectopic insertion of the knockout construct (Ect) (Fig. S1C) did not (Fig. 3A and S4). We hypothesized that this phenotype could be caused by a chromatin regulatory effect on transcription of the *hph* hygromycin resistance gene inserted at the *fub1* locus. In line with this hypothesis, we found that *hph* transcript levels were between 30 and 50 times higher in Ect than in *fub1*Δ and *fub1*Δ^C (Fig. 3B). LaeA is a global regulator of secondary metabolite gene

clusters in different fungi (Bok & Keller, 2004, Wiemann *et al.*, 2010, Butchko *et al.*, 2012, Lopez-Berges *et al.*, 2013) and have been previously reported to regulate FA production in *Fusarium* (Niehaus et al., 2014, Lopez-Berges et al., 2013). Transcript levels of *fub1* in the wild-type and the *laeA*Δ^{C} strains were between 300 and 500 times higher than in the *laeA*Δ mutant (Fig. 3C). We next examined the role of LaeA in chromatin remodeling and transcriptional regulation at the *F. oxysporum fub1* locus, using real-time qPCR with promoter- and gene-specific primers (Fig. 4A) on genomic DNA obtained from mycelia treated with micrococcal nuclease (MNase) (Fig. S5). Relative chromatin accessibility, calculated as the ratio of amplification from untreated versus MNase-treated mycelia, was about 6 times higher in wild-type and *laeA*Δ^{C} compared to *laeA*Δ (Fig. 4B). Moreover, relative chromatin accessibility was significantly lower at pH 7 in comparison with pH 5, and in a *pacC*Δ strain at both pH values compared to the wild-type (Fig. 4C and 4D), in line with the previous finding that *fub1* transcript levels are lower at pH 7 and in *pacC*Δ (Fig. 2A and 2C). We conclude that chromatin accessibility and transcription at the *fub1* locus, as well as production of FA and derivatives, are positively regulated by LaeA, moderate acidic pH and PacC.

fub1 and FA are not required for growth of *F. oxysporum* under Cu, Fe or Zn limiting conditions

The ability of FA to chelate metal ions like iron or copper has been known for a long time (Tamari & Kaji, 1952, Malini, 1966, Lakshminarayanan & Subramanian, 1955, Pan *et al.*, 2010). In a recent study, FA was shown to chelate different metal ions including Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+} and Zn^{2+} (Ruiz et al., 2015, Yin et al., 2015). We thus asked whether production of FA is required for growth of *F. oxysporum* under metal limiting conditions. Depletion of copper, iron or zinc was achieved by the addition of the specific chelators bathocuproinedisulfonic acid disodium salt (BCS), bathophenanthrolinedisulfonic acid disodium salt (BPS) and N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), respectively (Fig. 5A). Unexpectedly, although copper is an essential micronutrient in most living organisms (Vulpe & Packman, 1995), we observed no detectable growth defect in *F. oxysporum* grown under copper limitation (Fig. 5A), even in the presence of BCS concentrations up to 1 mM (data not shown). A similar result was previously reported in *Aspergillus fumigatus* (Park *et al.*, 2014). By contrast, depletion of iron and zinc resulted in severe growth defects as expected. In any case, inactivation of Fub1 had no additional effect on growth (Fig. 5A). Next, we asked whether Fub1 was required for fungal growth at toxic concentrations of

Cu, Fe or Zn. When the wild-type and *fub1*Δ strains were grown on GMM with up to 5 mM of the different metal ions, no significant differences were observed between strains (Fig. 5B). These results demonstrate that FA is not essential for growth under limiting or toxic concentrations of copper, iron or zinc. However, we noted that transcript levels of *fub1* were significantly reduced in the presence of these three metal ions (Fig. 5C).

FA toxicity in tomato plants is reversed by exogenous addition of Cu, Fe and Zn

Production and phytotoxic properties of FA have been studied over the past 75 years (Yabuta et al., 1937, Dong *et al.*, 2014, Bacon *et al.*, 1996, Gäumann, 1957, Gäumann, 1958) and a number of mechanisms for FA toxicity have been suggested, most of them related to modifications in the plant cell membrane (D'Alton & Etherton, 1984). To test FA toxicity, roots of 3-week-old tomato plants were immersed in sterile water with or without FA. Plants maintained in the presence of 0.5 to 1 mM FA exhibited a progressive depigmentation of the stem, most likely due to anthocyanin degradation, followed by a general loss of turgor and finally wilting of the entire plant (Fig. 6A). Importantly, external addition of copper, iron or zinc either to the FA solution or by foliar spraying, a process by which leaves can take up ions through the stomata and distribute them throughout the plant (Neumann & Prinz, 1975, Eddings & Brown, 1967), rendered plants more resistant to FA and significantly increased stem strength and pigmentation (Fig. 6B-D). Furthermore, the phytotoxic effect of FA was partially recapitulated by immersion of the roots in a solution containing the membrane-permeable chelator TPEN (Fig. 7), but not of the membrane-impermeable chelators BPS or BCS (data not shown). Collectively, these results suggest that phytotoxicity of FA is mediated by to chelation of metal ions inside the plant.

FA is a virulence factor of *F. oxysporum* on tomato plants and immunodepressed mice

We noted that expression of *fub1* in *F. oxysporum* was markedly upregulated during early stages of plant infection (Fig. 8A) and therefore tested the role of FA production in virulence. Tomato plants whose roots were inoculated with conidia of the *F. oxysporum* wild-type or *fub1*Δ^{C} strains showed progressive wilt symptoms and usually died before day 25 post inoculation (dpi) (Fig. 8B). In contrast, plants inoculated with the *fub1*Δ mutant displayed a significantly reduced mortality rate (Fig. 8B) and most survived the

assay developing only mild disease symptoms. Moreover, the amount of fungal biomass in roots and stems was markedly reduced in *fub1*Δ in comparison to the wild-type and complemented strains (Fig. 8C). Thus, FA is required for full virulence of *F. oxysporum* in tomato plants.

Since the tomato pathogenic *F. oxysporum* strain can also infect and kill immunosuppressed mice (Ortoneda et al., 2004), we tested the role of FA production during infection of a mammalian host. Inoculation with 10^7 conidia of the wild-type or *fub1*Δ^C strains resulted in killing of all animals before 15 dpi (Fig. 9A), and transcripts of *fub1* were detected inside the host (Fig. S6). However, animals inoculated with two independent *fub1*Δ mutants showed significantly delayed mortality (Fig. 9A). In contrast to the plant infection, fungal burden in kidney, liver and lung of surviving mice did not differ significantly between the strains (Fig. 9B). These results suggest that FA contributes to virulence of *F. oxysporum* on mammals, but is not required for dissemination in the host.

DISCUSSION

FA was discovered almost 80 years ago (Yabuta et al., 1937) and was the first fungal toxin whose production was detected *in planta* (Gäumann, 1957). Its strong phytotoxicity (Niehaus et al., 2014, Stipanovic et al., 2011), moderate toxicity in animals (Yin et al., 2015, Porter et al., 1995) and bacteria (Bacon et al., 2006, Ruiz et al., 2015), and its pharmacological properties (Wang & Ng, 1999, Song & Yee, 2001) make the study of FA biosynthesis and regulation of high interest. Moreover, FA inhibits growth of fungi, including its producer *Fusarium*. However, FA producing strains use a variety of strategies, such us active export or enzymatic modification, to protect themselves from the toxin (Studt et al., 2016, Crutcher *et al.*, 2015). Since the recent discovery of the FA biosynthetic gene cluster, different components of the cluster have been characterized (Brown et al., 2012, Niehaus et al., 2014, Studt et al., 2016, Brown et al., 2015). Inactivation of Fub1, the PKS acting in the first step of the FA biosynthetic pathway, completely abolishes the production of FA and its derivatives in different *Fusarium* species (Brown et al., 2012, Niehaus et al., 2014) (this work). Here, we use targeted deletion of *fub1* to demonstrate, for the first time, a role of FA in virulence of the cross-kingdom pathogen *F. oxysporum* on plant and mammalian hosts.

Chromatin-mediated regulation of FA production

The regulation of FA production has been studied for close to 80 years. Initially it was proposed that FA is mainly produced under alkaline conditions (Yabuta *et al.*, 1939), while later studies suggested that nitrogen sufficiency and slightly acidic media are optimal for FA production (Pitel & Vining, 1970). Here we compared two different media, potato dextrose (PD) and czapek-dox (CD), both in solid and liquid versions. Although PD is a richer and more complex medium, we found that production of FA was higher in CD, a medium that has been known for a long time to promote FA production (Löffler & Mouris, 1992). By contrast, Bik and Bea were preferentially produced in PD. The exact reason for this difference is currently unknown. We hypothesized that pH could act as a key regulatory factor, and observed significantly higher *fub1* expression and FA production at pH 5 compared to pH 7. Our results are in contrast to those reported in *F. fujikuroi* showing a higher expression of *fub1* at pH 8 in comparison to pH 4, requiring the alkaline pH regulator PacC (Niehaus et al., 2014). We also confirmed that PacC is required for full expression of *fub1* at both pH 5 and 7. The seemingly contradictory results between *F. oxysporum* and *F. fujikuroi* could be explained by the different experimental conditions used in the two studies, MES-buffered versus unbuffered media, respectively (Niehaus et al., 2014). It is known that pH of an unbuffered culture can change rapidly during fungal growth. On the other hand, the optimum pH for *fub1* expression and FA production in *F. oxysporum* could be around pH 5 or higher, a range where PacC is still active. In line with this hypothesis, *pacC* transcript levels are similar at pH 5 and 7*,* but almost undetectable at pH 4 (Caracuel et al., 2003). The global regulator of secondary metabolism LaeA (Bok & Keller, 2004, Wiemann et al., 2010, Butchko et al., 2012, Lopez-Berges et al., 2013) has been previously shown to regulate FA production in *Fusarium* (Niehaus et al., 2014, Lopez-Berges et al., 2013). LaeA contains a conserved *S*-adenosylmethionine-(SAM-) binding site essential for its function, contributes to histone H3 lysine 9 trimethylation (Reyes-Dominguez *et al.*, 2010) and links transcriptional and epigenetic control of gene expression (Sarikaya-Bayram *et al.*, 2014). In line with previous reports suggesting a positive role of LaeA in FA biosynthesis (Niehaus et al., 2014, Lopez-Berges et al., 2013), we show here that inactivation of LaeA leads to a significant decrease in chromatin accessibility at the FA gene cluster. These findings, together with the reduced expression of the *hph* gene when inserted at the place of *fub1*, suggest a major regulatory function of LaeA in remodeling chromatin structure at the *F. oxysporum* FA locus. In addition, we showed that moderate acidic pH and PacC contribute to an increase in chromatin accessibility at the *fub1* locus. Although our data suggest that this contribution requires LaeA, this remains to be confirmed experimentally. The fact that inactivation of LaeA has by far the strongest effect on

expression of the FA gene cluster and FA production, suggests that other stimuli like nutrients or pH may converge on this master regulator of secondary metabolism to regulate expression of the gene cluster.

FA has been long known for its ability to chelate metal ions (Tamari & Kaji, 1952, Malini, 1966, Lakshminarayanan & Subramanian, 1955, Pan et al., 2010). However, the regulation of FA biosynthesis by metals has not been studied so far. Here we show that transcript levels of *fub1* are negatively regulated by copper, iron or zinc. Similarly, transcript levels of *sidC*, a LaeA-regulated gene functioning in the biosynthesis of the siderophore ferricrocin, are also downregulated in the presence of iron (Perrin *et al.*, 2007, Lopez-Berges et al., 2013, Eisendle *et al.*, 2004). While this suggests that FA might function in metal uptake, we found that *fub1* was not essential for growth of *F. oxysporum* during copper, iron or zinc limiting conditions, most likely because more specific and efficient uptake mechanisms are present in filamentous fungi such as high affinity copper and zinc transporters (Vicentefranqueira *et al.*, 2005, Park et al., 2014), and siderophore-assisted iron uptake (Schrettl & Haas, 2011). Alternatively, metal-chelating FA might be used by *Fusarium* to inhibit microbial competitors in the soil or to improve growth under toxic metal concentrations. Indeed, FA is exported in *F. fujikuroi* and *F. oxysporum* f. sp. *vasinfectum* via the Major Facilitator Superfamily (MFS) transporters Fub11 and FubT, respectively (Crutcher et al., 2015, Studt et al., 2016). However, we found that lack of FA production was not detrimental during fungal growth under toxic copper, iron or zinc conditions.

Mechanism of FA phytotoxicity and role in virulence

Early studies established the phytotoxic activity of FA and its role in the induction of wilt symptoms in plants (Yabuta et al., 1937, Gäumann, 1957, Gäumann, 1958). Our study confirmed that tomato seedlings develop typical wilt symptoms when their roots are exposed to FA. The fact that wilting was observed in cotyledons and lower leaves suggests that FA is transported and distributed throughout the entire plant. Similar wilt symptoms were reported in water melon seedlings (Hong-Sheng *et al.*, 2008).

The precise mechanism of phytotoxicity of FA remains unknown. A number of studies suggested that could be related to its ability to chelate different metal ions (Tamari & Kaji, 1952, Lakshminarayanan & Subramanian, 1955, Gäumann, 1958, Ruiz et al., 2015). Here we show that addition of copper, iron or zinc to FA-treated plants significantly reduces wilting. Importantly, inhibition of FA toxicity was also functional

when the metal ions and FA were applied to different parts of the plant (leaves and roots, respectively) indicating that the chelating mechanism occurs inside the plant. This is further supported by the fact that the membrane-permeable metal chelator TPEN, but not the membrane-impermeable chelators BPS or BCS, was able to exert a toxic effect similar to that of FA. Although additional mechanisms of FA toxicity cannot be ruled out, our results clearly support a causal link between FA phytotoxicity and metal chelation.

FA has been one of the first fungal toxins for which a functional role in virulence has been proposed (Gäumann, 1957, Gäumann, 1958) and several studies have provided circumstantial evidence linking FA production to plant pathogenicity (Venter & Steyn, 1998, Gapillout *et al.*, 1996, Dong et al., 2014). However, so far no formal proof for such a role has been provided. Here we demonstrate that mutants lacking *fub1,* which are unable to produce FA or its derivatives, are significantly reduced in their capacity to cause mortality in tomato plants. Interestingly, these mutants also caused less mortality in immunosuppressed mice, showing for the first time the relevance of FA production during fungal infection of mammals. Although the pH of mammalian blood is around 7.3, which is not favorable for FA production, low amounts of FA might be sufficient to promote fungal virulence on mammals. In addition, it cannot be ruled out that FA production is under positive regulation inside the host. Indeed, FA was previously shown to be produced in blood cultures in a LaeA- and VeA-dependent manner (Lopez-Berges et al., 2013). Previously, the mycotoxin beauvericin was also shown to contribute to infection of *F. oxysporum* in plants and mice. This suggests that the production of secondary metabolites, many of which are regulated by the Velvet complex and LaeA, could play a role in the capacity of *F. oxysporum* to attack both plant and animal hosts. In line with this idea, mutants lacking VeA or LaeA are significantly attenuated in virulence on tomato plants and mice (Lopez-Berges et al. 2013), as are Velvet complex mutants in other human and plant pathogenic fungi (Bok *et al.*, 2005, Webster & Sil, 2008, Laskowski-Peak *et al.*, 2012, Jiang *et al.*, 2011, Lee *et al.*, 2012, Merhej *et al.*, 2012, Wiemann et al., 2010, Myung *et al.*, 2009, Lopez-Berges et al., 2013). Additional studies, including investigations on combinatory/synergic effects of co-occurrence mycotoxins, are required to fully understand the role of SM production in cross-kingdom pathogenicity of *F. oxysporum*.

EXPERIMENTAL PROCEDURES

Fungal isolates and culture conditions

Fusarium oxysporum f. sp *lycopersici* race 2 wild-type isolate 4287 (FGSC 9935) was used in all experiments. Fungal strains were stored as microconidial suspensions at -80°C with 30% glycerol. For extraction of genomic DNA and microconidia production, cultures were grown in PDB at 28°C (Di Pietro & Roncero, 1998). For analysis of gene expression and relative chromatin accessibility, freshly obtained microconidia were germinated for 14-16 h in PDB or GMM. Germlings were harvested by filtration, washed three times in sterile water and transferred to fresh PDB, CDL or GMM with or without 50 µM $CuSO_4$, $FeSO_4$ or $ZnSO_4$ for the indicated time periods. pH 5 and pH 7 buffered conditions were achieved using 100 mM 4-Morpholineethanesulfonic (MES), when indicated. For determination of colony growth, 2 x 10^4 microconidia were spotted onto PDA, CDA, MMA or GMM with or without FA (0-0.75 mg/ml), with or without 200 µM BPS, 200 µM BCS or 4 µM TPEN and with or without $CuSO_4$, $FeSO_4$ or $ZnSO_4$ (0.05-5 mM). Plates were incubated at 28°C for the indicated time periods. All experiments included two replicates and were performed at least three times with similar results.

Fungal strains

PCR reactions were routinely performed with VELOCITY™ DNA Polymerase (Bioline) using a MJ Mini™ Personal Thermal Cycler (Bio-Rad) (see Table S1 for a complete list of primer sequences used in the study). All fungal transformations and purification of the transformants by monoconidial isolation were performed as described (Di Pietro & Roncero, 1998). The cassette for targeted replacement of the entire coding region of the *F. oxysporum fub1* gene with the hygromycin B-resistance marker (Punt *et al.*, 1987) was assembled by a fusion PCR method (Szewczyk *et al.*, 2006). DNA fragments flanking the *fub1* coding region were amplified from genomic DNA of *F. oxysporum* wild-type with primers *fub1*-F1 + *fub1*-R1 and *fub1*-F2 + *fub1*-R2, respectively, while the hygromycin B-resistance marker, under control of the *Aspergillus nidulans gpdA* promoter and *trpC* terminator, was amplified from pAN7-1 plasmid (Punt et al., 1987) with primers *fub1-hph*-F + *fub1-hph*-R. The three DNA fragments were then PCR fused with primers *fub1*-F1n + *fub1*-R2n. The obtained *fub1*Δ allele was used to transform protoplasts of the *F. oxysporum* wild-type strain to hygromycin B-resistance (Fig. S1B). Transformants showing homologous insertion of the construct were genotyped by PCR of genomic DNA with primers *fub1*-F1 + *fub1*-R2 (not shown) and by Southern blot analysis (Fig. S1C). To generate a construct for complementation of the *fub1*Δ strain, a 9645 bp fragment spanning from 1077 bp

upstream of the wild-type *F. oxysporum fub1* translation initiation codon to 1092 bp downstream of the translation termination codon was amplified by PCR with primers *fub1*-F1 + *fub1*-R2. The amplified fragment was used to cotransform protoplasts of the *fub1*Δ strain with the phleomycin B–resistance gene under control of the *A. nidulans gpdA* promoter and *trpC* terminator, amplified from pAN8-1 plasmid (Mattern *et al.*, 1988) with primers *gpdA*-15b + *trpC*-8b (Fig. S2A) Several phleomycin-resistant cotransformants were analyzed for the presence of a functional *fub1* allele by PCR with gene-specific primers *fub1*-F3 + *fub1*-R3 (Fig. S2B). Among the different complemented strains we selected one in which FA and derivatives production (Fig. 1) and *fub1* transcript levels returned to wild-type values (Fig. S2C and S2D).

Nucleic acid manipulations and quantitative real-time RT-PCR analysis

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelia following previously reported protocols (Raeder & Broda, 1985, Chomczynski & Sacchi, 1987). Quality and quantity of extracted nucleic acids were determined by running aliquots in ethidium bromide-stained agarose gels and by spectrophotometric analysis in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) respectively. Routine nucleic acid manipulations were performed according to standard protocols (Sambrook & Russell, 2001). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul *et al.*, 1990). RT-qPCR was performed as described (Lopez-Berges *et al.*, 2010, Lopez-Berges *et al.*, 2012) using FastStart Essential DNA Green Master (Roche) in a CFX Connect Real-Time System (Bio-Rad). Gene-specific primers (see Table S1) were designed to flank an intron, if possible. Transcript levels were calculated by comparative ΔCt and normalized to *act1*.

Analysis of chromatin structure

Mycelia of *F. oxysporum* strains grown under the indicated conditions were harvested by filtration, lyophilized and ground to a fine powder in a Mini-BeadBeater 8 (BioSpec Products). Nuclease digestion was performed as described (Basheer *et al.*, 2009, Gonzalez & Scazzocchio, 1997, Lopez-Berges et al., 2013). Briefly, 20 mg of lyophilized mycelium was suspended in 1 ml of micrococcal nuclease (MNase) buffer (250 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM $CaCl_2$, 3 mM $MgCl_2$), and 300 ml of the suspension were treated for 5 min with 3 U of MNase (Sigma) at 37°C. The reaction was terminated by adding stop buffer (2% SDS, 40 mM EDTA). DNA was

obtained by phenol/chloroform extraction, precipitated, washed with 70% ethanol, dissolved in water and treated with RNAse (see Fig. S5). Quantitative real-time PCR was performed as described above using promoter- and gene-specific primers (see Table S1). Chromatin accessibility was expressed by comparative ΔCt as the ratio between amplification levels from untreated gDNA relative to those obtained from MNase digested gDNA. Values were represented relative to those of the wild-type strain.

Mycotoxin quantification

FA and derivatives quantification was performed as described (Lopez-Berges et al., 2013). Samples were obtained from fungal colonies grown for 3 days at 28°C on PDA or CDA and from mycelia and supernatant of the wild-type strain germinated in PDB for 16 h and then transferred for 3 h to fresh PDB or CDL buffered to pH 5 or pH 7. Samples were homogenized in acetonirile/water/glacial acetic acid (79:20:1; v:v:v) with a Homogenizer Workcenter T10 basic (IKA®) for 1 min at a rate of 4 ml solvent per gram of sample. The mix was re-homogenized after 2 min of repose, filtered, centrifuged 10 min at 12000 *g*, and the supernatant was lyophilized. Dry crude extracts were reconstituted in the solvent, and mycotoxin detection and quantification was performed with a QTrap 5000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1290 Series UPLC System (Agilent, Waldbronn, Germany), as described (Malachova et al., 2014). Supernatant samples were directly lyophilized and then reconstituted in the solvent for quantification.

Determination of FA toxicity on tomato plants

3-week-old seedlings of tomato plants (cultivar Monika) were individually root immersed in inoculum tubes containing pH 6 sterile water with different FA concentrations or 200 µM BPS, 200 µM BCS and 4 µM TPEN and placed in a greenhouse for the indicated time periods. Copper, iron and zinc foliar spray was performed, when indicated, 2 h before the root immersion. Briefly, plant roots were carefully covered with cling film and leaves spayed two times with 0.025% $CuSO_4$, $FeSO_4$ or $ZnSO_4$ in 0.1% tween 20 solutions. When the leaves were completely dry, roots were washed 3 times in sterile water before the immersion in the indicated

solutions. Symptoms were monitored daily and scored 3 and 6 d after the FA or chelant treatment.

Plant infection assays

Tomato root inoculation assays were performed as described (Di Pietro & Roncero, 1998), using 2-week-old tomato seedlings (cultivar Monika). Severity of disease symptoms and plant survival was recorded daily for 30 days. Ten plants were used for each treatment. Virulence experiments were performed at least three times with similar results. Plant survival was calculated by the Kaplan-Meier method and compared among groups using the log-rank test. Data were analysed with the software GraphPad Prism 4. Quantification of fungal biomass in planta was performed as described (Pareja-Jaime *et al.*, 2010) using total genomic DNA extracted from tomato roots or stems infected with *F. oxysporum* strains at 3 or 7 dpi. Relative amounts of fungal gDNA were calculated by comparative ΔCt of the *Fusarium act1* gene normalized to the tomato *EFα1* gene (see Table S1).

Animal infection assays

Mice were cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series, No. 123; http://conventions. coe.int/Treaty/en/Treaties/Html/123.htm). Experimental conditions were approved by the Animal Welfare Committee of the Faculty of Medicine, Universitat Rovira i Virgili. Infection assays with immunodepressed mice were performed as described (Ortoneda et al., 2004). Briefly, groups of 10 Oncins France (OF) 1 male mice (Charles River, Criffa S.A.) were immunosuppressed with an intraperitoneal 200 mg/kg dose of cyclophosphamide (Laboratorios Funk S.A.) 2 days before the inoculation, and then every 5 days, and infected by injecting 0.2 ml of an inoculum of 10^7 conidia into a lateral vein of the tail. Survival was recorded daily for the indicated time periods. Infection experiments with each individual strain were performed at least three times. Survival was estimated by the Kaplan-Meier method and compared among groups using the log-rank test. To determine fungal tissue burden, randomly chosen surviving mice inoculated with 10^7 conidia were sacrificed 5 dpi. Kidneys, livers and lungs were aseptically removed, weighed, and homogenized in sterile saline, and 10-fold serial dilutions were spread onto PDA. Plates were incubated at 28°C, colonies were counted

after 3 d, and the number of colony forming units (CFU) per gram of organ was calculated. Fungal colony counts were converted to $\log_{10}$ and compared using the analysis of variance test. Data were analyzed with the software GraphPad Prism 4.

Accession numbers

Sequence data can be found in the GenBank/EMBL database or in the Fusarium Comparative Genome database under the following accession numbers: Fub1, FOXG_15248; Act1, FOXG_01569; EFα1, NC_015443; pAN7-1 (PgpdA-hygr-TtrpC), Z32698; pAN8-1 (PgpdA-phleor-TtrpC), Z32751.

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SUPPORTING INFORMATION LEGENDS

Figure S1. Identification of the *F. oxysporum* FA gene cluster and *fub1* knockout strategy

(A) Conserved synteny between *F. fujikuroi* and *F. oxysporum* FA gene clusters. Note that the two genes inserted between *fub3* and *fub4* in *F. oxysporum* are present in *F. fujikuroi* in another chromosome. **(B)** *F. oxysporum fub1* locus and targeted gene disruption construct. **(C)** Southern blot analysis. gDNA of the indicated strains was treated with *Xho*I, separated on a 0.7% agarose gel, transferred to a nylon membrane and hybridized with the DNA probe indicated in **(B)**.

Figure S2. Generation and selection of the *fub1*Δ complemented strain (*fub1*Δ^{C})

(A) Strategy of *fub1*Δ complementation by co-transformation with a *fub1* wild-type allele and the phleomycin resistance marker. Relative position of the PCR primers used for genotyping are indicated. *phleo*^{r}, phleomycin resistance gene. **(B)** PCR amplification of gDNA of the indicated strains using primers F3 and R3. The complemented strains, *fub1*Δ^{C} and *fub1*Δ^{C} #2, produce a banding pattern consistent with the integration of an intact *fub1* allele. wt, wild-type strain. **(C)** Quantitative real-time RT-PCR performed in the indicated strains germinated for 16 h in potato dextrose broth (PDB) and then transferred for 3 h to czapec-dox liquid (CDL). Transcript levels of *fub1* are expressed relative to those in the wild-type strain. Bars represent standard errors from two independent biological experiments with three technical replicates each. **(D)** Fusaric acid (FA) and fusarinolic acid (FnA) in cultures of the indicated strains grown for 3 d on czapek-dox agar (CDA) was quantified by liquid chromatography/tandem mass spectrometry and expressed in ng/ml of extract.

Figure S3. Mycelial growth on potato dextrose agar (PDA) with or without FA

Growth of the indicated strains cultured for 3 d at 28ºC.

Figure S4. Mycelial growth in czapek-dox agar (CDA) with or without hygromycin B

Growth of the indicated strains cultured for 3 d at 28ºC.

Figure S5. Nucleosomal repeat length in *F. oxysporum*

Genomic DNA was extracted from lyophilized mycelium of the indicated strains, treated with MNase for 5 minutes at 37ºC (T), separated in a 2% agarose gel, stained with ethidium bromide and visualized under UV light. DNA extracted from untreated mycelium was loaded as a control (UT). M, DNA marker.

Figure S6. *F. oxysporum fub1* is expressed during infection of mice

(A, B) Melt curves in quantitative real-time RT-PCR experiments of the indicated samples. Note the detection of a non-specific amplicon in mice samples which makes quantification impossible.

Table S1. Primers used in this study

FIGURE LEGENDS

Figure 1. Fub1 is required for production of fusaric acid and its derivatives in *F. oxysporum*

(A) Fusaric acid (FA) and fusarinolic acid (FnA) in cultures of the indicated strains grown for 3 d on potato dextrose agar (PDA) or czapek-dox agar (CDA) was quantified by liquid chromatography/tandem mass spectrometry and expressed in ng/ml of extract. **(B)** Quantification of beauvericin (Bea) and bikaverin (Bik) was performed as in **(A)**. wt, wild-type strain. Bars represent standard errors from two independent fungal cultures. **P* < 0.05; ***P* < 0.001; ns, not significant.

Figure 2. pH and medium composition regulate *fub1* transcript levels and fusaric acid production

(A) Quantitative real-time RT-PCR was performed in the wild-type strain germinated for 16 h in potato dextrose broth (PDB) and then transferred for 3 h to fresh PDB or czapek-dox liquid (CDL) buffered at the indicated pH with 100 mM MES. Transcript levels of *fub1* are expressed relative to those in PDB at pH 7 (see numbers upon the columns for the exact data). Bars represent standard errors from two independent

biological experiments with three technical replicates each. **(B)** Quantification of FA in culture supernatants (upper panel) and mycelia (lower panel) of the wild-type strain grown as in **(A)** was performed by liquid chromatography/tandem mass spectrometry. Bars represent standard errors from two independent fungal cultures. **(C)** Quantitative real-time RT-PCR was performed in the indicated strains germinated for 16 h in Glutamine minimal medium (GMM) and then transferred for 3 h to fresh GMM buffered at the indicated pH with 100 mM MES. Transcript levels of *fub1* are expressed relative to those of the wild-type at both pH values. Bars represent standard errors from two independent biological experiments with three technical replicates each. *P < 0.05; **P < 0.001; ***P < 0.0001; ns, not significant.

Figure 3. Transcript levels of *fub1* are controlled by LaeA

(A) Colonies of the indicated strains grown on potato dextrose agar (PDA) with or without 50 mg/ml hygromycin B for 3 d at 28ºC. **(B, C)** Quantitative real-time RT-PCR was performed in the indicated strains germinated for 16 h in potato dextrose broth (PDB) and then transferred to fresh PDB for 1 h. Transcript levels of the *hph* **(B)** and *fub1* **(C)** genes are expressed relative to those of the ectopic transformant and the wild-type strain, respectively. wt, wild-type strain; Ect, ectopic transformant. Bars represent standard errors from two independent biological experiments with three technical replicates each. ***P < 0.0001.

Figure 4. LaeA, pH and PacC regulate chromatin modifications at the *fub1* locus

(A) Physical map of the promoter region of the *fub1* gene located in the FA gene cluster. Primers used for chromatin analysis are indicated. **(B-D)** Real-time quantitative PCR performed on gDNA of the indicated strains grown as in Fig. 3B and 3C. Relative chromatin accessibility was calculated as the ratio of amplification levels obtained with gDNA from untreated mycelia versus gDNA from MNase-treated mycelia, and represented relative to that of the willd-type **(B)**, the wild-type at pH 5 **(C)** or the wild-type at different pH values **(D)**, with each of the indicated primer pairs (see Table S1). wt, wild-type strain. Bars represent standard errors from two independent biological experiments with three technical replicates each. *P < 0.05; **P < 0.001; ***P < 0.0001.

Figure 5. Expression of *fub1* is repressed by copper, iron and zinc

(A) Growth of the indicated strains on minimal medium agar (MMA) with or without the indicated metal chelators. Plates were cultured for 3 d at 28ºC. **(B)** Growth of the indicated strains on solid Glutamine minimal medium (GMM) supplemented with the indicated concentrations of Cu, Fe or Zn. Plates were cultured for 3 d at 28ºC. **(C)** Quantitative real-time RT-PCR was performed in the wild-type strain germinated for 16 h in GMM without Cu, Fe or Zn and then supplemented or not with 50 µM $CuSO_4$, $FeSO_4$ or $ZnSO_4$ for 2 h. Transcript levels of *fub1* are expressed relative to those in GMM. wt, wild-type strain; BCS, Bathocuproinedisulfonic acid disodium salt; BPS, Bathophenanthrolinedisulfonic acid disodium salt hydrate; TPEN, N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine. Bars represent standard errors from two independent biological experiments with three technical replicates each. ** $P < 0.001$; *** $P < 0.0001$.

Figure 6. Phytotoxic effect of fusaric acid on tomato plants is remediated by exogenous copper, iron or zinc

(A) Roots of 3-week-old seedlings of tomato plants (cultivar Monika) were immersed in sterile water with the indicated concentrations of FA for 3 and 6 d. **(B-D)** Leaves of plants were sprayed with 0.025% $CuSO_4$, $FeSO_4$ or $ZnSO_4$ solutions before immersing roots in the FA solution. Boxed areas are shown at double magnification.

Figure 7. The membrane-permeable zinc chelant TPEN causes similar phytotoxicity symptoms in tomato plants as fusaric acid

Leaves of tomato plants were pre-treated or not with a 0.025% $ZnSO_4$ solution and roots were immersed in sterile water containing 4 µM of the zinc chelant TPEN for 3 and 6 d. Boxed areas are shown at double magnification.

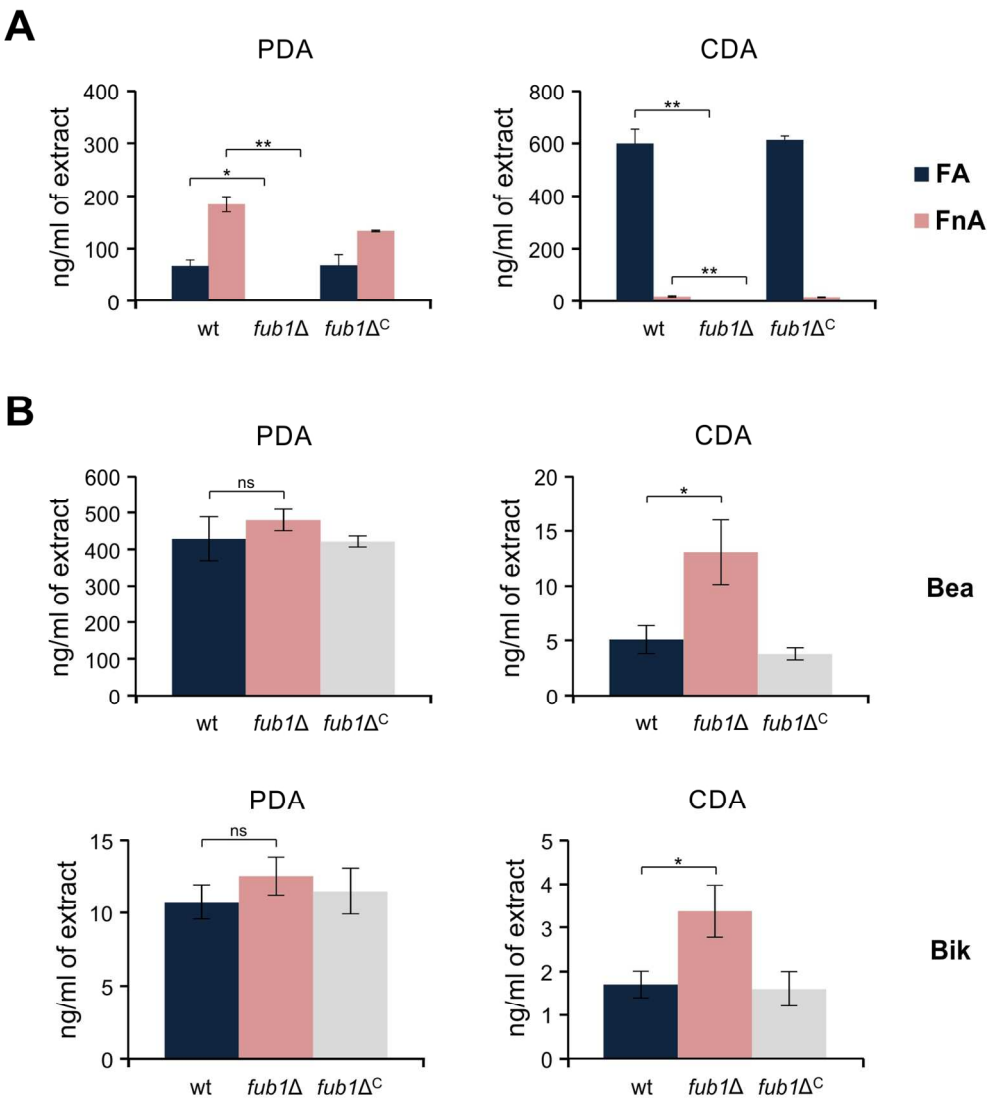
Figure 8. Fusaric acid production is required for full virulence of *F. oxysporum* on tomato plants

(A) Quantitative real-time RT-PCR was performed in the wild-type strain germinated for 16 h in potato dextrose broth (PDB) and then transferred to czapek-dox liquid (CDL) for 3 h or from inoculated tomato roots and stems at 3 days post inoculation (dpi). Transcript levels of *fub1* are expressed relative to those in CDL. **(B)** Groups of 10 tomato plants (cultivar Monika) were inoculated by dipping roots into a suspension of 5

x 10^6 freshly obtained microconidia/ml of the indicated fungal strains. Percentage survival was plotted for 30 d. Data shown are from one representative experiment. Experiments were performed 3 times with similar results. **(C)** Quantitative real-time PCR was used to measure the relative amount of fungal DNA in total genomic DNA extracted from tomato roots and stems at 3 and 7 dpi with the indicated strains. Amplification levels are expressed relative to those of plants infected with the wild-type strain. wt, wild-type strain. Bars represent standard deviations from two independent biological experiments with three technical replicates each.*$P < 0.05$; **$P < 0.001$.

Figure 9. Fusaric acid is a virulence factor on mice

(A) Groups of 10 immunosuppressed Oncins France 1 male mice were inoculated with 10^7 microconidia of the indicated strains by lateral tail vein injection. Percentage survival was plotted for 20 d. Data shown are from one representative experiment. Experiments were performed 3 times with similar results. **(B)** Four randomly chosen surviving mice inoculated with 10^7 microconidia of the indicated strains were sacrificed 5 days post inoculation (dpi) and homogenates obtained from the indicated organs were quantitatively cultured on potato dextrose agar (PDA). wt, wild type strain. *$P < 0.05$; **$P < 0.001$; ns, not significant.



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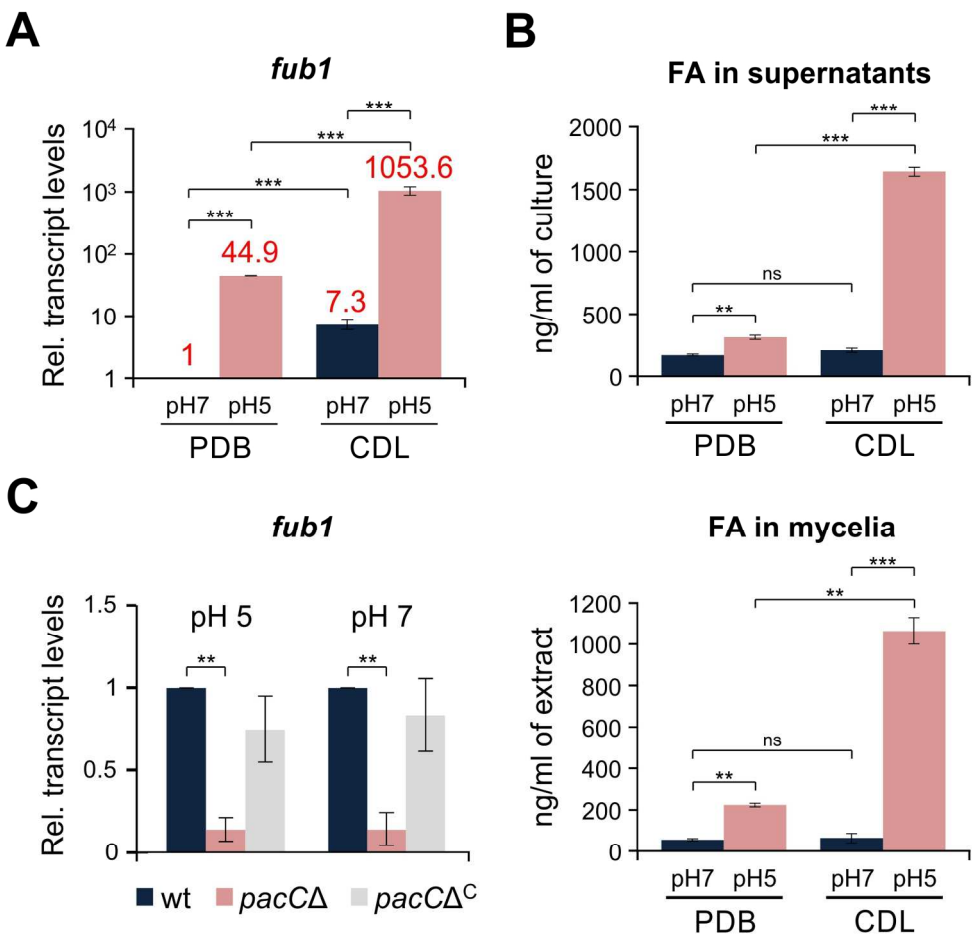

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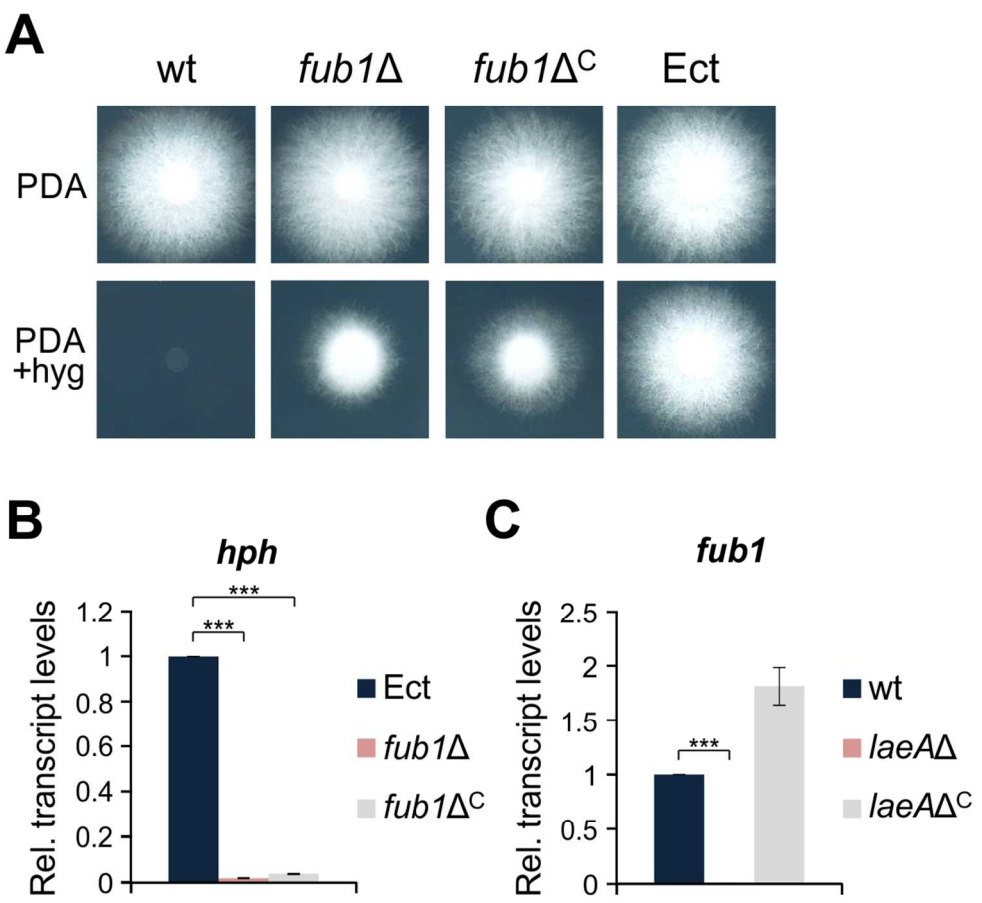

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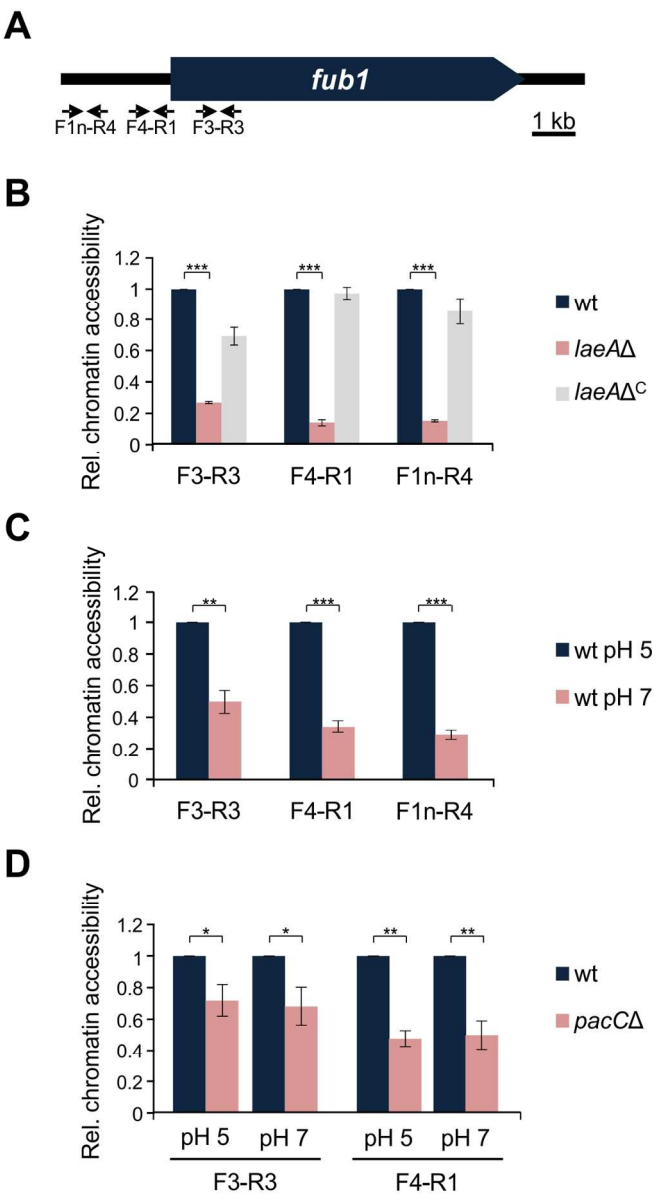

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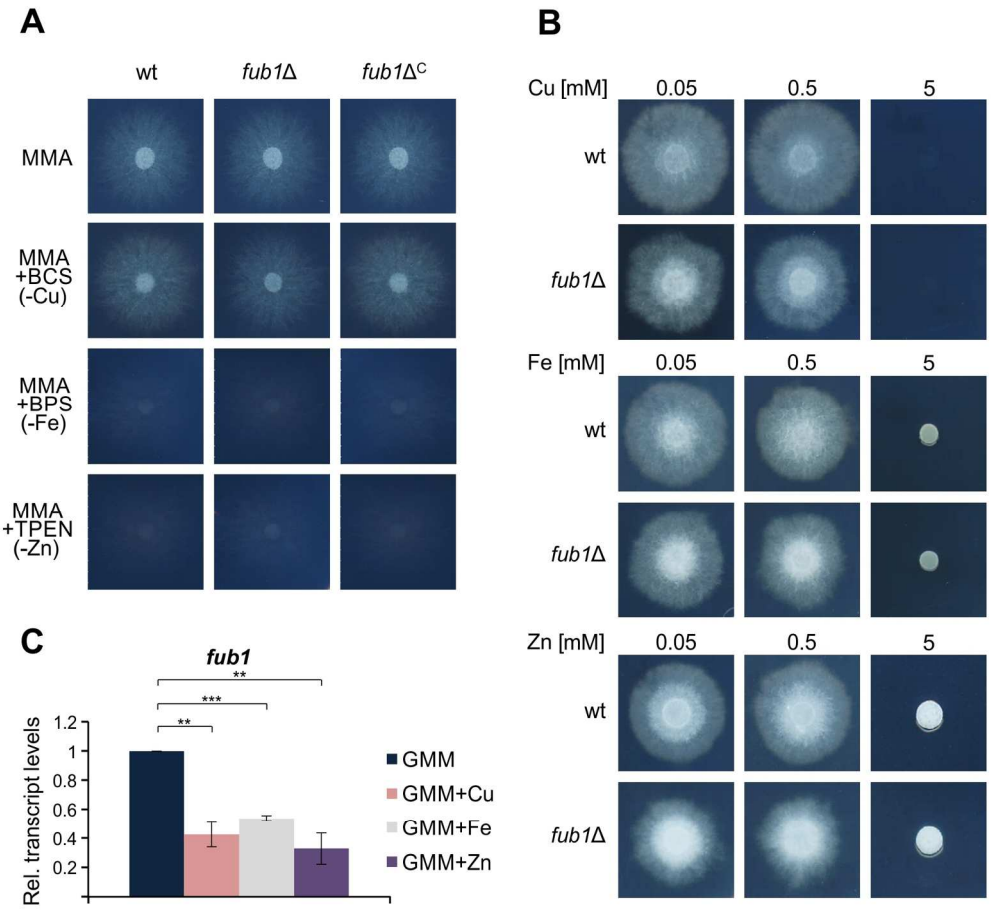

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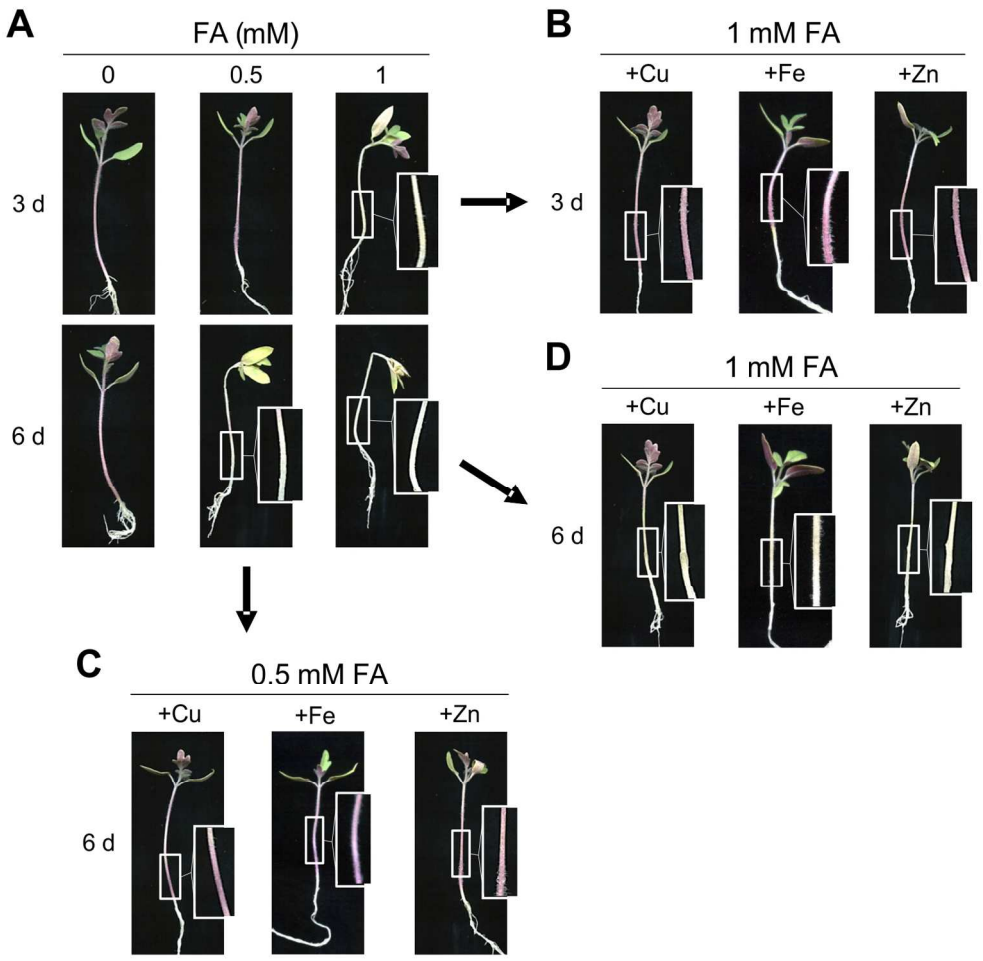

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151x148mm (300 x 300 DPI)

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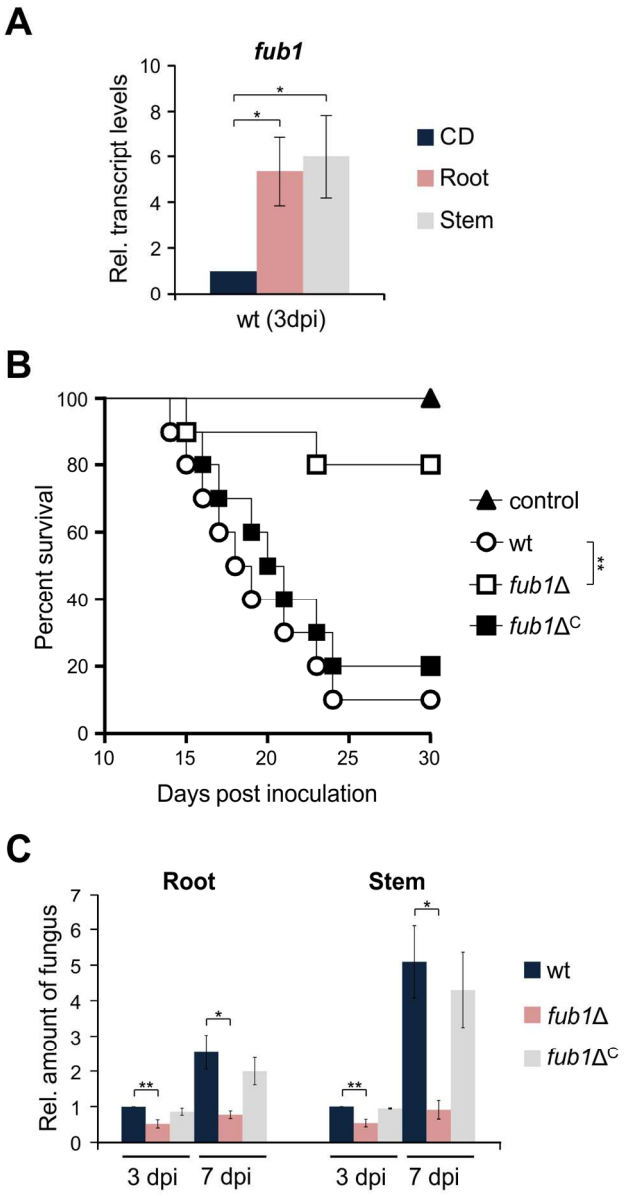

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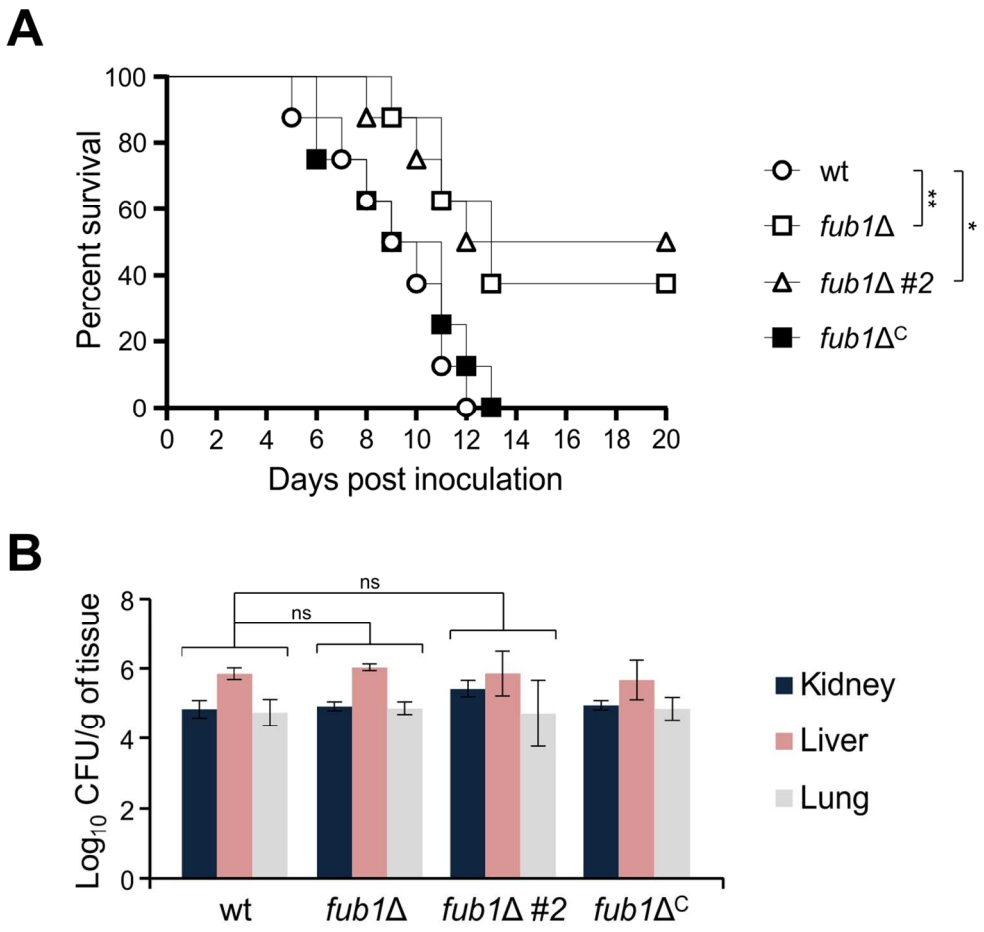

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