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Characterization of novel mutants with an altered gibberellin spectrum in comparison to different wild-type strains of *Fusarium fujikuroi*

Sabine Albermann · Tino Elter · Andreas Teubner · Wolfgang Krischke · Thomas Hirth · Bettina Tudzynski

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Abstract The rice pathogen *Fusarium fujikuroi* is known for producing a wide range of secondary metabolites such as pigments, mycotoxins, and a group of phytohormones, the gibberellic acids (GAs). Bioactive forms of these diterpenes are responsible for hyperelongation of rice stems, yellowish chlorotic leaves, and reduced grain formation during the *bakanae* disease leading to severely decreased crop yields. GAs are also successfully applied in agriculture and horticulture as plant growth regulators to enhance crop yields, fruit size, and to induce earlier flowering. In this study, six *F. fujikuroi* wild-type and mutant strains differing in GA yields and the spectrum of produced GAs were cultivated in high-quality lab fermenters for optimal temperature and pH control and compared regarding their growth, GA production, and GA gene expression levels. Comparative analysis of the six strains revealed that strain 6314/Δ*DES*/Δ*PPT1*, holding mutations in two GA biosynthetic genes and an additional deletion of the 4'-phosphopantetheinyl transferase gene *PPT1*, exhibits the highest total GA amount. Expression studies of two GA biosynthesis genes, *CPS*/*KS* and *DES*, showed a constantly high expression level for both genes under production conditions (nitrogen limitation) in all strains. By cultivating these genetically engineered mutant strains, we were able to produce not only mixtures of different bioactive GAs (GA_3, GA_4, and GA_7) but also pure GA_4 or GA_7. In addition, we show that the GA yields are not only determined by different production rates, but also by different decomposition rates of the end products GA_3, GA_4, and GA_7 explaining the varying GA levels of genetically almost identical mutant strains.

Keywords *Fusarium fujikuroi* · Gibberellic acid · Gene expression analyses · Fermentation

Introduction

The filamentous fungus *Fusarium fujikuroi* (*Gibberella fujikuroi*) was first described in 1917 as the causative agent of the *bakanae* disease of rice in Japan (Sawada 1917). Infected rice plants show hyper elongated internodes and develop yellowish chlorotic leaves and empty grains due to the fungus' ability to produce gibberellic acids (GAs), a group of phytohormones (Desjardins et al. 2000; Sun and Snyder 1981; Yabuta 1935). These symptoms result in crop losses up to 40 %, indicating the wide impact of the fungus on agriculture.

Beside their negative effects on rice plants, GAs are biotechnologically produced in large scale (about 100 t per year) as plant growth regulators to enhance crop yields, plant biomass, and fruit size, to induce earlier flowering or to positively influence other processes of plant development in general (Rademacher 1997; Tudzynski and Sharon 2002). In the fruit-growing sector, for instance, GAs are applied externally to loosen up seed heads and prevent fruit rotting.

Tino Elter and Sabine Albermann contributed equally to the work.

S. Albermann · B. Tudzynski (✉)
Institute of Plant Biology and Biotechnology, Westfälische Wilhelms Universiät Münster, Schlossplatz 8,
48143 Münster, Germany
e-mail: tudzynsb@uni-muenster.de

T. Elter · A. Teubner · T. Hirth
Institute for Interfacial Engineering, University of Stuttgart, Nobelstraße 12,
70569 Stuttgart, Germany

W. Krischke
Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Nobelstr. 12,
70569 Stuttgart, Germany

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Currently, only GA_3 and a mixture of two other bioactive gibberellins, GA_4 and GA_7, are available on the international market.

Beside GAs, the fungus is also famous for its capability to produce a wide spectrum of other polyketide synthase (PKS)- or nonribosomal peptide synthetase (NRPS)-derived secondary metabolites, such as the pigments neurosporaxanthin (Avalos and Cerdá-Olmedo 1986; Linnemannstöns et al. 2002a), the pigments bikaverin (Kjær et al. 1971; Linnemannstöns et al. 2002b; Wiemann et al. 2009) and fusarubins (Studt et al. 2012), and mycotoxins such as fusarins (Barrero et al. 1991; Kleigrewe et al. 2012) or fusaric acid (Bacon et al. 1996). However, the production of GAs is the characteristic trademark of *F. fujikuroi*, since only some isolates of the closely related *Fusarium* species, such as *Fusarium proliferatum*, *Fusarium konzum*, and *Fusarium sacchari*, are able to produce small amounts of GAs despite the presence of the entire GA gene cluster (Malonek et al. 2005; Troncoso et al. 2010; Tsavkelova et al. 2008).

GAs belong to the class of cyclic diterpenoids. In 1938, Yabuta and co-workers succeeded in crystallization of the first two GAs from culture fluid named "gibberellin A and B" and their chemical and biological properties were studied (Yabuta and Sumiki 1938). Until now, 136 different GAs (GA_1–GA_{136}) are known, all exhibiting a characteristic gibberellin ring structure (MacMillan 2002). Out of these compounds, only a few possess bioactive properties, such as the main product of GA biosynthesis in *F. fujikuroi*, GA_3, its precursors GA_4 and GA_7, as well as GA_1, a minor GA in the fungus (Bömke and Tudzynski 2009; reviewed by Hedden and Thomas 2012; MacMillan 2002; reviewed in Tudzynski 2005).

The major steps of the fungal GA biosynthesis were discovered by incorporation studies applying ^{14}C-labeled acetate and mevalonate to wild-type and UV mutants with a genetic block at a certain step of the biosynthetic pathway and subsequent analysis of accumulated intermediates by combined gas chromatography and mass spectrometry (Bearder et al. 1974, 1983; Binks et al. 1969; Birch et al. 1958; MacMillan et al. 1967).

In the mid-1990s, the GA biosynthetic genes in *Arabidopsis thaliana* were cloned and shown to be distributed on all five chromosomes in the plant genome (Hedden and Phillips 2000; Lange et al. 1994; Phillips et al. 1995; Sun and Kamiya 1994; reviewed in Yamaguchi 2008), while the first fungal GA biosynthetic genes were identified from *F. fujikuroi* and a second GA-producing species, *Phaeosphaeria* spp., in the late 1990s (Kawaide et al. 1997; Tudzynski et al. 1998; Tudzynski and Hölter 1998). Today, all seven genes belonging to the GA biosynthetic cluster in *F. fujikuroi* are well characterized regarding their function and regulation (for review see Bömke and Tudzynski 2009; Tudzynski et al. 2003; Tudzynski 2005). Although higher plants and fungi produce structurally identical GAs, there are profound differences in the character of GA biosynthetic enzymes (cytochrome P450 monooxygenases in the fungus, 2-oxoglutarate-dependent dioxygenases (ODDs) in the plant), indicating that higher plants and fungi have evolved GA biosynthetic pathways independently and that fungi did not acquire the plant genes by horizontal gene transfer (Hedden et al. 2001).

In fungi, GAs are built via the mevalonate pathway starting with the condensation of two acetyl-coenzyme A (acetyl-CoA) molecules resulting in the formation of hydroxymethylglutaryl coenzyme A (Hmg-CoA). This compound is further reduced to mevalonate by the hydroxymethylglutaryl-coenzyme A reductase (HmgR) (Woitek et al. 1997). Genetic engineering of the HmgR-encoding gene was recently shown to result in significant increase of GA production yields in *F. fujikuroi* (Albermann et al. 2013).

Mevalonate is subsequently converted to geranyldiphosphate, farnesyldiphosphate (FPP), and geranylgeranyldiphosphate (GGDP) by the action of the farnesyldiphosphate synthase and the GGDP synthase (GGS) (Homann et al. 1996; Mende et al. 1997). In *F. fujikuroi* two different GGDP synthase-encoding genes exist, *GGS1* (Mende et al. 1997) and *GGS2* (Tudzynski and Hölter 1998), both metabolizing FPP to GGDP. Ggs1 is responsible for ubiquinone and neurosporaxanthin biosynthesis, whereas Ggs2 constitutes the first enzyme of the GA biosynthesis pathway (Tudzynski and Hölter 1998). Deletion mutants of *ggs2* exhibit a total loss in GA production, while deletion of *ggs1* was found to be lethal. Thus, these two iso-enzymes cannot functionally complement each other (S. Albermann and B. Tudzynski, unpublished data).

For GA biosynthesis, GGDP is converted to *ent*-kaurene via *ent*-copalyldiphosphate by the bifunctional diterpene cyclase Cps/Ks, encoded by *cps*/*ks*, in a two-step cyclization reaction (Tudzynski et al. 1998). In the following sequential oxidation steps, *ent*-kaurene is oxidized to *ent*-kaurenoic acid by the *ent*-kaurene oxidase P450-4 (Tudzynski et al. 2001). The next four sequential biosynthetic steps from *ent*-kaurenoic acid to GA_{14} (7β-hydroxylation, contraction of ring B by oxidation at C-6, 3β-hydroxylation, and oxidation at C-7) are catalyzed by a single cytochrome P450 monooxygenase, the GA_{14} synthase P450-1 (Rojas et al. 2001). The first bioactive gibberellin, GA_4, is generated by sequential C20-oxidation catalyzed by the 20-oxidase P450-2 (Tudzynski et al. 2002). Next, the GA_4 desaturase, Des, converts GA_4 to GA_7 by formation of a carbon-1, 2 double bond (Tudzynski et al. 2003). Recently, the nature of this unusual oxidase was cleared up by expressing the cDNA of the fungal desaturase (*des*) in *Escherichia coli*: Des is a 2-oxoglutarate-dependent dioxygenase with putative iron and 2-oxoglutarate binding residues, typical of such enzymes, but with very low amino acid sequence homology to known ODDs (Bhattacharya et al. 2012).

Finally, GA_7 is converted to the main product of *F. fujikuroi* wild-type strains, GA_3, by the 13-hydroxylase P450-3 (Tudzynski et al. 2003). Generation of the minor side product GA_1 via 13-hydroxylation of GA_4 is also carried out by the enzyme P450-3 (reviewed in Bömke and Tudzynski 2009; Tudzynski et al. 2003) (Fig. 1).

The aim of this work was the genetic manipulation of the fungal GA pathway to (1) improve the gibberellin yield and (2) to gain mutant strains that do not produce GA_3, but its bioactive precursors, GA_4 or GA_7, that are not available on the international market yet. By deleting the *PPT1* gene encoding the 4'-phosphopantetheinyl transferase, the flux of acetyl-CoA was channeled into the isoprenoid pathway in order to improve the overall GA production yield. The PPT1 enzyme is essential for activating PKS and NRPS enzymes by transfering a 4'-phosphopantetheine moiety onto conserved serine residues of PKSs and NRPSs (Crawford et al. 2008; Lambalot et al. 1996), and its deletion results in loss of all PKS-and NRPS-derived metabolites.

To better compare the production yields and GA spectra, all wild-type and mutant strains were cultivated in parallel multi-fermenters under standardized pH, temperature, and aeration conditions applying a fed-batch fermentation approach. We show that the production yields of GA-producing strains are not only determined by the production rate but also by differing chemical stability of the single GAs.

Material and methods

Fungal strains and culture conditions

F. fujikuroi strains IMI58289 (Commonwealth Mycological Institute, Kew, UK), m567 (Culture Collection Weimar, Germany), 6314, 6314/Δ*PPT1*, 6314/Δ*DES* (Tudzynski et al. 2003), and 6314/Δ*DES*/Δ*PPT1* were pre-cultivated in 100 ml Darken medium (Darken et al. 1959) at 28 °C for 72 h on a rotary shaker at 180 rpm using 300 ml Erlenmeyer flasks. Subsequently, the mycelium was washed thrice by centrifugation (5 min at 3,000×*g*) and was re-suspended in saline solution. In the case of pellet formation in the shake flask, the mycelium was homogenized with a blender (Ultra-Turrax T25, IKA, Germany) before the washing step. After washing, the dry weight was measured and the fermenters were inoculated with 0.05 g/l mycelium. For fermentation, a modified ICI medium containing 5 g/l glucose, 1 g/l $MgSO_4$ 7 H_2O, 0.5 g/l KH_2PO_4, 1.49 g/l $(NH_4)_2SO_4$, 1.25 ml/l 1 M lysine, and 2 ml/l of trace element solution (Geissmann et al.

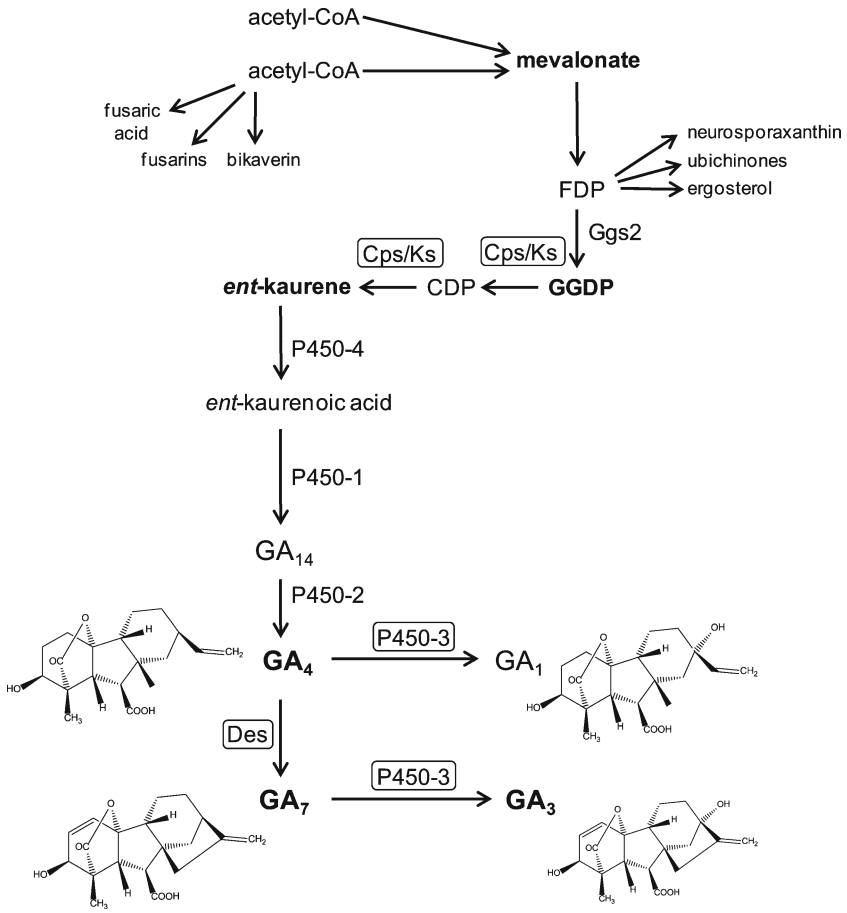

Fig. 1 Schematic overview of the GA biosynthesis pathway in *F. fujikuroi*. Enzymes investigated in this study are highlighted by *boxes*, main intermediates or end products of the pathways are indicated in *bold letters*. *Broken arrows* display successive biosynthesis steps to simplify the diagram. Acetyl-CoA on the one hand constitutes a precursor for secondary metabolites like fusaric acid, fusarins, or bikaverin. On the other hand, it can be converted successively into mevalonate. The mevalonate pathway finally ends in farnesyl diphosphate (FDP) formation which can then be utilized for gibberellin biosynthesis

1966) was used. The concentration of glucose was kept constant at 5±1 g/l through feeding of feed medium containing 400 g/l glucose, 10 g/l $MgSO_4$ $7H_2O$, 5 g/l KH_2PO_4, and 10 ml/l of trace elements to circumvent substrate inhibition (Brückner and Blechschmidt 1991). Cultivation was performed in the multifermenter system Bioblock TC4SC (DASGIP, Germany) consisting of four vessels exhibiting a working volume of 1 l each. pH values were constantly measured (405-DPAS-SCK8S, Mettler Toledo, Germany) and kept at 4±0.05. The aeration was kept constant at 40 l/h. To prevent foaming and associated attachment of the fungus on the fermenter vessel, head space aeration was used at the beginning. After reaching the set point of dissolved oxygen (DO) at 30 % saturation, gas entered the reactor through the sparger at the bottom. DO was measured online using a Clark electrode (InPro 6820, Mettler Toledo, Germany) and controlled by a cascade varying the stirrer speed. The fermenters have been stirred with two Rushton type impellers at a speed between 750 and 1,400 rpm, ensuring the DO never dropped below 30 % saturation.

Plasmid construction and fungal transformation

E. coli strain TOP 10 F′ (Invitrogen Life Technologies, Darmstadt, Germany) was used for cloning and amplification of plasmids, and transformation of *E. coli* cells was performed according to Hanahan (1983). For *F. fujikuroi* transformation, protoplasts were prepared from fungal mycelium according to Tudzynski (1999). About 1 10^7 protoplasts of *F. fujikuroi* strains were transformed with 10 μg of the appropriate plasmid. Regeneration of transformed protoplasts was carried out for 4 to 5 days at 28 °C in complete regeneration medium (RM: 0.7 M sucrose, 0.05 % yeast extract, 2 % agar) containing either 100 μg/ml nourseothricin (Werner-Bioagents, Jena, Germany) or 100 μg/ml geneticin (InvitrogenLife Technologies, Darmstadt, Germany), respectively. Resistant transformants were purified by single spore isolation to homokaryons and integration of the deletion construct was confirmed by diagnostic PCR (for primer sequences see Table 1).

Strain 6314/Δ*DES*/Δ*PPT1* was generated by transformation of 6314/Δ*DES* protoplasts with the plasmid pΔppt1-nat (Wiemann et al. 2012) containing a *PPT1* deletion cassette including *nat1* for nourseothricin resistance. Transformants were selected on regeneration medium containing 100 μg/ml nourseothricin, and positive colonies were proved by diagnostic PCR using primers ppt1-dia-rev and pLOF-Oli-P for the 3′ flank, ppt1-dia-for and Tub-T for the 5′ flank. Absence of the wild-type fragment was verified by primers ppt1_R and ppt1_F.

Generation of 6314/Δ*PPT1* was performed by transformation of protoplasts from *F. fujikuroi* strain 6314 with the plasmid pRS426-Δ*PPT1* (Albermann et al. 2013), containing a geneticin resistance cassette that would allow further genetic engineering of this strain by transformation of already existing plasmid holding different resistance cassettes. Transformants were selected on regeneration medium containing 100 μg/ml geneticin and homologous integration of the deletion construct was verified by diagnostic PCR using primers pKSGen_gpd_P and ppt1_dia_r for the 3′ flank, pKSGen_tub_T and ppt1_dia_f for the 5′ flank and ppt1_F and ppt1_R for amplification of the wild-type fragment, respectively.

Table 1 List of sequences and designation of all primers used in this study

Primer designation	Sequence (5′–3′)
pLOF-Oli-P	GGTACTGCCCCACTTAGTGGCAGCTCGCG
ppt1-dia-for	GGAGTTTTGTTGTGCCCACAC
ppt1-dia-rev	GATCAGACTCTTCAGGGAGCTGACATTC
Tub-T	GGTCCTCGGAGTGGAGAGGG
ppt1_R	GGTGATGAGCAGGTGGTGAGAGG
ppt1_F	ATAGTATTGCTGGGGTGGCAAGGTCGTTGC
pKSGen_gpd_P	GGTGATGAGCAGGTGGTGAGAGG
pKSGen_tub_T	CCTGTCAGACACTCTAGTTGTTGAC
ppt1_dia_r	TTGTCAACCTTGAGACTCAG
ppt1_dia_f	GCACAGTAGCAATGCCTCA

Standard molecular methods

For DNA and RNA analyses, standard techniques were applied (Sambrook et al. 1989). Fungal DNA was prepared by pestling lyophilized mycelium. Afterwards, the mycelium was dissolved in an extraction buffer according to Cenis (1992). DNA for Southern hybridization experiments was prepared as described by Doyle and Doyle (1990).

Isolation of total *F. fujikuroi* RNA was performed using the RNAgents total RNA isolation kit (Promega GmbH, Mannheim, Germany). Samples of 20 μg 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). Subsequent Northern blot hybridizations were carried out using the method of Church and Gilbert (1984).

For DNA amplification, PCR samples containing 1 μl DNA, 5 pmol of each primer, 200 nM dNTPs, and 1 U of BioTherm™DNA polymerase (GeneCraftGmbH, Lüdinghausen, Germany) were prepared. Application of the BioTherm™DNA polymerase was carried out by initial preheating of the sample at 94 °C for 4 min followed by 36 cycles of 1 min at 94 °C, 1 min at 56 to 65 °C, 1–3 min at 70 °C, and a final elongation step for 10 min at 70 °C. Primers used for PCR were obtained from Eurofins GmbH (Ebersberg, Germany) or Biomers.net GmbH (Ulm, Germany).

Offline measurements

Sampling included the concentration measurements of mycelial dry weight (X), glucose, ammonia, and gibberellins. The sample was filtered through a glass fiber disc (Macherey-Nagel, Germany GS85/70; d=47 mm). The retained mycelium was washed twice with de-ionized water and was frozen immediately in liquid nitrogen. Subsequently, the mycelium was freeze dried for gravimetric determination of dry matter and for gene expression analysis.

Glucose and GA concentrations were measured on the same HPLC system (Perkin Elmer Flexar FX-10) applying different methods. The system consisted of a high pressure gradient system, an auto injector, a column oven, a refractive index, and a diode array detector. For glucose analysis, an isocratic method with a flow rate of 0.6 ml/min at 45 °C, with 5 mM sulfuric acid, an Aminex HPX-87H column (Biorad, Germany), and a RI detector were used.

Prior to GA quantification, GAs were extracted from the culture filtrate. This was done twice with equal volumes of ethyl acetate. The extract fractions were pooled, evaporated, and re-dissolved in acidified 40 % methanol solution. To account for loss and dilution of GAs during the sample preparation, GA_3 was used as an internal standard for 6314-derived strains. The average retrieval of the internal standard in samples of strain 6314 was used to correct the measurements of m567 strains. GA analysis was performed via gradient elution with 0.05 % (*v*/*v*) acetic acid (HPLC grade, AppliChem, Germany) in double distillated water and methanol (HPLC Gradient Grade, J.T. Baker, USA). The methanol concentration was increased linearly from 10 % at 0 min, 70 % at 13 min, 70 % at 23 min, 100 % at 25 min to 100 % at 30 min under a constant flow rate of 0.5 ml/min. The column Prontosil 120-5-5 Phenyl (Bischoff Chromatography, Germany) was kept at 35 °C, and the chromatogram was recorded using UV adsorption at 205 nm. Calibration standards were graciously provided by Lewis Mander of the Australian National University, Canberra.

Determination of the decomposition of GA_4 and GA_7

In order to quantify the loss of GAs through spontaneous decomposition, approximately 200 mg/l of each GA_3-, GA_4-, and GA_7-standard have been dissolved in 0,1 M disodium citrate buffer at pH 4 and sterile filtered with FP 30/0.2 CA-S membrane filter disks (Whatman, Maidstone, U.K.) into sterile 100 ml screw cap shake flasks. The flasks were incubated at 100 rpm and 20 °C, 30 °C, or 55 °C, respectively. Sampling took place under sterile conditions and the frequency depended on the velocity of the decomposition reaction. The samples were analyzed for GA concentration by HPLC analysis.

The kinetic of GA decomposition was described as a first-order reaction by Pérez et al. (1996). Therefore, the time course of the GA concentration was fitted to an exponential. The reaction constant of the fitted function represents the constant of decay (k). In order to estimate the rate of decomposition at different temperatures, the rate constants were fitted to Arrhenius' law (Eq. 1) (A: pre-exponential factor; E_A: activation energy; R: universal das constant).

$$k_{GA} = A \cdot e^{\left(-\frac{E_A}{R \cdot T}\right)} \quad (1)$$

Data pretreatment, curve fitting, and calculation of confidence intervals

For a better comparison and for calculating kinetic constants, all fermentations were aligned. This was done by shifting the time, so that nitrogen limitation occurred at 40 h. For depicting the time course of each strain, data points close to each other have been aggregated by calculating the average and standard deviation of each measured variable. For fitting the measurements to a mathematical model, the combined data of each strain have been used.

All nonlinear regressions have been performed with Matlab® 2011b curve fitting toolbox (Mathworks, Natick, United States). Specifically, we used the functions nlinfit for parameter estimation, nlparci to estimate the 95 % confidence interval of the parameters, and nlpredci to estimate the 95 % confidence interval of values predicted by the model. A more detailed description on parameter estimation using curve fitting toolbox was given by Sin et al. 2008.

Simulation of GA production with simultaneous decomposition

The simulation of GA concentration is based on a mass balance comprising fungal production and chemical decomposition of GAs (see Eq. 2).

$$\frac{dc_{GA}}{dt} = r_{GA} - k_{GA} \cdot c_{GA} \quad (2)$$

Since nitrogen is needed for cell division, it can be concluded that the concentration of the biocatalyst is constant throughout the whole production phase and thus the GA production rate (r_{GA}) should be constant. In this study, loss of active cells by cell death is not considered. Due to their chemical structure, specific GAs exhibit different constants of decay (k_{GA}). The rate of GA decomposition is proportional to the concentration of GA c_{GA} in the culture filtrate and thus should increase with time. When decomposition reaches rates equal to the fungal production of GA, the concentration of GA in the culture will stagnate.

Equation 2 can be solved analytically. The resulting equation (Eq. 3) is time dependent and is valid from the time of nitrogen exhaustion (t_N). The constant k_{GA} was determined experimentally, while r_{GA} had to be obtained by curve fitting.

$$c_{GA}(t) = \frac{r_{GA}}{k_{GA}} \cdot \left(1 - e^{-k_{GA} \cdot (t - t_N)}\right) \quad (3)$$

Calculation of fungal biomass

The growth rate (μ) and the initial biomass ($m_{X,0}$) were obtained by fitting the measured, total biomass (m_X) to a time-dependent exponential function (Eq. 4).

$$m_X = m_{X,0} \cdot e^{\mu \cdot t} \quad (4)$$

Calculation of glucose consumption and the glucose feed profile

The turnover of any given component i can be calculated from a modified mass balance (Eq. 5). Thus, the consumption of glucose results from the change in concentration and volume given by $m_i(t) - m_{i,0}$ minus the mass added through feeding ($m_{i,F}$), plus the mass that was removed through sampling ($m_{i,S}$).

$$\Delta m_i(t) = m_i(t) - m_{i,0} - m_{i,F}(t) + \sum_0^t m_{i,S} \quad (5)$$

In order to keep the glucose concentration at 5 g/l, we applied an adaptive feed profile that factors in the two staged nature of the fermentation process. Depending on the availability of nitrogen, the fermentation process can be divided into an exponential phase and a production phase. During the first stage, glucose is consumed exponentially and an exponential feed profile has been applied. After all the nitrogen has been consumed, the glucose uptake rate is relatively constant and a constant feed rate was applied (Eq. 6).

$$F = \frac{r_{Glc}}{c_{Glc,\,F}} \quad \text{with} \quad r_{Glc}(t, c_{NH_4}) = \begin{cases} r_{Glc,\,Exp} \cdot e^{\mu \cdot t}, & \text{if } c_{NH_4} > 0 \\ r_{Glc,\,Prod}, & \text{if } c_{NH_4} = 0 \end{cases} \quad (6)$$

After each sampling, the glucose concentration has been determined and its consumption was calculated. Finally the consumption curve was fitted to the integrated form of Eq. 6, in order to revise the rate constants. Typical values for the growth rate (μ) and the glucose uptake rates $r_{Glc,\,Exp}$ and $r_{Glc,\,Prod}$ were 0.12 h^{-1}, 0.067 and 0.22 g/h, respectively.

Results

Generation of mutant strains with altered GA spectrum and GA yields

Most of the *F. fujikuroi* wild-type strains like IMI58289 and m567 used in this work, produce GA_3 as the major product together with lower amounts of their precursors GA_7 and GA_4 (Fig. 1). Previously, a GA_7-producing strain was generated by UV mutagenesis of strain m567. This UV mutant, 6314, was shown to carry a point mutation in the 13-hydroxylase-encoding gene *P450-3* (Tudzynski et al. 2003). Due to this mutation, strain 6314 and all mutants derived from this strain lack the ability to convert GA_7 to GA_3 as well as GA_4 to GA_1 (Fig. 1). Therefore, the main product of these strains is GA_7 accompanied by low amounts of its precursor GA_4. To create GA_4-producing strains, the GA_4 desaturase-encoding gene *DES* was deleted in strain 6314 generating mutant 6314/Δ*DES* whose only product is GA_4 (Fig. 1), thus enabling us to identify parameters and kinetics of GA biosynthesis that lead to differences in final GA amounts of these strains.

Recently, we have shown that deletion of the 4'-phosphopantetheinyltransferase 1-encoding gene *PPT1*, which is essential for activating PKS and NRPS enzymes (Crawford et al. 2008; Lambalot et al. 1996) abolished the formation of PKS-derived red pigments (bikaverin and fusarubins) and NRPS-derived mycotoxins and siderophores (Wiemann et al. 2012). To show whether the deletion of *PPT1* also results in increased GA_7 and GA_4 yields of mutant strains 6314 and 6314/Δ*DES*, respectively, we created the GA_7-producing double mutant 6314/Δ*PPT1* and the GA_4-producing triple mutant 6314/Δ*DES*/Δ*PPT1*. By this approach, we wanted to increase the intracellular acetyl-CoA pool and divert more carbon flux to the GA biosynthesis. Additionally, GA purification is considerably improved in the absence of pigments and other disturbing metabolites.

Fermentation of six *F. fujikuroi* strains reveals differences in the GA spectrum and product yield

So far, GA production was investigated by use of shaking cultures without regulation of pH and aeration conditions. In contrast to shaking cultures, lab fermenters allow advanced process control leading to more reliable and reproducible results. This includes control of pH and dissolved oxygen as well as the avoidance of glucose repression, pellet growth, and attachment of the fungus on the fermenter vessel.

Thus, the two *F. fujikuroi* wild-type strains, IMI58289 and m567, the two GA_7-producing mutants, 6314 and 6314/Δ*PPT1*, as well as two GA_4-producing mutants 6314/Δ*DES* and 6314/Δ*DES*/Δ*PPT1*, were cultivated in identical lab scale bioreactors and compared regarding their

growth rates, biomass formation, and GA yields. We used a fed-batch fermentation approach with continuous feeding of glucose to avoid carbon limitation. Since it is well known that the presence of consumable nitrogen represses the expression of GA biosynthetic genes and subsequently inhibits GA production (Mihlan et al. 2003), the nitrogen concentration at the beginning of fermentation was adjusted to 25 mM using $(NH_4)_2SO_4$ and lysine. During exponential growth, the concentration of ammonia decreased exponentially and was exhausted at about 40 h (Fig. 2).

Growth of all six *F. fujikuroi* strains in the lab fermenter could be roughly divided into two phases: an exponential growth phase and a production phase. The exponential phase lasted until the nitrogen source was exhausted at about 40 h past inoculation (Fig. 2). In this phase, the fungal dry weight increased exponentially, and the growth rate of all compared strains ranged between 0.12 and 0.2 h^{-1} (Fig. 3). After nitrogen exhaustion, the fungal strains grew with a linear growth rate for about 2 days before the dry weight finally stagnated (Fig. 2). All strains converged to a final biomass concentration of about 15 g/l.

The produced GA levels of all six strains grown under identical fermentation conditions showed significant differences. GA analysis of the two wild-type strains, which produce mainly GA_3, revealed about 55 % higher GA_3 production levels of strain m567 (216 mg/l) compared to IMI58289 which produced only about 140 mg/l GA_3 (Figs. 2 and 4). The GA_4-producing strains 6314/Δ*DES* (390 mg/l) and 6314/Δ *DES*/Δ*PPT1* (420 mg/l) showed a prolonged linear production phase resulting in 80–100 % higher GA titers compared to strains m567 and the GA_7-producing strains 6314 (234 mg/l) and 6314/Δ*PPT1* (195 mg/l) (Fig. 2).

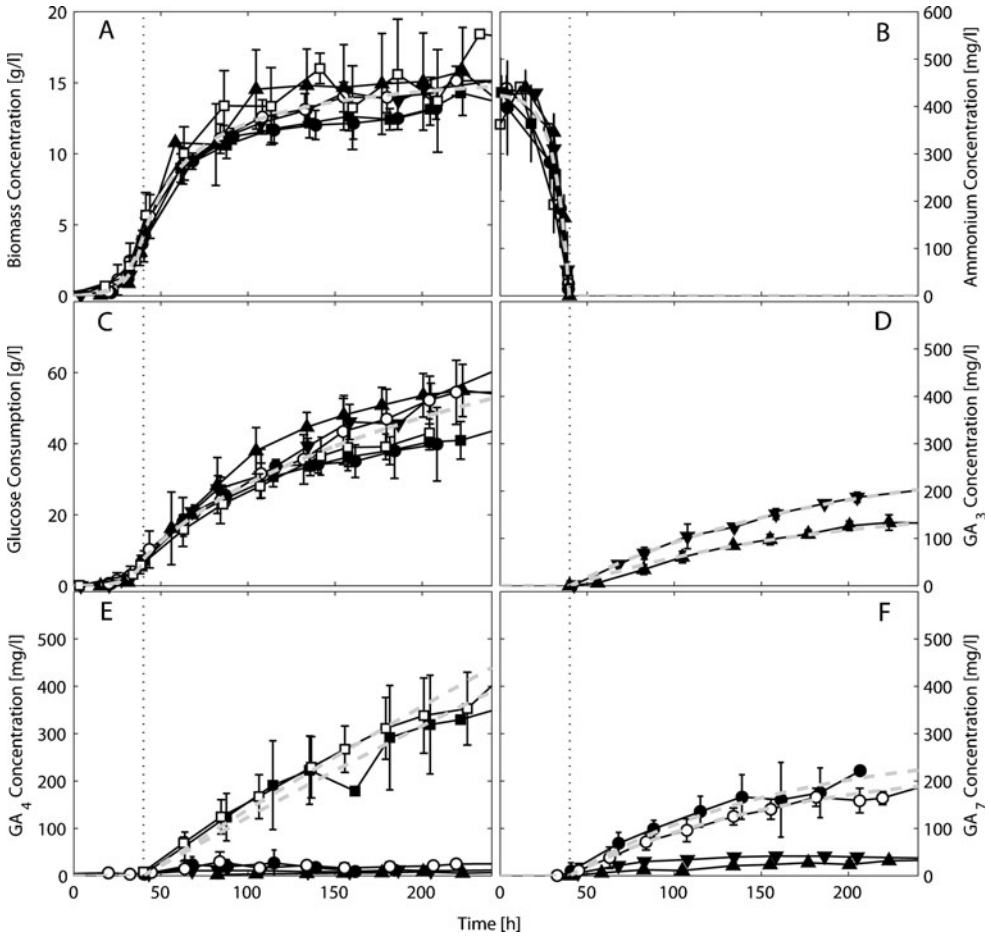

Fig. 2 Time course of the fermentation of *F. fujikuroi* strains IMI58289 (*filled triangle*), m567 (*inverted filled triangle*), 6314/Δ*DES* (*filled square*), 6314/Δ*DES*/Δ*PPT1* (*empty square*), 6314 (*filled circle*), and 6314/Δ*PPT1* (*empty circle*) at 28 °C, pH 4 and 750 rpm, 5±1 g/l glucose, an initial biomass concentration of 0.05 g/l and 25 mM initial nitrogen. *Markers* and *error bars* denote average and standard deviations of the measurement of at least two fermentations and slim dotted lines indicate the time point of nitrogen limitation. For the sake of clarity, measurements with value zero have been omitted. Biomass concentration (**a**), ammonium concentration (**b**), and glucose consumption (**c**): *Thick dotted lines* indicate the general behavior during growth (–- -) and storage phase (—). Concentrations of specific gibberellins (**d**–**f**): *Thick dotted lines* show simulated GA concentrations assuming a constant production rate and taking into account the decay of GAs using the following rates: $k_{GA_3} = 4.55 \cdot 10^{-3} \cdot h^{-1}$, $k_{GA_4} = 1.71 \cdot 10^{-3} \cdot h^{-1}$ and $k_{GA_7} = 6.42 \cdot 10^{-3} \cdot h^{-1}$

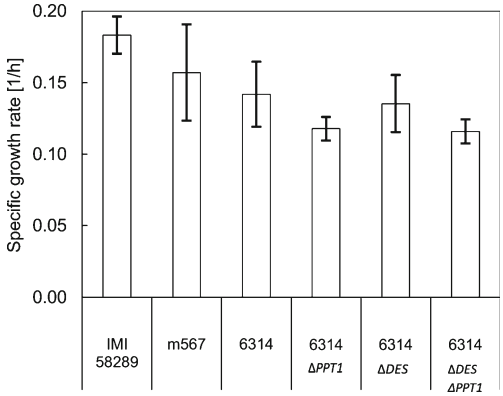

Fig. 3 Average rates and estimated 95 % confidence interval of growth rate during exponential growth. The strains were grown at 28 °C, pH 4 and 750 rpm up to 240 h in synthetic medium. Glucose concentration was held at 5±1 g/l. The initial biomass concentration was 0.05 g/l. Data of at least two fermentations per strain were used to determine growth rates

Specific GAs show different rates of decomposition

Beside the production rate, the level of degradation of a certain fermentation product is also an important factor for estimating the final production yield. Therefore, we examined the decomposition rates of GA_3, GA_4, and GA_7 at pH 4, the optimal pH value for GA biosynthesis. Figure 5 displays the time course of the decomposition at different temperatures. The data of each temperature and GA has been fitted to an exponential function and the rates of decay are shown in Table 2. These measurements revealed that GA_7 is less stable than GA_3 or GA_4 assuming that the C_1–C_2 double bond is responsible for the reduced stability of GA_3 and GA_7. This difference in product stability between GA_7 and GA_3 on one hand, and GA_4 on the other hand, might be an explanation for the extended linear GA_4 accumulation. In comparison to Pérez et al. (1996), who only studied GA_3 at 30 °C, the coefficients of decay obtained in this study are in the same order of magnitude. The values given by Pérez et al. range from 0.0089 h^{-1} at pH 4 to 0.016 h^{-1} at pH 9, while we observed rate constants between 0.003 to 0.010 h^{-1} at pH 4 and 30 °C.

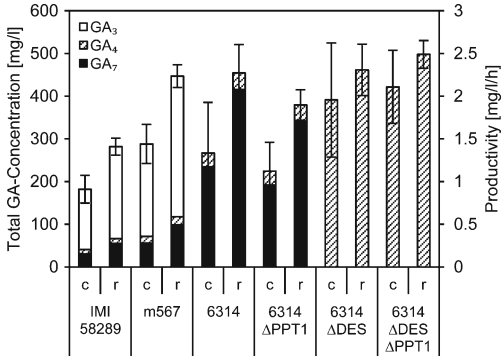

Fig. 4 Average GA titers after 240 h of cultivation (*c*, *left*) and estimated volumetric production rates (*r*, *right*) of at least two fermentations per strain. *Error bars* denote estimated 95 % confidence intervals. The strains were grown at 28 °C, pH 4, and 750 rpm up to 240 h in synthetic medium. Glucose concentration was held at 5±1 g/l. The initial biomass concentration was 0.05 g/l. Data of at least two fermentations per strain were used to determine the depicted constants

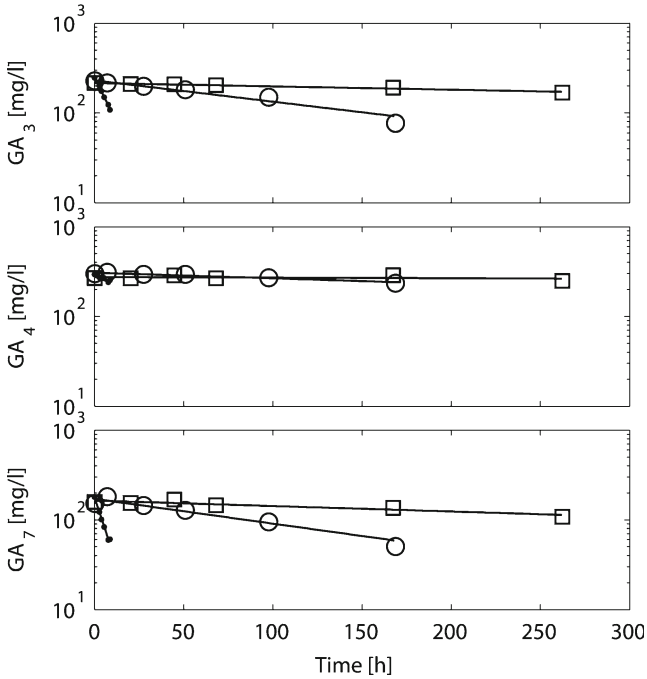

Fig. 5 Time course of gibberellin decomposition at 20 °C (*empty square*), 30 °C (*empty circle*), and 55 °C in water. *Solid lines* show the results of exponential fitting

Gene expression of the GA cluster genes *DES* and *CPS/KS* is induced upon nitrogen limitation

As the six analyzed strains IMI58289, m567, 6314, 6314/Δ*DES*, 6314/Δ*PPT1*, and 6314/Δ*DES*/Δ*PPT1* severely differ in GA production yields of synthesized GAs (Fig. 4), we studied the expression of genes *CPS*/*KS* encoding the key enzyme of the GA pathway, Cps/Ks, and *DES*, encoding the GA_4 desaturase, by Northern analysis in the time course of the fermentation process. However, expression levels of *CPS*/*KS* and *DES* did not significantly differ between the low and higher GA-producing strains. As expected, samples harvested under nitrogen sufficient conditions show very low expression levels for both genes, whereas expression levels rapidly rise when nitrogen becomes limited in the medium after 40 h past inoculation (Fig. 6). These observations fit well with the production rates of all strains increasing simultaneously with the onset of GA gene expression upon nitrogen exhaustion (Figs. 2 and 4). Although the yields of GA_7 and GA_3 tend to stagnate at the end of the fermentation process, probably due to degradation of these GAs, the transcript levels of GA genes did not decrease in time (Figs. 2 and 6).

Discussion

Loss of function of P450-3, Des, and Ppt1 results in varying growth rates, product yields, and GA spectra, but similar gene expression patterns

The maximum growth rate of a fungus is genetically determined and can be affected by mutations. Differences in the growth rate between isolates of the same species illustrate the strain's fitness and often have an impact on primary and secondary metabolism.

In our studies, wild-type strain IMI58289 exhibited the highest growth rate followed by the second wild-type isolate m567, and those mutants still expressing *PPT1* (Fig. 3). Despite smaller differences in growth rate, neither inactivation of the gene *P450-3*, nor disruption of *DES*, both encoding GA biosynthetic genes, has a significant impact on the strain's fitness. In contrast, deletion of *PPT1*, encoding the 4'-phosphopantetheinyl transferase, affects not only secondary metabolism but also primary metabolism due to its involvement in lysine biosynthesis (Wiemann et al. 2012). Although lysine was added to the culture media, mutants 6314/Δ*PPT1* and 6314/Δ*DES*/Δ*PPT1* tend towards a decreased growth rates compared to the reference strains with a functional *PPT1* gene (Fig. 3). The effect of *PPT1* deletion on growth behavior suggests that either the growth rate is limited by an ineffective lysine or iron uptake, or that one of the missing secondary metabolites would be beneficial for the fungal growth.

With regard to the GA spectra, all genetic modifications showed the expected effects. The loss of 13-hydroxylase P450-3 precludes both GA_3 and GA_1 formation in strains 6314 and 6314/Δ*PPT1* and led to the formation of GA_7 as the main product in these strains, while the deletion of *DES* in the 6314 background resulted in an exclusive production of GA_4 (Tudzynski et al. 2003). Interestingly, the deletion of *DES* led to a prolonged production of GA_4 and thus to higher total GA yields compared to the GA_7 production of the reference strain 6314 and GA_3 production of both wild-type strains (Figs. 2 and 4). As GA_4 did not accumulate during the fermentation of 6314 fermentation, a bottle neck caused by a low conversion rate of GA_4 to GA_7 can be excluded.

Expression studies confirmed our previous data that transcript levels of GA biosynthetic genes (shown for *CPS*/*KS* and *DES*) significantly increase upon nitrogen depletion and stay on a constant high level during the whole production phase (Fig. 6). The obtained nitrogen repression of GA genes during the growth phase is in accordance with low expression of the positive nitrogen regulator AreA under nitrogen sufficient conditions. AreA was shown to be essential for GA gene expression upon nitrogen limitation (Schönig et al. 2008; Tudzynski 1999; Wagner et al. 2010).

As GA gene expression did not differ in all tested strains (Fig. 6), the reason for the prolonged production phase in Δ*DES* mutants cannot be attributed to differences in gene regulation.

Different decomposition rates of specific GAs lead to divergent GA concentration curves

The main product of Δ*DES* mutants, GA_4, is chemically more stable than GA_3 and GA_7, the main products of the wild-type and 6314-derived strains expressing *DES*. This difference in stability can be attributed to an additional double bond in GA_3 and GA_7 which makes the molecule more reactive. Since strains with a functional *DES* gene and Δ*DES* mutants showed initially identical slopes in total GA concentration (Fig. 2; 40–80 h), it has been assumed that they exhibit the same productivity. Therefore, the deviation of GA concentration later on in the fermentation process is presumably caused solely by different decomposition kinetics. To prove this hypothesis, we interpolated the rates of decomposition for GA_3, GA_4, and GA_7 at 28 °C using Arrhenius' law. Thereafter, we used these rates, $k_{GA_3} = 4.55 \cdot 10^{-3} \cdot h^{-1}$, $k_{GA_4} = 1.71 \cdot 10^{-3} \cdot h^{-1}$ and $k_{GA_7} = 6.42 \cdot 10^{-3} \cdot h^{-1}$, to estimate the volumetric production rate for each GA of each strain according to Eq. 2. As shown in Fig. 2, the dashed simulated curves match the measurements. The agreement of measurements and simulated values is also confirmed by high coefficients of determination. The fitting of the major GAs resulted in R^2 values of 0.96 for IMI58289, 0.99 for m567, 0.74 for 6314 0.9 for 6314/ΔPPT1, 0.92 for 6314/ΔDES, and 0.88 for 6314/ΔDES/ΔPPT1. Hence, the non-linear behavior

Table 2 Constants of decay with estimated 95 % confidence intervals of GA3, GA4, and GA7 at 20 °C, 30 °C, and 55 °C including coefficients of Arrhenius' law

	Constant of decay k [10^{-3}/h] (R^2)											
T [°C]	GA_3				GA_4				GA_7			
20	0.9	±	0.1	(0.97)	0.3	±	0.6	(0.51)	1.3	±	0.6	(0.94)
30	6.6	±	3.6	(0.97)	2.2	±	0.46	(0.94)	8.7	±	4.0	(0.93)
55	92.9	±	10.1	(0.99)	19.3	±	11.3	(0.85)	139.0	±	19.1	(0.99)
	Arrhenius coefficients											
A [1/h]	$2.45 \cdot 10^{15}$				$2.48 \cdot 10^{12}$				$5.95 \cdot 10^{15}$			
EA [kJ/mol]	103				88				104			

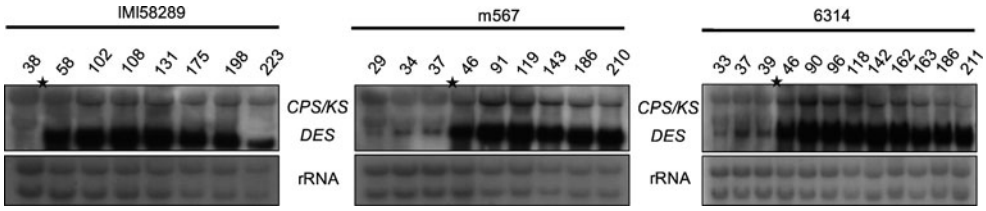

Fig. 6 Northern analysis on the expression of two GA pathway genes, *CPS*/*KS* and *DES*. Strains IMI58289, m567, and 6314 were grown at 28 °C, pH 4 and 750 rpm up to 240 h in synthetic medium. Glucose concentration was held at 5±1 g/l. The initial biomass concentration was 0.05 g/l. As a loading control, mRNA was visualized by UV light. *Asterisks* indicate the time point of ammonium exhaustion. As probes, radio-labeled genomic DNA fragments of CPS/KS and DES were applied

of the GA production can be explained in terms of GA decomposition.

This result has profound implications on the process design of *F. fujikuroi* fermentations. As the rate of decomposition is proportional to the GA concentration, while production rate is constant, the GA concentration must converge to a constant value $GA_{max} = r_{GA}/k_{GA}$, where production equals decomposition. This value depends on the production strain, biomass concentration, and on the produced GA type. Under the given conditions, the theoretical maximum GA_3 or GA_7 concentration of m567-derived strains is approximately 330 mg/l, while GA_4 could potentially reach 1.3 g/l. Therefore, the cultivation time for GA_4-producers should be prolonged, while the GA_7-producers showed titers already close to the maximum concentration. To overcome the limitation in GA_3 and GA_7 concentration, either the volumetric production rate r_{GA}must be increased or the rate of decay $k_{GA} \cdot c_{GA}$ must be kept low. The volumetric productivity is the product of the mass specific productivity and the biomass concentrations. Thus, product levels could be increased by genetic engineering, while elevated biomass concentrations will certainly result in higher product levels. Yet, the rate of decomposition composed of the kinetic constant, which strongly depends on pH and temperature, and the GA concentration also restricts final GA amounts. By keeping the product concentration at low levels, the rate of decomposition can be slowed down. This can be achieved via continuous product removal, for instance by continuous cultivation or by extractive fermentation (Hollmann et al. 1995).

Furthermore, all estimated volumetric production rates are presented as stacks in Fig. 4. It shows that the total productivity of all strains, except for IMI58289, is nearly identical. Since these strains are all derived from m567, it can be concluded that none of the genetic manipulations affected the performance of the overall GA-synthesis.

Deletion of *PPT1* leads to decreased growth rates but no improvement in GA-productivity

Deletion of competing pathways in engineered production strains constitutes a powerful tool for strain improvement which was for instance performed for improvement of 1-butanol production in engineered *E. coli* strains, resulting in a product increase of about 220 mg/l (Atsumi et al. 2008) or down-regulation of *ERG9*, the gene encoding the first enzyme for the sterol biosynthesis pathway in *Saccharomyces cerevisiae*, leading to an increased intracellular FPP pool (Asadollahi et al. 2008).

For *F. fujikuroi*, it was previously shown that deletion of *PPT1* encoding the 4'-phosphopantheteinyl transferase resulted in loss of PKS- and NRPS-derived metabolites and slight increase of GA production in the low-producing wild-type strain C1995 (Wiemann et al. 2012). Deletion of *PPT1* in high-producing strains 6314 and 6314/Δ*DES*, respectively, was meant to result in a higher carbon yield, improved GA production rates, and easing of downstream processing for GA extraction and purification due to abolishment of pigmented side-products. These assumptions were partially approved as the culture broth of the Δ*PPT1* mutants remained white during fermentation, while strains expressing the *PPT1* wild-type gene accumulated the red pigment bikaverin. Hence, the production of side-products, at least of pigments, has been reduced due to the loss of PKS and NRPS activation. Therefore, the non-pigmented cultures allow considerably simplified downstream processes. However, in contrast to our expectations, GA productivity or carbon yield were not affected by the deletion of *PPT1*, as neither GA production nor glucose consumption showed a significant difference compared to the respective parental strains (Figs. 2 and 5).

The slightly reduced growth rate compared to the parental strains 6314 and 6314/Δ*DES* fits very well with the results of Wiemann et al. (2012) on the impact of *PPT1* deletion in the *F. fujikuroi* wild-type strain IMI58289. The growth defect appearing even in the presence of external lysine was attributed to a reduced iron uptake caused by the inability of Δ*PPT1* mutants to produce siderophores and cannot be completely overcome by lysine feeding during submerse cultivation (Wiemann et al. 2012).

Summarizing, we were able to directly compare genetically manipulated *F. fujikuroi* mutant strains and two wild-type strains regarding their GA spectrum, production yields, and GA gene expression. The six analyzed strains differed significantly in their GA yields, although the transcript levels of the GA biosynthesis genes were almost identical

and simultaneously increased upon nitrogen depletion in all strains. Wild-type strain IMI58289 revealed the lowest total GA amount but the highest growth rate. The second wild-type strain m567 and the GA_7-producing mutant strains 6314 and 6314/Δ*PPT1* derived from m567 produce about 65 % more GAs than IMI58289. The Δ*DES* strains, 6314/Δ*DES* and 6314/Δ*DES*/Δ*PPT1*, both producing GA_4 as the final product, reached about three-fold higher GA yields compared to IMI58289. We showed that the increased GA yields in these two GA_4-producing strains are due to the higher stability of GA_4 compared to GA_7 and GA_3 indicating that both, genetic capacity of a strain and product stability, are highly important parameters in long-term fermentation processes. Deletion of *PPT1* did not result in significantly elevated production yields but abolished formation of PKS-derived red pigments that would disturb GA purification from fermentation broth.

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