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Characterization of the fusaric acid gene cluster in *Fusarium fujikuroi*

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Abstract The "bakanae" fungus *Fusarium fujikuroi* is a common pathogen of rice and produces a variety of mycotoxins, pigments, and phytohormones. Fusaric acid is one of the oldest known secondary metabolites produced by *F. fujikuroi* and some other *Fusarium* species. Investigation of its biosynthesis and regulation is of great interest due to its occurrence in cereal-based food and feed. This study describes the identification and characterization of the fusaric acid gene cluster in *F. fujikuroi* consisting of the PKS-encoding core gene and four co-regulated genes, *FUB1–FUB5*. Besides fusaric acid, *F. fujikuroi* produces two fusaric acid-like derivatives: fusarinolic acid and 9,10-dehydrofusaric acid. We provide evidence that these derivatives are not intermediates of the fusaric acid biosynthetic pathway, and that their formation is catalyzed by genes outside of the fusaric acid gene cluster. Target gene deletions of all five cluster genes revealed that not all of them are involved in fusaric acid biosynthesis. We suggest that only two genes, *FUB1* and *FUB4*, are necessary for the biosynthesis. Expression of the *FUB* genes and production of fusaric acid and the two derivatives are favored under high nitrogen. We show that nitrogen-dependent expression of fusaric acid genes is positively regulated by the nitrogen-responsive GATA transcription factor AreB, and that pH-dependent regulation is mediated by the transcription factor PacC. In addition, fusaric acid production is regulated by two members of the fungal-specific *velvet* complex: Vel1 and Lae1. In planta expression studies show a higher expression in the favorite host plant rice compared to maize.

Keywords *Fusarium fujikuroi* · Fusaric acid · Polyketide synthase · Mycotoxin · HPLC–HRMS · Nitrogen regulation

Introduction

The rice pathogenic fungus *Fusarium fujikuroi* is well known for the production of gibberellic acids (GAs), a group of diterpenoid phytohormones. Beside GAs, the fungus produces some other secondary metabolites (SMs), such as the pigments bikaverin and fusarubins (Wiemann et al. 2009; Studt et al. 2012), as well as the mycotoxins fusarins (Díaz-Sánchez et al. 2012; Niehaus et al. 2013), fumonisins (Wiemann et al. 2013), and moniliformin (Cole et al. 1973; Wiemann et al. 2012). The recent sequencing of the *F. fujikuroi* genome revealed 17 polyketide synthases (PKS), 15 non-ribosomal peptide synthases (NRPS), two dimethylallyl tryptophan synthases (DMATS), and ten terpene cyclases (TC)-encoding genes indicating a much broader potential to produce yet unknown compounds (Wiemann et al. 2013).

One of the oldest known SMs of *F. fujikuroi* is fusaric acid (5-butylpicolinic acid, FA), a mycotoxin with low to moderate toxicity to animals and humans, but with high phytotoxic properties. Searching for the causing agent of the rice "bakanae" disease, Japanese scientists first isolated FA from the culture fluid. Later, the structure of GAs, the real causative agents of "bakanae" disease, has been elucidated (Yabuta et al. 1937). Since then, the formation of FA has been shown also for other *Fusarium* species belonging to the *Gibberella fujikuroi* species

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complex (GFC), e.g., *Fusarium verticillioides*, *Fusarium proliferatum*, and *Fusarium subglutinans*, but also for more distantly related *Fusarium* species, such as *Fusarium crookwellense*, *Fusarium sambucinum*, *Fusarium heterosporum*, *Fusarium oxysporum*, and *Fusarium solani* (Bacon et al. 1996). Due to the widespread production of FA by many *Fusarium* species, this compound was suggested to be used as a marker toxin for *Fusarium* contamination of food and feed. Compared to other FA producers, isolates of *F. fujikuroi* and the sibling species *F. proliferatum* were shown to produce high concentrations of FA (up to 1,000 μg/g of corn) (Bacon et al. 1996).

Up to now, several studies concerning the toxicity and mode of action of FA have been published. This metabolite is toxic to various plants, fungi, and bacteria, but has also pharmacological activities (Wang and Ng 1999). For example, FA was found to inhibit dopamine-β-hydroxylase and therefore cause hyposensitive effects in different animals (Hidaka et al. 1969). A later survey confirmed these data and indicated effects on brain and pineal neurotransmitters (Porter et al. 1995). Interestingly, it was also found to have beneficial health effects, for instance against *Acanthamoeba* (Boonman et al. 2012) or against HIV-1 dementia (Ramautar et al. 2012).

Only recently, the first fusaric acid biosynthetic gene, *FUB1*, encoding a reducing PKS, was identified in *F. verticillioides*. Deletion of this gene resulted in loss of FA production (Brown et al. 2012). Microarray analyses revealed that four genes adjacent to *FUB1* were co-regulated with *FUB1* suggesting that five genes belong to an assumed FA biosynthetic gene cluster (Brown et al. 2012).

In this paper, we describe the molecular characterization of the entire FA gene cluster in *F. fujikuroi* by targeted gene replacement of all postulated biosynthetic genes and subsequent product analysis. We demonstrate that only two of the five cluster genes are needed for the biosynthesis of FA. Furthermore, we studied the regulation of cluster gene expression by environmental conditions and revealed a strong dependency on nitrogen availability, the nitrogen responsive GATA transcription factor AreB, and the pH regulator PacC. Additionally, expression of *FUB* genes is positively regulated by two members of the fungal-specific *velvet* complex: Vel1 and the putative histone methyltransferase Lae1. We show that, depending on culture conditions, FA can be converted into fusarinolic acid and dehydrofusaric acid, and that these modifications are carried out by genes outside the FA gene cluster.

Materials and methods

Fungal strains, culture conditions, and plasmid constructions

The wild-type strain *F. fujikuroi* IMI58289 (Commonwealth Mycological Institute, Kew, UK) was used for the experiments and it was the parent strain for the knock-out mutants. For the regulation studies, the Δ*pacC* strain (Wiemann et al. 2009), Δ*areA* strain (Tudzynski et al. 1999), the Δ*areB* strain (Michielse et al. 2013), and the Δ*vel1*, Δ*vel2*, and Δ*lae1* strains (Wiemann et al. 2010) were used.

For RNA isolation and high-performance liquid chromatography (HPLC) measurements, the strains were preincubated in 300-mL Erlenmeyer flasks in 100 mL Darken medium (Darken et al. 1959) at 28 °C on a rotary shaker at 180 rpm in the dark. After 3 days, 500 μL of the starter culture was used as inoculums for cultivation in ICI (Imperial Chemical Industries Ltd, UK) media (Geissman et al. 1966). These media were in 300-mL Erlenmeyer flasks and contained 100 mL of either 6 mM or 60 mM glutamine or 6 mM or 120 mM $NaNO_3$. Protoplasting was conducted after Tudzynski et al. (1999). For pH shift experiments, the wild-type and the Δ*pacC* mutant were grown for 3 days in 60 mM glutamine ICI media at 28 °C. Then, the mycelia were harvested. After washing, the mycelia were shifted for 2 h into 60 mM glutamine which was either adjusted to pH 4 or pH 8 (Balan et al. 1970).

For DNA isolation, the different strains were grown for 2–3 days on cellophane sheets (Alba Gewürze, Bielefeld, Germany) on solid complete medium (CM) (Pontecorvo et al. 1953) at 28 °C in the dark. For RNA isolation, the fungus was grown in 300-mL Erlenmeyer flasks in 100 mL ICI media with different nitrogen qualities and quantities for 3–6 days on a rotary shaker in the dark. The harvested mycelium was used for the isolation.

Standard molecular methods

Fungal DNA was prepared by first grinding lyophilized mycelium into a fine powder with a mortar and pestle and then dispersing it in DNA extraction buffer as described (Cenis 1992). For Southern blot analysis, genomic DNA was digested with the indicated restriction enzymes (Fermentas GmbH, St. Leon-Rot, Germany), fractionated in 1 % (w/v) agarose gels, and transferred to Nytran® nylon transfer membranes (Whatman Inc., Sanford, ME, USA) by downward blotting. ^{32}P-labeled probes were prepared using the random oligomer-primer method and membranes were hybridized according to the protocol of Sambrook et al. (1989). After hybridization with ^{32}P-labeled probes overnight, the membrane was washed with 1× SSPE (0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7) and 0.1 % SDS.

Total *F. fujikuroi* RNA was isolated using the RNAgents total RNA isolation kit (Promega GmbH, Mannheim, Germany). Samples of 20 μg of total RNA were transferred to Hybond-N^+ membranes after electrophoresis on a 1 % (w/v) agarose gel containing 1 % (v/v) formaldehyde, according to Sambrook et al. (1989). Northern blot hybridizations

were accomplished by the method of Church and Gilbert (1984).

Polymerase chain reactions (PCR) contained 25 ng DNA, 5 pmol of each primer, 200 nM dNTPs, and 1 U of BioTherm™ DNA polymerase (GENECRAFT GmbH, Lüdinghausen, Germany) and were initiated with a 4-min soak at 94 °C followed by 36 cycles of 1 min at 94 °C, 1 min at 56 to 60 °C, 1–3 min at 70 °C, and a final soak for 10 min at 70 °C. The homologous integration events in transformants targeting replacement of *FUB1–FUB5*, respectively, with the hygromycin resistance marker were verified by PCR using either the fub1-5-5F-diag and pCSN44-hph-trpC-T primers (5′ flank) or the fub1-5-3R-diag primers in combination with the pCSN44-trpC-P2 primer (3′ flank). The absence of the wild-type gene in deletion strains was verified by PCR using primer pairs targeting the replaced coding region (fub1-5-WT-F and fub1-5-WT-R) (for primer sequences, see Table S1).

Vector cloning

The plasmids pΔFUB1–pΔFUB5 were assembled using yeast recombinational cloning (Schumacher 2012). The plasmid DNA of *Saccharomyces cerevisiae* was extracted with the yeast plasmid isolation kit (SpeedPrep; DualsystemsBio Tech). This extract was utilized for following PCR reactions. For amplification of the knock-out constructs of the genes *FUB1–FUB5*, the TaKaRa polymerase kit [TaKaRa Biotechnology (Dalian) Co., Ltd., Japan] and the fub1-5-5F and fub1-5-3R primers, respectively, were used. For generating overexpression vectors by PCR-based yeast recombinational cloning, a Taq polymerase with proofreading activity was used: 25 ng of genomic DNA, 5 pmol of each primer, and 1 U of Phusion® polymerase (Finnzymes; Thermo Fisher Scientific, Finland).

F. fujikuroi transformations

Generation of protoplasts and transformations were performed as described (Tudzynski et al. 1996). About 10^7 of these protoplasts were transformed with 10 μg of the overexpression plasmid pOE::FUB2, pOE::FUB3, pOE::FUB4, and/or pOE::FUB5. For generation of knock-out mutants, 10 μg of the gene replacement cassettes of the vectors pΔFUB1–pΔFUB5 or pΔFUB2–5, respectively, was transformed. The transformants were regenerated for 6–7 days at 28 °C in a complete regeneration agar (0.7 M sucrose, 0.05 % yeast extract) containing 120 μg/mL hygromycin B (Calbiochem, Darmstadt, Germany) or 100 μg/mL nourseothricin (Werner-Bioagents, Jena, Germany). For DNA isolation and following analysis, resistant transformants were used.

Stability of fusaric acid at pH 5 in a cell-free system

FA was dissolved in ammonium acetate buffer which was adjusted to pH 5 and stored overnight and afterwards analyzed for the formation of derivatives by HPLC–diode-array detection (DAD). Additionally, pure ICI media containing 60 mM glutamine or 120 mM sodium nitrate were spiked with FA and incubated at 28 °C for 6 days to determine the influence of the media on the formation of the derivatives.

Chemicals and solvents

The employed chemicals and solvents were purchased from Grüssing GmbH (Filsum, Germany) or Sigma-Aldrich (Deisenhofen, Germany). The water used for sample preparation and chromatography was purified with a MilliQ® Gradient A10 system (Millipore, Schwalbach, Germany). FA was purchased from Sigma (Deisenhofen, Germany). For the standard solutions for HPLC analysis, fusaric acid was dissolved in methanol/water (1:9, v/v) at a concentration of 10 μg/mL.

Sample preparation for HPLC analysis

Prior to HPLC analysis, the culture filtrates of the different fungi were filtrated through disposable syringe filters (RC Membrane, 0.45 μm, 4 mm Syringe Filters non-sterile, PP Housing, Luer/Slip; Phenomenex, Aschaffenburg, Germany) and without further cleanup directly used for HPLC–DAD and HPLC–high-resolution mass spectrometry (HRMS).

Analysis of fusaric acid and derivatives by HPLC–HRMS and HPLC–DAD analysis

For HPLC–HRMS analysis of the crude culture filtrate, a HPLC system (Accela LC with Accela Pump 60057-60010 and Accela Autosampler 60057-60020; Thermo Scientific, Dreieich, Germany) coupled to a Fourier-transform mass spectrometer with a heated electrospray ionization source (LTQ Orbitrap XL™; Thermo Scientific) was used. Ionization was carried out in the positive ionization mode using the following parameters: capillary temperature 275 °C, vaporizer temperature 350 °C, sheath gas flow 40 units, auxiliary gas flow 20 units, source voltage 3.5 kV, and tube lens 119 V.

Data were acquired and analyzed with the software Xcalibur™ 2.07 SP1 (Thermo Scientific). The chromatography was carried out on a 150 mm×2.00 mm i.d., 5 μm, Gemini® C18 with a 4 mm×2 mm Gemini® NX C18 guard column (Phenomenex). Solvent A was 1 % formic acid in methanol; solvent B, 1 % formic acid in water. The gradient was performed at 40 °C from 10 % A to 60 % A in 20 min followed by column flushing for 5 min at 100 % A and

equilibration at the starting conditions of 10 % A for 10 min. The flow rate was 250 μL/min and the injection volume 10 μL.

FA was identified by comparison to retention time and the MS spectrum of the standard solution [retention time (RT) 10.1 min*m/z* $[M+H]^+$ 180.1014]. Additionally, two similar compounds with RT of 3.4 min and 7.3 min (*m/z* $[M+H]^+$ 196.0964 and 178.0858) were identified and further characterized.

For the generation of MS^2 spectra, the chromatogram was divided into three segments, the first from 0 to 5.2 min, the second up to 9 min, and the third up to 35 min incorporating each one of the three peaks. As the compounds already showed insource fragmentation, for the fragmentation of the $[M+H]^+$ ions, all fragment ions were set on a reject mass list to exclude them from fragmentation. The spectra were generated via collision-induced dissociation with normalized collision energy of 35.00 %.

The HPLC–DAD analysis of the crude culture filtrates was carried out on a HPLC–DAD system (Shimadzu LC with $DGU\text{-}20A_3$ degasser, LC-10AT VP pumps, SIL-10AF autosampler, CTO-10AS VP column oven, SPD-M20A diode-array detector, and CBM-20A communication bus module; Shimadzu, Duisburg, Germany). Data were acquired and analyzed with the software LCSolution (Shimadzu). The chromatography was carried out on a 250 mm×4.60 mm i.d., 5 μm, Gemini® C18 with a 4 mm×3 mm Gemini® C18 guard column (Phenomenex).

Solvent A was 1 % formic acid in methanol (v/v) and solvent B was 1 % formic acid in water (v/v). The gradient was performed at 40 °C from 10 % A to 60 % A in 20 min followed by column flushing at 100 % A for 2 min and equilibration of the starting conditions of 10 % A for 10 min. The flow rate was 1 mL/min and the injection volume 30 μL. The retention time of FA with the column used for HPLC–DAD was 14.7 min, and of the two derivatives 6.1 and 11.3 min. DAD spectra of the three compounds were extracted and compared. For semi-quantitative estimation of the produced amount, the three peaks were extracted and integrated at a wavelength of 270 nm.

Isolation and identification of fusaric acid derivatives

The Miracloth-filtrated culture filtrate was firstly extracted on a Strata C18-E (55 μm, 70 Å) 10 g/60 mL SPE column using a method modified after Kleigrewe et al. (2012). Firstly, the column was activated by subsequent flushing with 50 mL methanol and 50 mL water under vacuum. Then, the aqueous culture filtrate was applied under vacuum and, finally, the column was washed with 100 mL of water to elute salts and sugars from the medium. The bound FA derivatives were eluted from the column with 50 mL 20 % methanol/water (v/v). The solvent was removed on a rotary evaporator (Rotavapor-R; Büchi Labortechnik GmbH, Essen, Germany) and the residue dissolved in about 3 mL of 10 % methanol (v/v). The dissolved extract was further purified on a preparative HPLC–UV system in two steps.

The purification was carried out on a preparative HPLC–UV system (Varian Polaris pumps with Rheodyne manual injection port and Varian ProStar UV detector; Varian, Europe). The software Galaxie 1.9.302.530 (Varian) was used for data acquisition. The column used was a 250×10.0 mm Varian Microsorb 100-5 C18 column with a 10.0×10.0 mm Gemini® C_6-Phenyl guard column. Solvent A was 1 % formic acid in methanol (v/v), and solvent B was 1 % formic acid in water (v/v).

The first preparative run was from 10 % A to 10 0 % A in 30 min followed by equilibration at 10 % A for 5 min. The UV detector was set to 254 nm. The fraction between 8 and 11 min contains both FA derivatives and was further purified in a second preparative HPLC–UV run at 18 % isocratically. The first derivative eluted at about 7 min and the second at about 14 min.

The two isolated derivatives were analyzed by nuclear magnetic resonance (NMR) using a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany) NMR spectrometer. The signals are given in parts per million and are referenced to the solvent signals.

For signal assignment, additional to ^1H and ^{13}C NMR data, 2D NMR experiments, such as (H,H)-correlated spectroscopy (H,H-COSY), heteronuclear multiple-quantum correlation, and heteronuclear multiple bond correlation, were performed. Pulse programs for these experiments were gathered from the software library. Fusarinolic acid was dissolved in d_6-DMSO and dehydrofusaric acid in $CDCl_3$.

The ^1H and ^{13}C NMR data were compared to data published for known FA derivatives (Song and Yee 2001; Boonman et al. 2012; Abraham and Hanssen 1992). The first peak was identified as fusarinolic acid and the second peak as 9,10-dehydrofusaric acid.

Fusarinolic acid: ^1H NMR (d_6-DMSO, 400 MHz): δ 8.54 (1H, d, *J*=1.3 Hz, H-6), 7.96 (1H, dd, *J*=7.6 Hz, 2.1 Hz, H-3), 7.79 (1H, dd, *J*=8.0 Hz, 2.2 Hz, H-4), 3.58 (1H, h, *J*=6.2 Hz, H-9), 2.81–2.63 (2H, m, H-7), 1.68–1.56 (2H, m, H-8), 1.08 (3H, d, *J*=6.2 Hz, H-10); ^{13}C NMR (d_6-DMSO, 100 MHz): δ 166.2 (C, C-11), 149.4 (CH, C-6), 146.1 (C, C-2), 141.7 (C, C-5), 136.9 (CH, C-4), 124.4 (CH, C-3), 65.1 (CH, C-9), 40.1 (CH_2, C-8), 31.9 (CH_2, C-7), 23.6 (CH_3, C-10). For the spectra, see Figs. S3–S7.

Note: This numeration is not consistent with the one used initially by Pitel and Vining (1970) but adapted to the numeration common for 9,10-dehydrofusaric acid.

9,10-Dehydrofusaric acid: ^1H NMR ($CDCl_3$, 400 MHz): δ 8.63 (1H, s, H-6), 8.18 (1H, d. *J*=7.9 Hz, H-3), 7.78 (1H, dd, *J*=8.0 Hz, 2.0 Hz, H-4), 5.87–5.69 (1H, m, H-9), 5.04–5.01 (1H, m, H-10a), 5.01–4.97 (1H, m, H-10b), 2.84 (2H, t, *J*=7.6 Hz, H-7), 2.42 (2H, q, *J*=6.8 Hz, H-8); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.1 (C, C-11), 148.0 (CH, C-6),

144.9 (C, C-2), 142.3 (C, C-5), 138.8 (CH, C-4), 136.4 (CH, C-9), 124.4 (CH, C-3), 116.5 (CH_2, C-10), 34.7 (CH_2, C-8), 32.4 (CH_2, C-7). For the spectra, see Figs. S8–S12.

In planta gene expression quantification

Rice or maize plants were infected as previously described by Wiemann et al. (2013). Three infected plants were collected every 2 days and the roots were lyophilized. Total RNA was isolated from the roots with the RNAgents total RNA isolation kit (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. Then 1.5 μg total RNA was used to synthesize cDNA using SuperScript II reverse transcriptase (Invitrogen, Groningen, The Netherlands). cDNA was checked for absence of genomic DNA by PCR.

Quantitative RT-PCR (qPCR) was performed using iTaq Universal SYBR Green Supermix (BioRad) and cDNA as template, in an iQ5 Biorad thermocycler. In all cases, the qPCR efficiency was between 90 and 110 % and the annealing temperature was 58–60 °C. Every sample was run twice (technical repeats) from two independent biological experiments. The results were calculated according to the delta-delta-Ct (Pfaffl 2001). As reference genes, the related actin gene (primers FRACRTPCRFW and FRACRTPCRRV), GDP-mannose transporter (primers FUJGMTRTPCRFW and FUJGMTRTPCRRV), and ubiquitin gene (primers FUBRTPCRFW and FUBRTPCRRV) were used (Table S1). The following primers were used for amplification of the indicated genes (Table S1): FFUB1RTPCRFW and FFUB1RTPCRRF for *FUB1* gene, and FFUB5RTPCRFW and FFUB5RTPCRRV for *FUB5* gene.

Results

Five genes belong to the fusaric acid gene cluster

BlastN analysis with *FUB1* from *F. verticillioides* (Brown et al. 2012) against the *F. fujikuroi* genome sequence (Wiemann et al. 2013) resulted in the identification of the *F. fujikuroi FUB1* homologue (PKS6 according to the *Fusarium* PKS nomenclature). Adjacent to *FUB1*, we identified the homologous genes to the postulated *F. verticillioides FUB* genes *FUB2–FUB5* (Fig. 1). To elucidate if these genes are co-regulated and if their expression depends on nitrogen availability as shown for other secondary metabolites of *F. fujikuroi* (Wiemann et al. 2013), we grew the wild-type strain for 3 days in synthetic ICI medium with low (6 mM) or high (60 mM) levels of glutamine. The five genes, *FUB1–FUB5*, are co-expressed under nitrogen-sufficient and not expressed at all under nitrogen-limiting conditions. In contrast, the left (*FFUJ_02104*) and right (*FFUJ_02110*) border genes are not expressed under either condition, suggesting that they do not belong to the FA gene cluster (Fig. 2). The five putative FA cluster genes encode proteins with similarities to a polyketide synthase (PKS, *FUB1*), a hypothetical protein (*FUB2*), an aspartate kinase (*FUB3*), a serine hydrolase (*FUB4*), and a homoserine-*O*-acyltransferase (*FUB5*) (Table 1).

Production of fusaric acid and derivatives by *F. fujikuroi*

To study if the high expression of FA biosynthetic genes under nitrogen-sufficient conditions correlates with product formation, the fungus was grown in synthetic ICI medium with 60 mM glutamine for 5 days. Analysis of the culture filtrate revealed the presence of FA and two more compounds with similar fragmentation pattern, suggesting those compounds being FA derivatives (Fig. 3). Published fragments for FA generated on a triple–quadrupole mass spectrometer are those with a loss of H_2O and combined loss of H_2O and CO (van Pamel et al. 2011). The fragments obtained in our study by using an ion trap also arose from loss of H_2O or CO for all three compounds. In addition, the first compound shows the concomitant loss of H_2O and CO (Fig. 3). To further characterize the additional products, they were isolated and analyzed by NMR spectroscopy (Figs. S3–S12). By comparing the spectroscopic data with those of known FA derivatives (Abraham and Hanssen 1992; Song and Yee 2001; Boonman et al. 2012), the two additional compounds were identified as fusarinolic acid (**1**) and 9,10-dehydrofusaric acid (**2**) (Fig. 3). For fusarinolic acid, the broad singlet between 4.30 and 4.80, described in Song and Yee (2001), could not be identified due to the low signal intensity. The H-4 in both derivatives and the H-3 in fusarinolic acid show an additional coupling constant of 2 Hz due to a coupling with H-6 (Figs. S3 and S8).

To demonstrate if the newly identified compounds are intermediates or side products of the FA pathway, and if their appearance always correlates with the formation of FA, we performed a time course experiment and cultivated the fungus with either 120 mM $NaNO_3$ (alkaline pH) or 60 mM glutamine (acidic pH). Interestingly, the amounts of fusarinolic acid and 9,10-dehydrofusaric acid depend on the quality of the nitrogen source and the respective pH in these media. With

Fig. 1 Fusaric acid gene cluster (*FUB1–FUB5*) and its border genes *FFUJ_02104* and *FFUJ_02110*. The *arrows* indicate the direction of transcription and the *white bars* present the introns

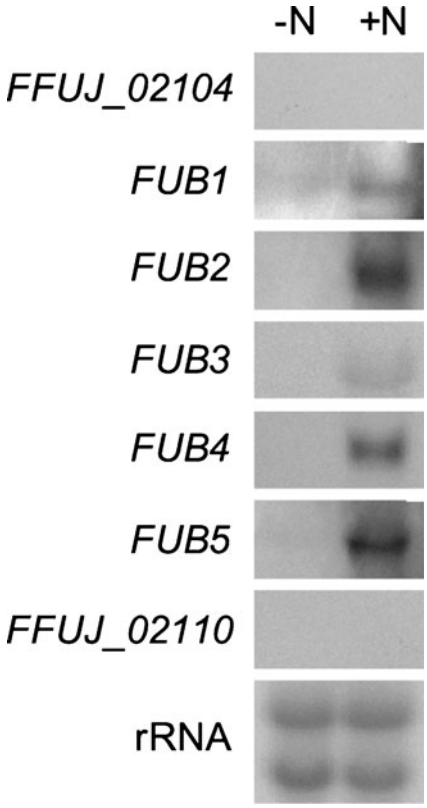


Fig. 2 Co-regulation of the *FUB* cluster genes. The wild type was grown for 3 days in 6 mM (−N) and 60 mM (+N) glutamine. After harvesting, RNA was isolated from lyophilized mycelia. Northern blot analysis was performed as described in "Materials and methods". As probes, the *FUB* genes *1–5* and the border genes *FFUJ_02104* and *FFUJ_02110* were used

$NaNO_3$ as nitrogen source, the main metabolite is FA. In contrast, with glutamine as sole nitrogen source, all three products accumulated in the culture fluid (Fig. 4).

To find out if the conversion of FA into the two derivatives is a pH-dependent non-enzymatic process, we stored FA for 1 day in a cell-free ammonium acetate buffer at pH 5 (corresponding to the pH in glutamine medium), or in cell-free ICI media with 120 mM $NaNO_3$ or 60 mM glutamine. Under none of these conditions were detectable levels of fusarinolic acid and 9,10-dehydrofusaric acid observed (data not shown), indicating that the formation of the two FA derivatives is due to enzymatic activities.

Targeted gene replacement of FA cluster genes and their role in FA biosynthesis

To study the involvement of the five cluster genes *FUB1–FUB5* in the FA biosynthetic pathway and the formation of the two derivatives, we performed targeted gene replacement of all genes (Fig. S1, shown for *FUB1*). The single deletion mutants (Δ*FUB1* to Δ*FUB5*) were grown under nitrogen-sufficient conditions (120 mM $NaNO_3$) for 6 days. HPLC–DAD analyses of culture filtrates revealed the total loss of FA production in Δ*FUB1* and Δ*FUB4*, while the Δ*FUB2*, Δ*FUB3*, and Δ*FUB5* mutants were still able to produce FA (**3**) though with reduced amounts (60–80 % of the wild type) (Fig. 5a). These data suggest that only *FUB1* and *FUB4* are directly involved in FA biosynthesis.

To confirm this result, we generated mutants expressing only the PKS-encoding gene *FUB1* and one or two more *FUB* genes. To do so, we first deleted genes *FUB2–FUB5* in the wild type resulting in mutant strain Δ*FUB2–5*. This multiple deletion strain was transformed either with single *FUB2*, *FUB3*, *FUB4*, or *FUB5* overexpression vectors, or simultaneously with both vectors of *FUB3* and *FUB4*. The generated mutant strains express either only *FUB1* and *FUB3* (Δ*FUB2–5*/OE::*FUB3*), *FUB1* and *FUB4* (Δ*FUB2–5*/OE::*FUB4*), or *FUB1, FUB3*, and *FUB4* together (Δ*FUB2–5*/OE::*FUB3*/OE::*FUB4*). For completeness, we also

Table 1 Fusaric acid biosynthetic genes and its border genes—shown are the gene name, the length of the genes, the domains and motifs, and its predicted function

Gene name (accession number)	Length (bp)	Domains and motifs	Predicted function
FFUJ_02104	785	Uncharacterized protein	Unknown function, probably not belonging to the fusaric acid gene cluster
FUB1 (*FFUJ_02105*)	7525	β-Ketoacyl synthase Acyl transferase domain Dehydratase domain Enoyl reductase domain Keto reductase domain Acyl carrier protein-like	Polyketide synthase; seems to be responsible for the condensation of three acetate units
FUB2 (*FFUJ_02106*)	315	Uncharacterized protein	Unknown function
FUB3 (*FFUJ_02107*)	1,716	Aspartate kinase	Unknown function
FUB4 (*FFUJ_02108*)	856	Serine hydrolase	Unknown function
FUB5 (*FFUJ_02109*)	1,433	Homoserine *O*-acyltransferase	Unknown function
FFUJ_02110	6,303	Ankyrin repeat	Unknown function, probably not belonging to the fusaric acid gene cluster

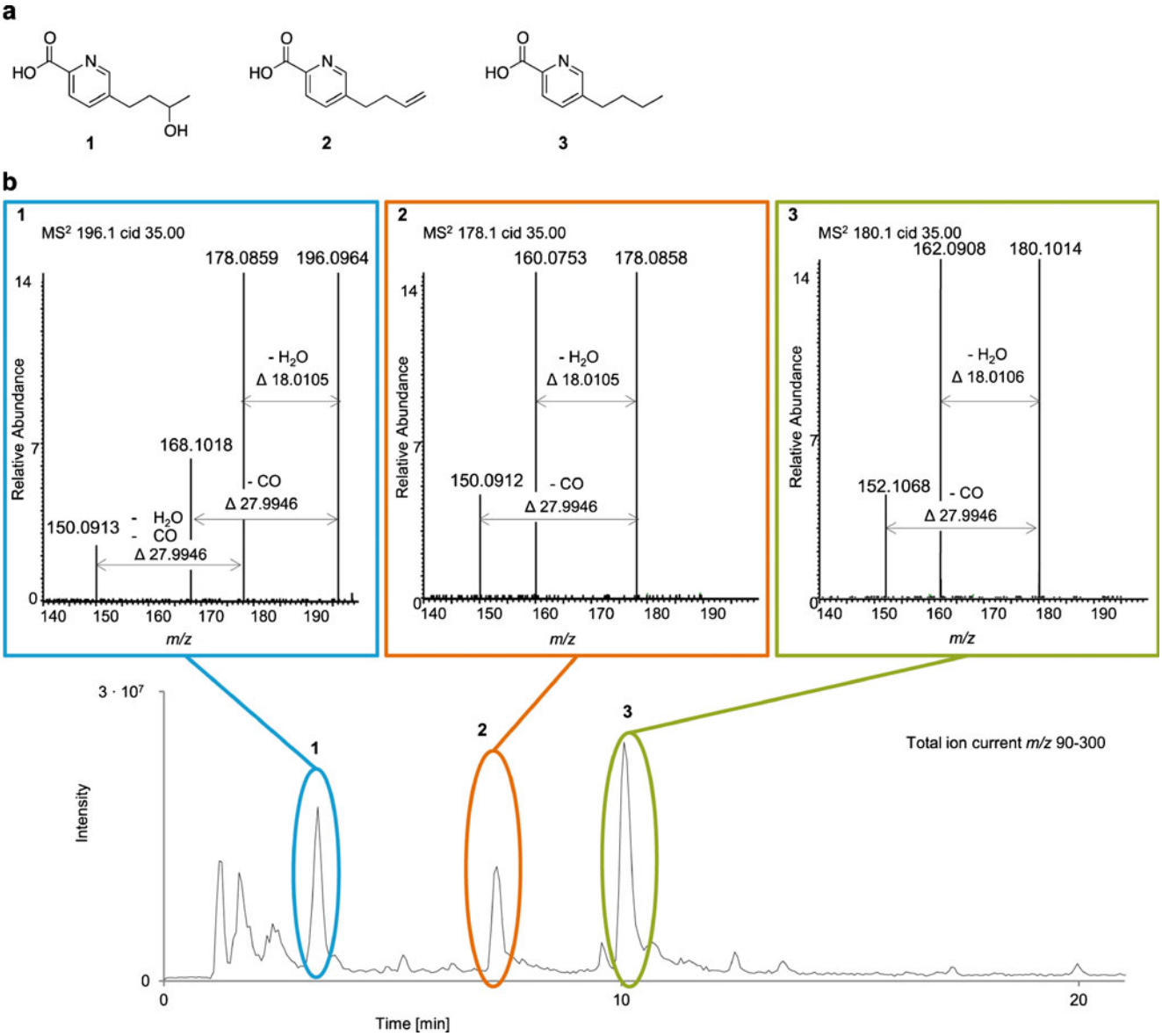


Fig. 3 HPLC–HRMS analysis of the wild type in 60 mM glutamine as nitrogen source. **a** Three derivatives are produced by *F. fujikuroi*: fusarinolic acid (**1**), dehydrofusaric acid, (**2**) and fusaric acid (FA) (**3**). **b** The product analysis was performed as described in "Materials and methods". The total ion current *m/z* 90.0–300 and fragment ion spectra generated with collisional induced dissociation are shown. As depicted, there are three peaks with a similar fragmentation. After isolation and NMR spectroscopy, they could be identified as fusarinolic acid (**1**), dehydrofusaric acid (**2**), and fusaric acid (**3**)

created mutants that have either *FUB1* and *FUB2* (Δ*FUB2*–5/OE::*FUB2*) or *FUB1* and *FUB5* (Δ*FUB2*–5/OE::*FUB5*) expressed. The fusaric acid production was only restored in mutants expressing *FUB1* and *FUB4*, though with significantly lower amounts than the wild type (Fig. 5b). Fusaric acid was also produced by mutants carrying *FUB1*, *FUB3*, and *FUB4*, but not by mutants expressing only *FUB1* and either *FUB2*, *FUB3*, or *FUB5* (Fig. 5b). These data strongly support our assumption that only FUB1 and FUB4 are essential for biosynthesis of fusaric acid (Fig. 5b).

To figure out if the FA cluster genes are involved in conversion of FA into the derivatives fusarinolic acid and 9,10-dehydrofusaric acid, the single deletion mutants were also grown in 60 mM glutamine. While Δ*FUB1* and Δ*FUB4* mutants do not produce any FA or FA derivatives as shown for the medium with 120 mM $NaNO_3$, the other deletion mutants produce a mixture of all three compounds similarly to the wild type (Fig. 5c). These data together with those from the cell-free experiment mentioned above clearly indicate that the conversion of FA into the derivatives depends on the presence of fungal mycelium and is therefore an enzymatic reaction. However, the formation of fusarinolic acid and 9,10-dehydrofusaric acid seems to be catalyzed by enzymes other than those encoded by the *FUB* genes.

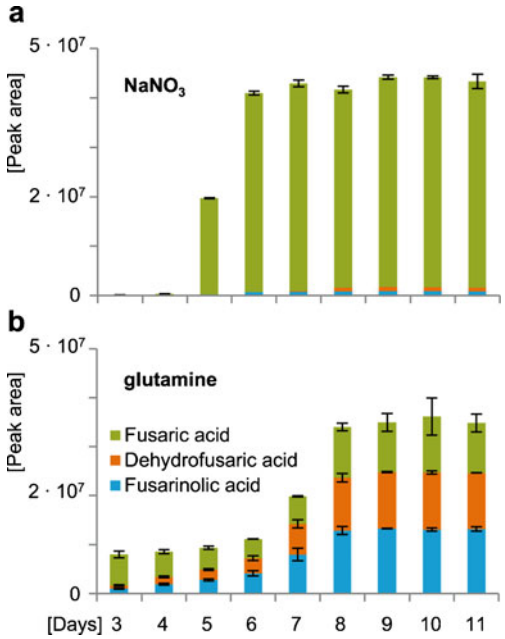


Fig. 4 The production of fusaric acid and its derivatives depends on the nitrogen source. The wild type was grown in a duplicate in a time course for 11 days in 120 mM $NaNO_3$ and 60 mM glutamine, respectively. The supernatant was analyzed by HPLC–DAD. The peaks of fusarinolic acid, dehydrofusaric acid, and fusaric acid were integrated at a wavelength of 270 nm and depicted in a column diagram

To show if the deletion of one *FUB* gene affects the expression of the remaining cluster genes, we performed northern blot analyses with all mutant strains after 5 days in the medium with 120 mM $NaNO_3$. Surprisingly, the deletion of *FUB1* and *FUB4* resulted in complete down-regulation of all other *FUB* genes (Fig. 5d). While *FUB* gene expression remained undetectable also for longer cultivation periods, expression of *FUB* genes was significantly delayed in the Δ*FUB1* mutant: transcripts of *FUB2–FUB5* were detectable only after 6 days (data not shown). The deletion of the other *FUB* genes had no impact on the expression of the remaining genes (Fig. 5d).

Regulation of the *FUB* genes

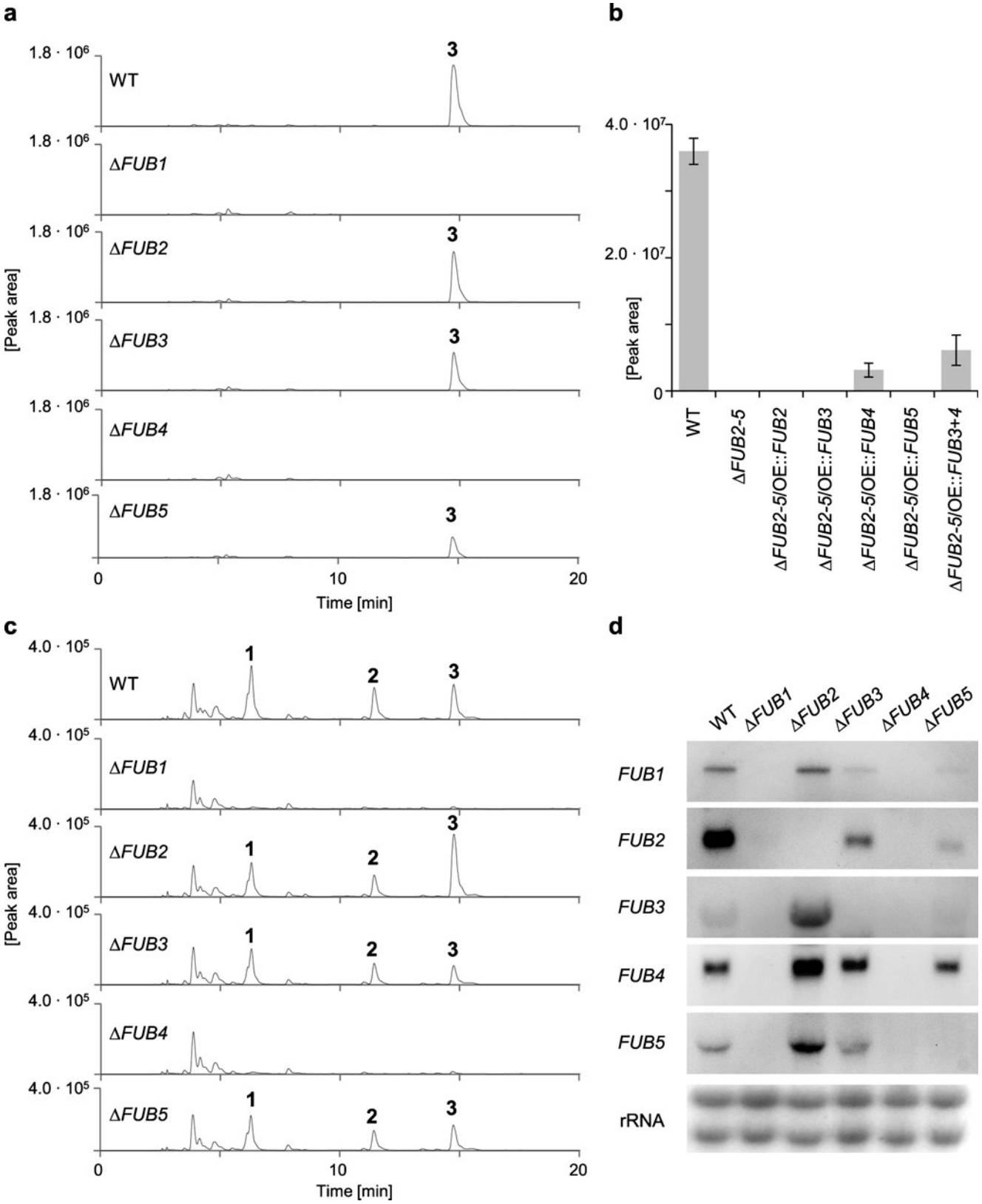
To get more insight into the mechanism of the aforementioned nitrogen regulation and the dependence of *FUB* gene expression on the pH, the *F. fujikuroi* wild-type strain was grown for 6 days in synthetic medium with low and high nitrogen concentrations and acidic (6 and 60 mM glutamine) or alkaline (6 and 120 mM $NaNO_3$) pH values. Analyses of the culture filtrates confirmed that FA is only produced under nitrogen-sufficient conditions (Fig. 6a). This result was supported by northern blot analysis: the *FUB* genes are expressed under high amounts of nitrogen (Fig. 6b). The inducing effect of nitrogen on *FUB* gene expression suggests the involvement

Fig. 5 Production of fusaric acid (FA) and its derivatives in the *FUB* deletion mutants and the influence of the single *FUB* gene deletion of the remaining genes. *FUB1* and *FUB4* are sufficient for FA biosynthesis. **a** HPLC–DAD analysis of the mutants grown for 6 days with 120 mM $NaNO_3$. Shown are the chromatograms at a wavelength of 270 nm; fusaric acid (**3**). **b** Relative amounts of FA (measured by HPLC–DAD) in the wild type (WT), Δ*FUB2–5* (only *FUB1* is expressed), Δ*FUB2–5*/OE::*FUB2* (*FUB1* and *FUB2* are expressed), Δ*FUB2–5*/OE::*FUB3* (*FUB1* and *FUB3* are expressed), Δ*FUB2–5*/OE::*FUB4* (*FUB1* and *FUB4* are expressed), Δ*FUB2–5*/OE::*FUB5* (*FUB1* and *FUB5* are expressed), and Δ*FUB2–5*/OE::*FUB3+4* (*FUB1*, *FUB3*, and *FUB4* are expressed). The WT and the mutants were grown in a triplicate for 6 days in 120 mM $NaNO_3$. **c** HPLC–DAD analysis of the mutants grown for 6 days with 60 mM glutamine. Shown are the chromatograms at a wavelength of 270 nm; fusarinolic acid (**1**), dehydrofusaric acid (**2**), and fusaric acid (FA) (**3**). **d** Northern blot of the mycelium of the mutants grown for 5 days with 120 mM $NaNO_3$. The blot was hybridized with indicated probes (*FUB1–FUB5*)

of the two nitrogen-responsive GATA transcription factors, AreA and/or AreB, both shown to be responsible for nitrogen regulation of GA biosynthetic genes (Mihlan et al. 2003; Schönig et al. 2008, Michielse et al. (2013)). Therefore, we cultivated the wild type and the regulatory mutants (Δ*areA*, Δ*areB*) for 4 days in 60 mM glutamine. HPLC–DAD analysis of the culture filtrates revealed that the expression of *FUB* genes and production of FA and its derivatives are reduced in the Δ*areA* and almost totally abolished in the Δ*areB* deletion mutants (Fig. 7).

Previous studies showed that components of the fungal-specific *velvet* complex, Vel1, Vel2, and Lae1, significantly affect biosynthesis and gene expression of some secondary metabolites in *F. fujikuroi* (Wiemann et al. 2010; Niehaus et al. 2013). Here we show that Vel1 and Lae1 act as positive regulators of the *FUB* gene cluster: both the *FUB* gene expression and production of FA and the two derivatives were significantly reduced in the Δ*vel1* and Δ*lae1* mutants compared to the wild type (Fig. 7a). Interestingly, Vel2 does not play a role, neither in the production of FA and its derivatives nor in regulation of *FUB* gene expression (Fig. 7).

Furthermore, although the *FUB* gene expression was almost identical in media with glutamine (acidic pH) and nitrate (alkaline pH) in long-term cultivations (5–6 days), we investigated also the short-term response of *FUB* gene expression to changes of the pH value. The fungus was grown for 3 days in synthetic ICI medium with 60 mM glutamine. The mycelium was washed and transferred into the same medium adjusted to either pH 4 or pH 8. After 2 h, the *FUB* genes are only expressed under alkaline conditions (Fig. 8). To show if the pH regulator PacC is involved in this short-term response to different pH conditions, we compared the *FUB* gene expression between the wild type and the Δ*pacC* mutant.


a
WT
ΔFUB1
ΔFUB2
ΔFUB3
ΔFUB4
ΔFUB5
[Peak area]
Time [min]
b
[Peak area]
WT
ΔFUB2-5
ΔFUB2-5/OE::FUB2
ΔFUB2-5/OE::FUB3
ΔFUB2-5/OE::FUB4
ΔFUB2-5/OE::FUB5
ΔFUB2-5/OE::FUB3+4
c
[Peak area]
Time [min]
d
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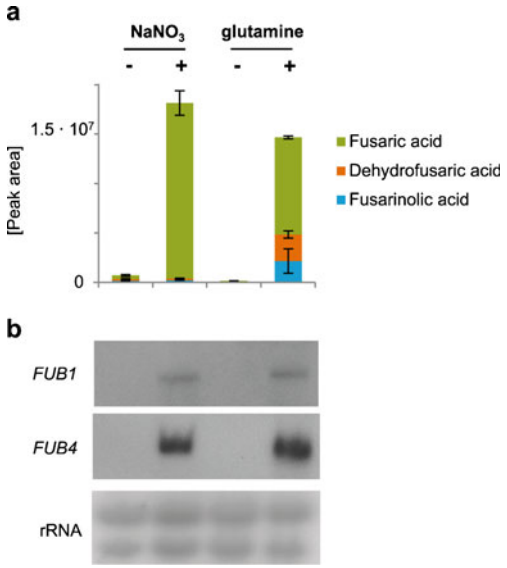

Fig. 6 Fusaric acid and its derivatives are produced under high nitrogen conditions. **a** Peak areas of fusarinolic acid, dehydrofusaric acid, and fusaric acid after HPLC–DAD analysis and integration at a wavelength of 270 nm referenced to the dry weight and normalized to the wild-type level. **b** Northern blot of the wild type (WT). The WT was grown in a triplicate under different nitrogen conditions (6 mM and 60 mM glutamine, 6 mM and 120 mM $NaNO_3$) for 6 days. After harvesting, the supernatant of the WT was analyzed. The northern blot was prepared from the mycelium. *FUB1* and *FUB4* were used as probes for the northern blot

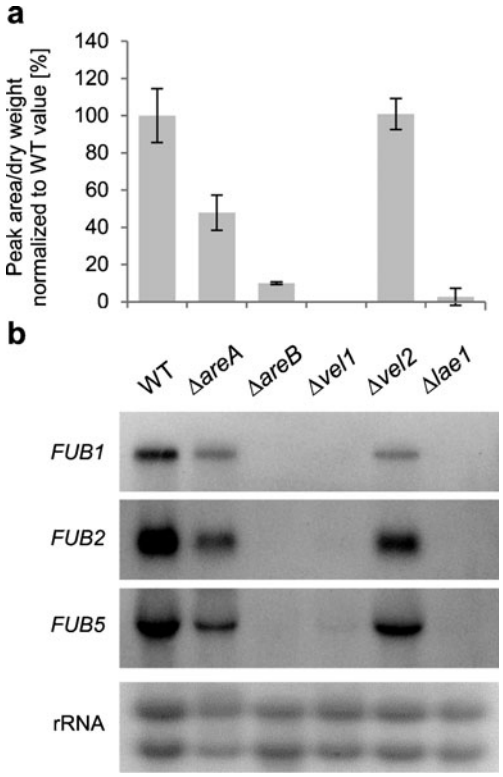

Fig. 7 Regulation of the *FUB* genes. **a** Peak areas of fusarinolic acid, dehydrofusaric acid, and fusaric acid after HPLC–DAD analysis and integration at a wavelength of 270 nm referenced to the dry weight and normalized to the wild-type level. **b** For northern blot analysis, the wild type and the Δ*areA*, Δ*areB*, Δ*vel1*, Δ*vel2*, and Δ*lae1* mutants were grown for 4 days in 60 mM glutamine. After harvesting, the supernatant of a triplicate was used for HPLC analysis, and the mycelium was used for RNA isolation. *FUB1*, *FUB2*, and *FUB5* were used as probes for northern blot analysis

Surprisingly, the *FUB* genes are not expressed in the Δ*pacC* mutant, neither at pH 4 nor at pH 8 (Fig. 8). These data indicate that the pH regulator PacC acts as activator of *FUB* gene expression after the pH shift to alkaline ambient conditions.

Host-specific in planta expression of *FUB* genes

Previously, we have shown that the expression of some secondary metabolites such as GAs and fumonisins is host specific (Wiemann et al. 2013). To show if the *FUB* genes are differentially expressed on the preferred host plant rice compared to maize, we infected the roots of rice and maize seedlings with wild-type microconidia. After 2, 4, 6, 8, and 10 days, the infected roots were collected, and qRT-PCR was performed with total RNA isolated from lyophilized root material. The expression of *FUB1* and *FUB5* showed similar in planta expression pattern (Fig. 9): both genes are higher expressed in rice roots (Fig. 9a) compared to maize roots (Fig. 9b), indicating that rice provides a specific host signal that strongly activates the expression of FA cluster genes.

Discussion

FA is a well-known *Fusarium* mycotoxin that has both phytotoxic and antibiotic properties against bacteria, protozoa, and fungi. On the other hand, FA represses the production of the polyketide 2,4-diacetylphloroglucinol (DAPG), a key factor in the antimicrobial activity of the biocontrol strain

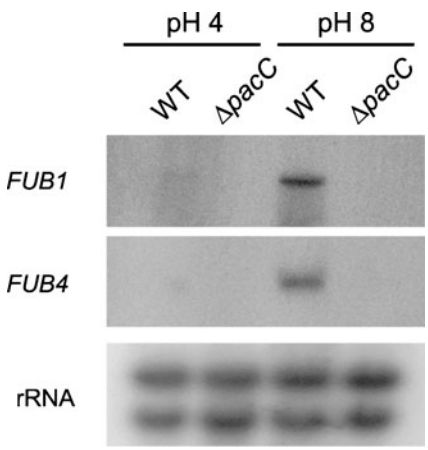

Fig. 8 The pH regulator PacC is an activator of the *FUB* genes. The wild type and Δ*pacC* mutant were grown for 3 days in 60 mM glutamine. The mycelium was harvested, washed, and then shifted into fresh ICI medium with 60 mM glutamine either adjusted to pH 4 or pH 8. After 2 h, the mycelium was harvested and a northern blot was performed from the isolated RNA. *FUB1* and *FUB4* were used as probes

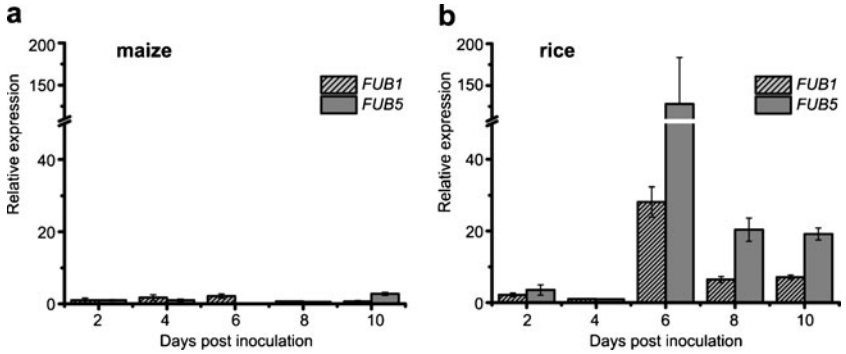

Fig. 9 Relative expression of *FUB1* and *FUB5* genes. **a** Rice and **b** maize plants were infected with *F. fujikuroi* conidia and three maize and three rice roots were collected every second day. Quantitative RT-PCR was performed using RNA from infected roots for cDNA synthesis. The gene expression pattern was studied using the delta-delta-Ct method. As reference, the expression of the sample after 2 days (in maize) was taken as 1, and the rest was compared to that value. Two independent experiments were done, all showing higher *FUB* gene expression in rice roots compared to maize roots

Pseudomonas fluorescens. DAPG is one of the most effective antimicrobial metabolites produced by strains of fluorescent *Pseudomonas* (Notz et al. 2002).

In the past years, the interest in FA increased due to the manifold proposed pharmacological activities and proposed therapeutic applications (Wang and Ng 1999). In particular, FA was shown to be a potent inhibitor of dopamine hydroxylase in vitro and in vivo and displayed antihypertensive activity. FA also exhibited marked antitumor activity on human colon adenocarcinoma cell lines (Song and Yee 2001).

Distribution of FA gene clusters among *Fusarium* spp.

While most of the typical *Fusarium* secondary metabolites are produced by a distinct group of closely related species, e.g., those of the *G. fujikuroi* species complex (GFC), FA production is widely distributed among the whole genus *Fusarium* (Bacon et al. 1996). The recent genome sequencing of three new members of the GFC, *F. fujikuroi*, *Fusarium mangiferae*, and *Fusarium circinatum*, confirmed the broad distribution of this gene cluster and allowed a comparison of FA cluster organization among all so far sequenced 15 *Fusarium* species (Wiemann et al. 2013). The cluster is present in all species inside the GFC, but also in the closely related species *F. oxysporum* outside the GFC. The only difference between these strains is that the *F. oxysporum* FA cluster has two additional genes whose function is not yet clear (Wiemann et al. 2013).

Fusarinolic acid and dehydrofusaric acid are not intermediates of the FA biosynthetic pathway

There are several early reports on formation of FA derivatives by *Fusarium* spp. beside FA as the main product. However, nothing was known about their origin. First hints for the formation of a more polar FA metabolite have been found by Braun (1960) and could be confirmed by the structure elucidation of fusarinolic acid (10-hydroxy fusaric acid) by Pitel and Vining (1970). Dehydrofusaric acid was firstly described by Stoll (1954). In 1970, the group of Pitel and Vining described dehydrofusaric acid and FA to be interconvertible in cultures of *G. fujikuroi* whereas fusarinolic acid is described as a more polar metabolite. The biosynthetic relationship between the three compounds could not be elucidated, but the study provided hints that FA might be an intermediate as it was converted faster to dehydrofusaric and fusarinolic acid as the other way round (Pitel and Vining 1970).

In this work, we have shown that in *F. fujikuroi* the ratio between FA and its two derivatives seems to be strongly influenced by the kind of nitrogen source in the medium and the respective pH value. In the alkaline $NaNO_3$-rich medium, the fungus produces mainly FA whereas all three compounds accumulate in the acidic glutamine-rich medium. Similar observations were described in a recent feeding study for *F. oxysporum* f. sp. *vasinfectum* that produced 9,10-dehydrofusaric acid in addition to FA in cultures supplemented with glutamine (Stipanovic et al. 2011). Deletion of the single biosynthetic genes either resulted in simultaneous loss of FA and FA derivatives production (Δ*FUB1* and Δ*FUB4*) or in wild-type-like ratio of FA and the produced derivatives (Δ*FUB2*, Δ*FUB3*, and Δ*FUB5*) (Fig. 5a, c).

To our knowledge, it was not known so far if any of the *FUB*-gene-encoding enzymes are involved in conversion of FA into its derivatives. Our results clearly show for the first time (1) that the conversion of FA to fusarinolic acid and dehydrofusaric is an enzymatic process and does not happen in cell-free systems, and (2) that the FA cluster genes and encoded enzymes are not involved in these reactions. We suggest that genes outside the FA gene cluster are responsible for these pH-dependent modifications.

The FA biosynthetic pathway and genes involved

Although the toxin is known for a long time, nothing was known about the biosynthetic pathway, potential

intermediates, and genes/enzymes involved. Only recently, the first biosynthetic gene has been identified in *F. verticillioides*, and a putative gene cluster adjacent to this PKS-encoding *FUB1* gene has been postulated based on their co-regulation in a microarray approach (Brown et al. 2012).

Already a long time ago, feeding experiments with ^{14}C-labeled acetate to cultures of *G. fujikuroi* were performed leading to the conclusion that FA is built out of acetate or closely related metabolites (Hill et al. 1966). Similar studies were performed with the fungus *F. oxysporum* Schlecht leading to corresponding results (Dobson et al. 1967).

Nothing is definitely known about the origin of nitrogen in the FA molecule. Additional feedings with ^{13}C- and ^{15}N-labeled precursors led to the assumption that the nitrogen is derived from the amino group of aspartate that is transferred by an amino transferase to oxalacetate (Desaty et al. 1968). Recent feeding experiments with *F. oxysporum* f. sp. *vasinfectum* using differently labeled precursors such as $^{13}C_2$-acetate, ^{13}C- and ^{15}N-labeled aspartate, and ^{15}N-glutamine confirmed the hypothesis that three acetate units and one derivative of the citrate (TCA) cycle are precursors for the FA biosynthesis (Stipanovic et al. 2011). Additionally, the results indicated the more ready incorporation of the nitrogen from glutamine than from aspartate (Stipanovic et al. 2011). Based on these experimental data, a hypothetical biosynthetic pathway has been suggested that is however not confirmed by experimental data (Brown et al. 2012).

To gain a better insight into the genes belonging to the FA gene cluster and probably being involved in FA biosynthesis, we identified the genes adjacent to the PKS-encoding gene *FUB1*. Northern blot analysis under different nitrogen conditions revealed five co-regulated putative FA cluster genes (*FUB1–FUB5*), similarly to what was found by microarray studies in *F. verticillioides* (Brown et al. 2012). However, despite the co-regulation of these five cluster genes, only the deletion of *FUB1* (encoding the PKS) and *FUB4* (similarity to a hydrolase) resulted in total loss of FA biosynthesis under nitrogen-sufficient conditions (60 mM glutamine and 120 mM $NaNO_3$). Δ*FUB2*, Δ*FUB3*, and Δ*FUB5* deletion mutants still produce FA (**3**), though in reduced amounts, as well as fusarinolic acid (**1**) and dehydrofusaric acid (**2**) (Fig. 5c). In summary, HPLC–DAD analysis of all five deletion mutants suggests that only the genes *FUB1* and *FUB4* are necessary for FA biosynthesis. This surprising result was confirmed by overexpressing each single *FUB* gene in the multiple Δ*FUB2–5* mutant which has only the *FUB1* gene left. Mutants carrying only *FUB1* and *FUB4* (Δ*FUB2–5*/OE:*FUB4*) were able to produce significant amounts of FA.

We had expected that the putative aspartate kinase gene *FUB3* would play a role in activation of aspartate which could be used as nitrogen source for FA. Aspartate kinases are common enzymes in bacteria, plants, and fungi that catalyze the first reaction in the aspartate pathway, the phosphorylation of aspartate to produce lysine, threonine, methionine, and isoleucine in a series of reactions known as the aspartate pathway.

As FUB3 was postulated to be an essential biosynthetic enzyme, we also overexpressed the *FUB3* gene, alone or simultaneously with *FUB4*, in the Δ*FUB2–5* background. However, expression of *FUB1* and *FUB3* alone did not result in the formation of any FA, confirming the result that the Δ*FUB3* mutant still produces FA. However, we cannot exclude that *FUB3* and/or the other non-essential *FUB* genes might be important for elevated production yields, e.g., by providing precursor molecules.

Searching for *FUB3* homologues in the *F. fujikuroi* genome by BLAST analysis revealed one additional gene, *FFUJ_02489* (score = 567 bits, E-value 3.7e−162), with similarity to aspartate kinases. It is possible that this second putative aspartate kinase is able to take over the function of *FUB3* in the FA biosynthetic pathway. Gene replacement of this gene is currently in progress.

This is the second example for a nitrogen-induced secondary metabolite gene cluster in *F. fujikuroi*, where only some of the strictly co-regulated genes are involved in biosynthesis or regulation. For the fusarin C cluster, it was shown that only four of the nine cluster genes are required for fusarin C biosynthesis (Niehaus et al. 2013).

Unfortunately, no intermediates could be identified in the *FUB4* deletion mutant, probably due to the fact that the expected intermediates are supposed to be quite small and might be typical products of the primary metabolism, e.g., an amino acid and acetyl-CoA (Stipanovic et al. 2011). These metabolites are probably common in both the wild type and the mutants. Therefore, an accumulation of these primary metabolites in the deletion mutant cannot easily be detected. In addition, the expression of all *FUB* cluster genes is down-regulated in the *FUB4* deletion mutant.

Taken together, our current data suggest that only two genes are essential for biosynthesis of FA. However, we cannot exclude that the remaining co-regulated cluster genes are involved somehow in fine-tune regulation of FA biosynthesis.

The regulation of FA gene expression and FA biosynthesis

With the identification of the co-regulated cluster genes, we were able to study the regulation of *FUB* gene expression on a molecular level. Already in the years after its discovery, FA was shown to be preferentially produced in alkaline (Yabuta et al. 1939) or slightly acidic media and under high nitrogen conditions (Pitel and Vining 1970). In the present study, we could confirm the essential role of nitrogen availability on the level of gene expression. Thus, FA is the second secondary metabolite in *F. fujikuroi* beside the fusarins (Díaz-Sánchez et al. 2012; Niehaus et al. 2013) whose production is induced by high nitrogen conditions. All the other so far studied secondary metabolites in this fungus, such as gibberellins

(GAs), bikaverin, fusarubins, and carotenoids, are repressed by nitrogen (Bömke and Tudzynski 2009; Wiemann et al. 2009; Studt et al. 2012; Rodríguez-Ortiz et al. 2009). However, the mechanism of nitrogen metabolite repression of these metabolites differs. Only for GAs an essential role of the nitrogen-responsive GATA transcription factors AreA and AreB in nitrogen starvation-induced gene expression has been shown: the deletion of either AreA or AreB resulted in loss of GA gene expression (Mihlan et al. 2003; Schönig et al. 2008; Michielse et al., 2013). In contrast, none of these regulators is essential for bikaverin gene expression under nitrogen-limiting conditions (Wiemann et al. 2009). Surprisingly, one of the two nitrogen-responsive GATA transcription factors, AreB, seems to play an essential role for induction of FA gene expression under nitrogen-sufficient conditions while AreA does not significantly affect the expression of *FUB* genes and FA production. These results could be confirmed by our recent microarray studies comparing genome-wide gene expression of the wild type with that of the Δ*areA* and Δ*areB* mutants (Michielse et al. 2013). Thus, FA is the first studied secondary metabolite which is induced by high amounts of nitrogen in an AreB-dependent manner demonstrating that this GATA transcription factor can regulate both nitrogen-repressed and nitrogen-induced genes/gene clusters.

In the last years, the importance of the fungal-specific *velvet* complex in regulating differentiation and secondary metabolite production has been shown for several fungi (Bayram and Braus 2012). In this work, we have demonstrated that FA production and expression of the *FUB* genes are influenced by two members of the complex, Vel1 and Lae1, while Vel2 does not play a major role in regulation of this gene cluster. Similarly to our findings for FA, Vel1 and Lae1 act as activators of the fusarin biosynthesis, and Vel2 has only a minor effect (Niehaus et al. 2013). In *F. verticillioides* and *F. oxysporum*, Lae1 was also shown to be essential for FA production: the expression of *FUB* genes was totally lost or significantly reduced, respectively, in the Δ*lae1* deletion mutant (Butchko et al. 2012; López-Berges et al. 2013).

Beside the regulation of *FUB* gene expression by nitrogen and components of the *velvet* complex, we found a strict dependency of *FUB2–FUB5* gene expression on the presence of *FUB4*: its deletion resulted in the complete down-regulation of all the other *FUB* genes. Deletion of the PKS-encoding gene *FUB1* also resulted in complete loss of *FUB2–FUB5* gene expression up to the fifth day. However, transcripts are clearly detectable after 6 days indicating that the expression of the remaining *FUB* genes is only delayed in the Δ*FUB1* mutant. This observation can be explained by the reduced growth rate of the Δ*FUB1* mutant compared to the wild type (Fig. S2).

Beside expression studies under in vitro conditions, we were interested if FA cluster genes are expressed in planta and if the in planta expression depends on the host plant. Recently, we have shown that the biosynthetic genes of several secondary metabolites in *F. fujikuroi* (e.g., GAs, PKS19, fumonisin) are preferentially expressed on the favored host plant rice while the genes are significantly less or not expressed in maize (Wiemann et al. 2013). The same has been found for the *FUB* genes in this work: the genes are higher expressed in rice roots than in maize roots suggesting a specific role of FA in the infection of rice. Years ago, it has been shown that *Fusarium lycopersici* produces FA during its parasitic life in tomato plants (Kern and Kluepfel 1956). In contrast to these findings, Lopez-Berges et al. did not find any FA production by *F. oxysporum* during specific stages of tomato root infection (López-Berges et al. 2013).

In summary, we identified a FA gene cluster in *F. fujikuroi* consisting of five co-regulated genes. Only two of them seem to be directly involved in biosynthetic steps. Under acidic conditions, FA can be converted into fusarinolic acid and dehydrofusaric acid. This derivatization is an enzymatic process catalyzed by enzymes not linked to the FA gene cluster. The *FUB* genes are regulated by nitrogen availability in an AreB-dependent manner and by the pH regulator PacC. Furthermore, Vel1 and Lae1, components of the *velvet* complex, are essential for full expression of FA cluster genes.

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