Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Evolutionary adaptation of *Aspergillus niger* for increased ferulic acid tolerance

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Keywords
adaptive evolution, aromatic metabolism, *Aspergillus*, cell factory, ferulic acid tolerance, mutagenesis.

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2019/1497: received 23 August 2019, revised 28 October 2019 and accepted 29 October 2019

doi:10.1111/jam.14505

Abstract

Aims: To create an *Aspergillus niger* mutant with increased tolerance against ferulic acid using evolutionary adaptation.
Methods and Results: Evolutionary adaptation of *A. niger* N402 was performed by consecutive growth on increasing concentrations of ferulic acid in the presence of 25 mmol l^{-1} D-fructose, starting from 0·5 mmol l^{-1} and ending with 5 mmol l^{-1} ferulic acid. The *A. niger* mutant obtained after six months, named Fa6, showed increased ferulic acid tolerance compared to the parent. In addition, Fa6 has increased ferulic acid consumption and a higher conversion rate, suggesting that the mutation affects aromatic metabolism of this species. Transcriptome analysis of the evolutionary mutant on ferulic acid revealed a distinct gene expression profile compared to the wild type. Further analysis of this mutant and the parent strain provided the first experimental confirmation that *A. niger* converts coniferyl alcohol to ferulic acid.
Conclusions: The evolutionary adaptive *A. niger* mutant Fa6 has beneficial mutations that increase the tolerance, conversion rate and uptake of ferulic acid.
Significance and Impact of the Study: This study demonstrates that evolutionary adaptation is a powerful tool to modify micro-organisms towards increased tolerance to harsh conditions, which is beneficial for various industrial applications.

Introduction

Micro-organisms have a powerful ability to adapt rapidly in order to overcome new or changing environmental conditions. This ability can be applied as a tool to improve strains to obtain increased concentrations of enzymes or improved metabolism. Through an adaptive evolutionary approach, an *Aspergillus niger* strain with improved cellulase production was created (Patyshakuliyeva *et al.* 2016). This strategy was also successful for the creation of an *Aspergillus oryzae* strain with improved inulinase production (Culleton *et al.* 2016). Moreover, evolutionary adaptation has also been proven to be efficient to obtain industrial micro-organisms with increased stress tolerance and substrate utilization. For example, a *Saccharomyces cerevisiae* strain was created with improved tolerance to higher temperature and hydrolysate-derived inhibitors for second generation ethanol production (Wallace-Salinas and Gorwa-Grauslund 2013). Another example was the *S. cerevisiae* strains that were created with increased acetic acid tolerance to withstand the negative effects of acetic acid released during hydrolysis of lignocellulosic feedstocks for bio-ethanol production (González-Ramos *et al.* 2016). Evolutionary adaptation depends on spontaneous mutation(s) that is beneficial under the selection condition (Schoustra *et al.* 2009; Schoustra and Punzalan 2012). By exposing consecutive generations of the strain to the same selective condition, beneficial mutations are maintained, while other random mutations occur at low frequencies. In contrast, UV and chemical mutagenesis approaches generate large numbers of random mutations, making evolutionary adaptation a more targeted approach.

The aromatic compound ferulic acid is present in plant biomass where it is ester-linked to L-arabinose in xylan, to D-galactose and L-arabinose in pectin and to D-xylose

in xyloglucan (Mäkelä *et al.* 2015). In addition, ferulic acid, which is synthesized from coniferyl alcohol, occurs in the aromatic plant cell wall polymer lignin. Ferulic acid dimers can link polysaccharides to each other and to lignin (Buanafina 2009), while feruloyl esterases release (di-)ferulic acid from plant cell wall polysaccharides (e.g. xylan and pectin) (Mäkelä *et al.* 2015). Currently, three feruloyl esterases have been described for *A. niger*: FaeA, FaeB and FaeC, which differ in their relative activity on ferulic acid containing polymers and are induced by different monomeric sugars and/or aromatic compounds (de Vries *et al.* 1997, 2002; Dilokpimol *et al.* 2017).

After being released from polysaccharides or lignin, the aromatic compounds are further transported and degraded intracellularly through aromatic metabolism. Ferulic acid has a strong antimicrobial property at low concentration, including on *Aspergillus flavus*, *A. niger* and *A. terreus* (Lattanzio *et al.* 1994; Bisogno *et al.* 2007; Sova 2012; Borges *et al.* 2013; Shi *et al.* 2016). To survive this toxicity, ferulic acid needs to be converted to less or nontoxic compounds by fungi. Currently, several ferulic acid metabolic pathways have been observed in fungi, but the genes involved in these pathways are mostly unidentified (Lubbers *et al.* 2019a). The most observed ferulic acid metabolic pathways in filamentous fungi are the non-oxidative decarboxylation of ferulic acid to *p*-vinylguaiacol and further to vanillic acid, and a direct conversion of ferulic acid to vanillic acid (Milstein *et al.* 1983; Baqueiro-Peña *et al.* 2010; Lubbers *et al.* 2019a). The aim of this study was to use adaptive evolution to obtain an *A. niger* strain that tolerates high concentration of ferulic acid as a tool to study the aromatic metabolic pathways.

Material and methods

Strains, media and culture conditions

Aspergillus niger strains used in this study are shown in Table 1. The fungi were grown on complete medium (CM) agar plates at 30°C for 4 days (de Vries *et al.* 2004). Spores were harvested with 10 ml N-(2-Acetamido)-2-aminoethanesulfonic acid buffer (ACES) and the minimal media (MM) plates were inoculated with 10^3 freshly isolated spores (de Vries and Visser 1999; de Vries *et al.* 2004). All aromatic compounds were purchased from Sigma Aldrich.

Table 1 Strains used in this study

Strain	CBS-accession number	Genotype	Description	Reference
N402	141247	*cspA1*	Parental strain	Bos *et al.* (1988)
Fa6	CBS 145948	*cspA1*	Ferulic acid adaptive evolution mutant	This study

Evolutionary adaptation

A total of 10^3 freshly harvested spores of *A. niger* N402 in 2 μl were inoculated on MM plates containing 0·5 mmol l^{-1} ferulic acid with 25 mmol l^{-1} D-fructose. After 1 week of growth, all spores were harvested with ACES, and diluted to a 10^6 spores per ml concentration of which 20 μl was inoculated on fresh MM plates again containing 0·5 mmol l^{-1} ferulic acid and 25 mmol l^{-1} D-fructose. After another week, spores were harvested and inoculated to MM plates containing 1 mmol l^{-1} ferulic acid and 25 mmol l^{-1} D-fructose. This was repeated the next week, after which a similar approach was used while increasing the ferulic acid concentration incrementally until a concentration of 5 mmol l^{-1} was reached. For the toxicity test, strains were grown on MM plate containing the desired aromatic compound and 25 mmol l^{-1} D-fructose as carbon sources. Plates were supplemented with D-fructose to avoid possible carbon catabolic repression (de Vries *et al.* 2002).

Consumption test

Pre-cultures of *A. niger* were made in 1 l flasks containing 200 ml MM with 2% D-fructose and inoculated with 2×10^8 freshly harvested spores and incubated at 30°C, 250 rev min^{-1} for 16 h. Mycelia was harvested on Miracloth (Sigma-Aldrich, Darmstadt, Germany) and washed with MM, after which equal portions of mycelia were transferred to 250 ml flasks containing 20 ml MM and 1·5 mmol l^{-1} aromatic compound. A no carbon source condition was used as control. The cultures were incubated at 30°C, 250 rev min^{-1} and the supernatant was harvested every 2 h. Supernatant was diluted 10 times with acetonitrile and analyzed with HPLC. The reduction of aromatic compounds or formation of the products were monitored by HPLC (Dionex ICS-5000 + chromatography system; Thermo Scientific, Sunnyvale, CA) equipped with an Acclaim Mixed-Mode WAX-1 LC Column (3 × 150 mm; Thermo Scientific) and a UV detector (225, 250 or 280 nm, Thermo Scientific; Dilokpimol *et al.* 2017).

RNA extraction and quantitative RNA sequencing

Pre-cultures of *A. niger* inoculated with 2×10^8 spores of N402 and Fa6 were cultured overnight at 30°C, 250 rev min^{-1} in plastic flasks containing 200 ml CM with D-fructose (20 g l^{-1}). Mycelia were harvested on Miracloth and washed with MM. Equal portions of

mycelia were transferred to flasks containing 50 ml MM and 1 mmol l^{-1} aromatic compound. The cultures were incubated in rotary shakers for 2 h at 250 rev min^{-1} 30°C. Mycelia was harvested, dried between a tissue paper and frozen in liquid nitrogen. Frozen mycelia were ground with a tissue lyser (QIAGEN, Hilden, Germany). Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA) and was purified using the NucleoSpin RNA Clean-up kit (Macherey-Nagel) according the manufactures recommendations. The quantity and quality of RNA were determined by gel electrophoreses, Nanodrop™ (Thermo Scientific) and RNA 6000 Nano chips using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clare, CA). RNA sequencing was performed/analyzed by BGI Tech Solutions Co., Ltd. (Hong Kong) using Illumina HiseqTM 2000 platform (Illumina Inc., San Diego, CA). Transcriptome analysis was performed as described in Kowalczyk *et al.* (2017). In short, raw reads were produced from the original image data by base calling. On average, ~13 million reads of 49 bp per sample were obtained. After data filtering, the adaptor sequences, highly 'N' containing reads (≥10% of unknown bases) and low quality reads (more than 50% bases with a quality value of ≤5%) were removed. After data filtering, on average, 99·6% clean reads remained and were mapped to the genome of *A. niger* NRRL3 (http://genome.jgi.doe.gov/Aspni_NRRL3_1) using Bowtie2 (Langmead *et al.* 2009) and HISET (Kim *et al.* 2017). On average, 90·2% of the total reads were mapped to the genome and multi-mapped reads were removed. The gene expression level was measured and normalized as fragments per kilobase of transcript per million mapped reads (FPKM) (Trapnell *et al.* 2011) using the software RSEM (Li and Dewey 2011). Transcript differential expressions were conducted using the software package DESeq2 (Love *et al.* 2014).

The transcriptome data were stored at the NCBI Gene expression omnibus, under the GEO accession number GSE135001. Transcriptome data of N402 on *p*-coumaric acid and no carbon source were obtained from GEO accession number GSE13499 (sample IDs: GSM3983427, GSM3983427, GSM3983434 and GSM3983435).

Identification of genomic variants from RNA-seq data

The software JACUSA (Piechotta *et al.* 2017) was used to detect the possible genomic variants from RNA-seq data using the default parameters. The sequences of mapped reads were compared to the genome assembly of *A. niger* NRRL3 for searching of variants. The genomic variants sites were annotated to gene and protein level by using the software SnpEff (Cingolani *et al.* 2012). The candidate mutant sites related to evolutionary adaptation were selected as following criteria. The variant sites lead to changes of protein sequence and were observed in more than one sample in evolutionary strain and were not detected in any sample of reference strain.

Results

Evolutionary adaptation of *A. niger* on ferulic acid resulted in improved growth on ferulic acid and vanillin

Aspergillus niger N402 was grown on ferulic acid starting with a concentration of 0·5 mmol l^{-1} and increasing it gradually to 5 mmol l^{-1} over 6 months. Harvested spores were re-inoculated weekly for 1 month to fresh media containing ferulic acid to confirm the stability of the mutation. After several re-inoculations, the growth rate of *A. niger* on ferulic acid improved. This evolution mutant derived from ferulic acid is referred as Fa6. *Aspergillus niger* N402 and the adaptive evolution mutant Fa6 were grown for 5 days on 1·5–15 mmol l^{-1} aromatic compound (Fig. 1). On ferulic acid and vanillin, growth of Fa6 was improved compared to the wild type. No growth improvement was observed on caffeic acid, *p*-coumaric acid, syringic acid or vanillic acid. To investigate the ferulic acid tolerance of Fa6 in more detail, a more extensive growth profile was performed, demonstrating a significant increase in tolerance (Fig. 2).

Increased consumption of coniferyl alcohol and ferulic acid by Fa6

To investigate whether Fa6 is more efficient in the uptake of ferulic acid, a consumption test was performed. A starting concentration of 1·5 mmol l^{-1} ferulic acid was used, since both N402 and Fa6 were not affected by this concentration (Fig. 1). The consumption of ferulic acid was significantly faster for Fa6 compared to N402 (Fig. 3a). After 10 h, almost all ferulic acid was consumed by Fa6, while the N402 cultures had a residual concentration of 0·4 mmol l^{-1} ferulic acid. After 4 h, vanillic acid was detected and this increased over time in Fa6 than in *A. niger* N402 (Fig. 3b). Maximum vanillic acid was detected up to 1·07 mmol l^{-1} at 8 h in Fa6 in the culture supernatant. No additional compounds were detected during ferulic acid consumption.

We previously suggested that coniferyl alcohol is converted through ferulic acid and further to vanillic acid in fungi (Lubbers *et al.* 2019a), and therefore we also performed a consumption test with coniferyl alcohol (Fig. 4a). Low amounts of ferulic acid was detected during growth on coniferyl alcohol, but no clear difference was observed between N402 and Fa6 (Fig. 4b). The production of vanillic acid was also observed in both strains

(Fig. 4c). Coniferyl alcohol consumption of Fa6 was faster after 2, 4, 6 and 8 h compared to *A. niger* N402 (Fig. 4a), but after 10 h, the consumption of coniferyl alcohol was similar for both strains. However, at 6, 8 and 10 h significantly more vanillic acid was produced by Fa6 than by N402 (Fig. 4c). In addition, an unidentified compound was observed in the HPLC profile of both strains grown on coniferyl alcohol that increased over time. Its retention time did not match with coniferyl aldehyde or vanillin. When grown on vanillic acid, the consumption rate of this compound was similar for both strains (Fig. S1).

Differential transcriptional response of Fa6 to ferulic acid and *p*-coumaric acid

To study the differential gene expression of Fa6 compared to N402, RNA sequencing was performed on both strains grown for 2 h in MM containing 1 mmol l^{-1} ferulic acid of *p*-coumaric acid and a no carbon source culture was used as reference. This concentration was selected to trigger the expression of putative aromatic metabolism genes while minimizing the stress response. The transcriptome data of two biological replicates were clustered by a principal component analysis, which revealed that the response of N402 and Fa6 to ferulic acid and *p*-coumaric acid clearly differentiated them (Fig. 5). There was no overlap observed between the strains on ferulic acid, *p*-coumaric acid and the no carbon source control indicating that each strain responded uniquely to the used conditions.

Increased induction of the feruloyl esterase and xylanolytic genes by Fa6 grown in ferulic acid and *p*-coumaric acid

The transcriptome data were compared with previous expression data of the feruloyl esterase encoding genes (*faeA*, *faeB*, *faeC*) since these are known to be induced by ferulic acid and *p*-coumaric acid (de Vries *et al.* 1997, 2002; de Vries and Visser 1999; Dilokpimol *et al.* 2017). In *A. niger* N402 and Fa6, all three feruloyl esterase genes were induced (FC $\geq$ 2, $P \leq 0{\cdot}01$) by ferulic acid (Fig. 6a), while only *faeB* was induced by *p*-coumaric acid

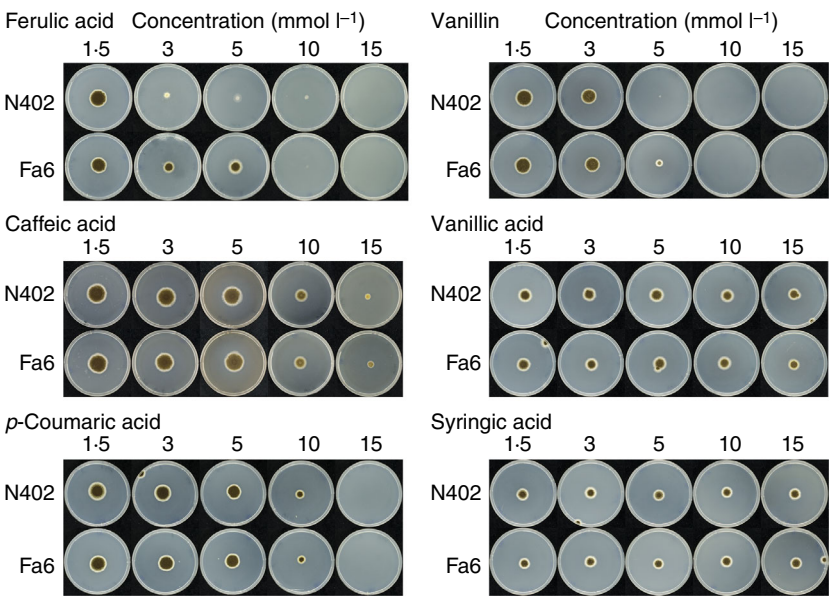

Figure 1 Growth profile of N402 and Fa6 on ferulic acid, caffeic acid, *p*-coumaric acid, vanillin, vanillic acid and syringic acid. Strains were grown for five days at 30°C on MM with the aromatic compound and 25 mmol l^{-1} D-fructose.

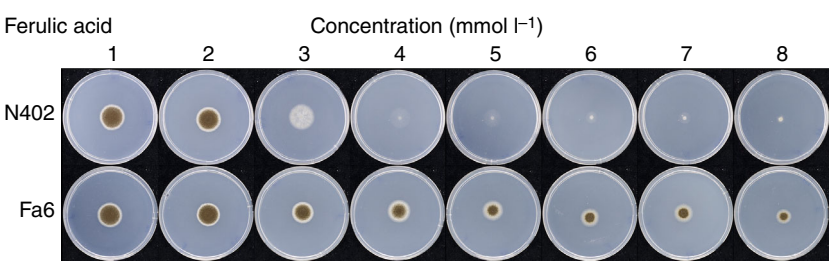

Figure 2 Detailed growth profile of N402 and Fa6 on ferulic acid. The strains were grown for six days on MM with ferulic acid and 25 mmol l^{-1} D-fructose.

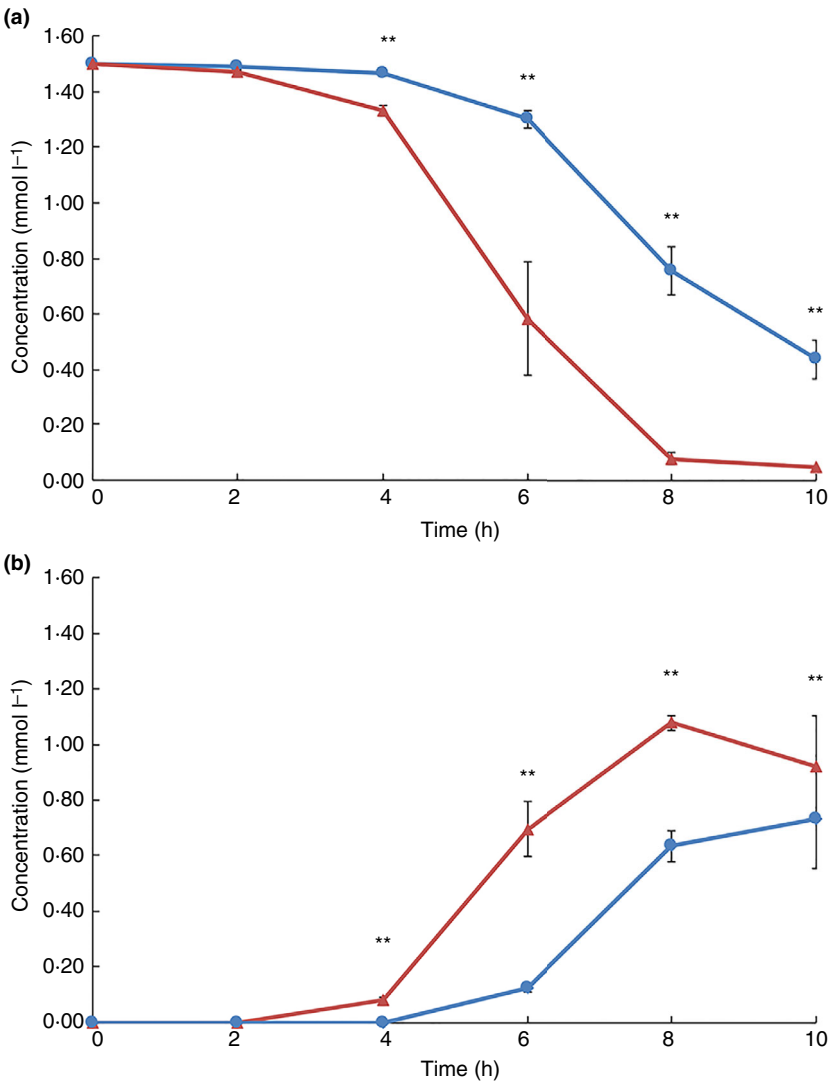

Figure 3 Conversion of ferulic acid by N402 and Fa6. (a) Consumption of ferulic acid. (b) Formation of vanillic acid. Lines with '●' represent N402 and lines with '▲' represent Fa6. Error bars represent the SD between two biological replicates. Statistical analysis was performed on two biological replicates with Student's *T*-test with Student's *T*-test, $^{**}P \leq 0{\cdot}01$.

(Fig. 6b). The expression of *faeA* on ferulic acid is 64-fold higher in Fa6 than in N402, while *faeC* was five-fold higher in Fa6. On *p*-coumaric acid, *faeB* was 1·6-fold higher expressed by Fa6 compared to N402. The transcriptome data set revealed that multiple xylanolytic genes are induced by ferulic acid in Fa6, but not in *A. niger* N402 (Table 2). In addition, two genes encoding putative lytic polysaccharide monooxygenases (LPMOs) (NRRL3_3929 and NRRL3_8994), from the CAZy Auxiliary Activities family 9 (Lombard *et al.* 2014), were induced by ferulic acid. In the genome, the LPMO NRRL3_3929 is co-localized to a GH11 xylanase gene (NRRL3_3928), which is also upregulated in Fa6. Similar patterns were observed for the putative LPMO NRRL3_8994 which is co-localized to *faeC* (NRRL3_8993) and an arabinoxylan arabinofuranohydrolase *Axh62A* (NRRL3_8707) which is co-localized to a xylanase *Xyn10B* (NRRL3_8708). The xylanolytic (XlnR) and arabinanolytic (AraR) regulators are known to regulate the xylanolytic genes (van Peij *et al.* 1998; Battaglia *et al.* 2011), but they were not induced by ferulic acid (Table 2).

Aromatic metabolic genes induced by ferulic acid and *p*-coumaric acid

Several ferulic acid metabolic pathways have been suggested for *A. niger* (Lubbers *et al.* 2019a). The non-β-oxidative decarboxylation of ferulic acid was suggested to be catalyzed by the ferulic acid CoA ligase (NRRL3_1060) (Srivastava *et al.* 2010). The putative gene encoding this enzyme was induced by ferulic acid and *p*-coumaric acid in N402, but not in Fa6 (Table 3). The gene encoding the second suggested enzyme of this pathway,

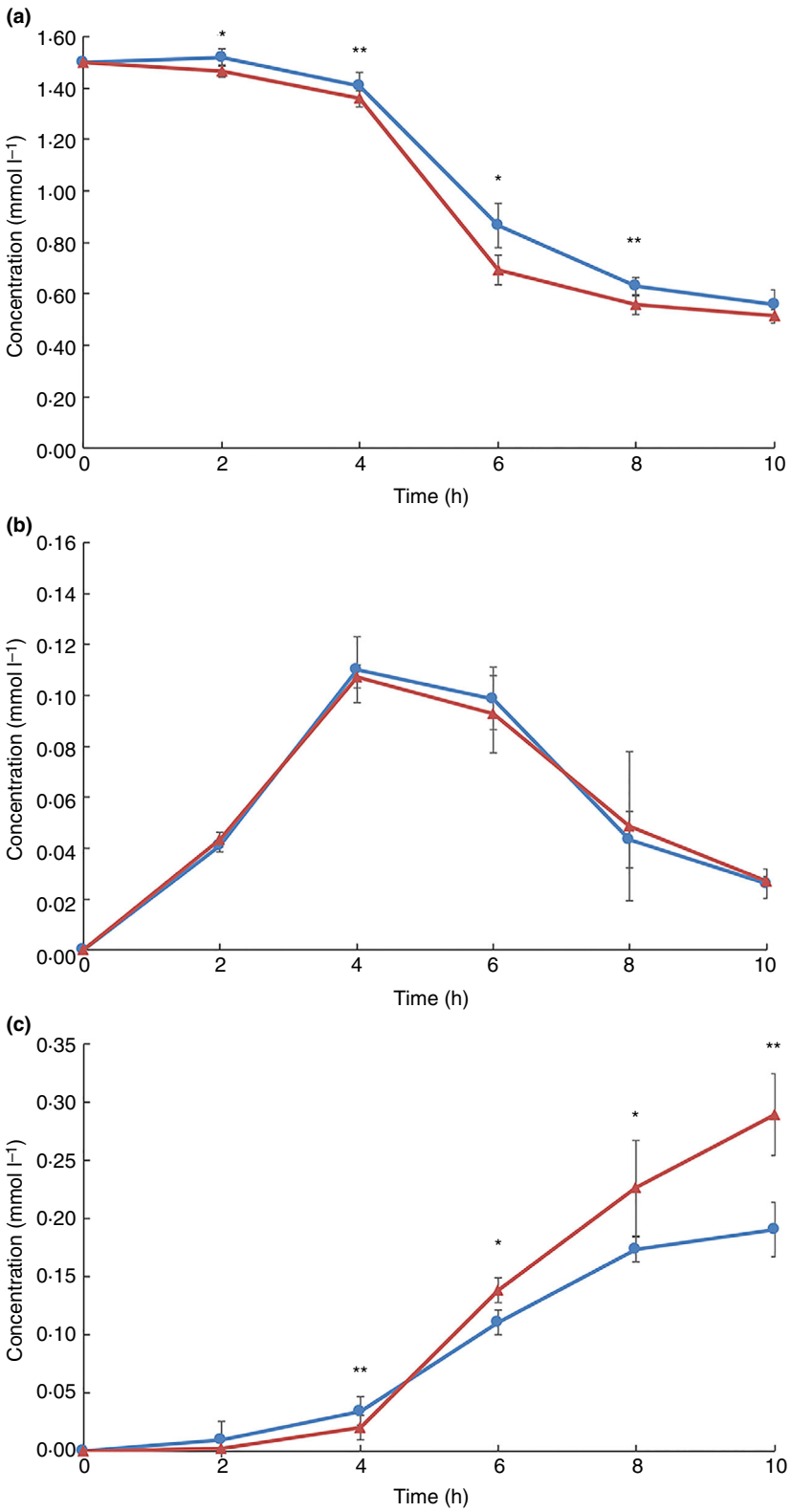

Figure 4 Conversion of coniferyl alcohol by N402 and Fa6. (a) Consumption of coniferyl alcohol. (b) Formation of ferulic acid. (c) Formation of vanillic acid. Lines with '●' represent N402 and lines with '▲' represent Fa6. Error bars represent the SD between two biological replicates. Statistical analysis was performed on two biological replicates with Student's *T*-test, $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$.

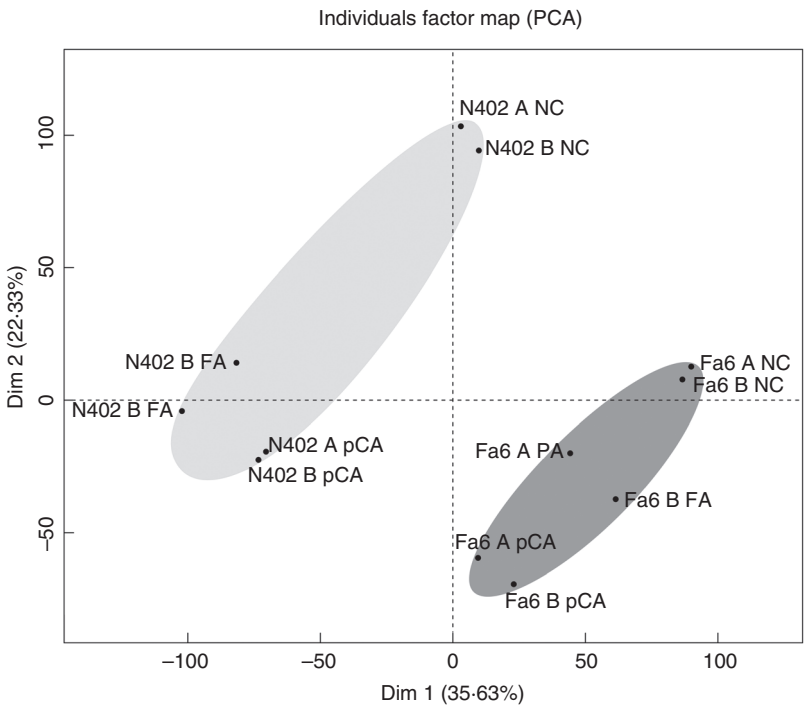

Figure 5 Principal component analysis of the complete transcriptome in *Aspergillus niger*. Two biological replicates of *A. niger* N402 and Fa6 on ferulic acid (FA), *p*-coumaric acid (pCA) and no carbon source (NC) are shown in a bi-dimensional matrix. N402 is highlighted in light gray and Fa6 is highlighted in dark grey.

hydroxycinnamoyl-CoA hydratase/ligase (NRRL3_6035) (Srivastava *et al.* 2010), was neither induced by ferulic acid nor *p*-coumaric acid. Non-oxidative decarboxylation is another observed ferulic acid metabolic pathway in *Aspergillus* species (Milstein *et al.* 1983; Baqueiro-Peña *et al.* 2010; Srivastava *et al.* 2010; Taira *et al.* 2018), involving the enzymes cinnamic acid decarboxylase (CdcA) and phenolic acid decarboxylase (AlPad). However, no induction of *cdcA* (NRRL3_8296) in *A. niger* N402 or Fa6 was observed. A close homolog of AlPad was suggested in *A. niger* (NRRL3_8440) (Taira *et al.* 2018), which was induced by *p*-coumaric acid and ferulic acid in N402, but not in Fa6 (Table 3). In contrast, the benzoic acid metabolic pathway genes benzoate-*p*-hydroxylase (*bphA*), hydroxyquinol 1,2-dioxygenase (*hqdA*), *p*-hydroxybenzoate-*m*-hydoxylase (*phhA*) and protocatechuate 3,4-dioxygenase (*prcA*) (R. J. M. Lubbers *et al.* unpublished data) were induced by *p*-coumaric acid in N402, and to a higher level in Fa6.

Genomic variant analysis on Fa6 transcriptome data

In order to find mutations responsible for the improved ferulic acid tolerance, genomic variant analysis was performed on the transcriptome data. In total, 41 single nucleotide polymorphisms (SNPs) sites were identified on 17 proteins (Table S1). Two SNPs caused new stop codons on a putative DnaJ chaperone protein (NRRL3_4111) and transcriptional regulator (NRRL3_6646). In Fa6, the expression of NRRL3_6656 was reduced by ferulic acid or *p*-coumaric acid compared to the no carbon source control (Table S2).

Discussion

Adaptive evolution is a powerful tool, which uses the ability of micro-organisms to adapt to environmental changes, in order to improve strains for various biotechnological applications (Wallace-Salinas and Gorwa-Grauslund 2013; Culleton *et al.* 2016; González-Ramos *et al.* 2016; Patyshakuliyeva *et al.* 2016). Most of the current filamentous fungi obtained through adaptive evolution were created for increased enzymatic activity. Evolutionary adaptation was also used to study antifungal resistance in *Aspergillus nidulans* and *Aspergillus fumigatus* (Schoustra *et al.* 2005, 2006; Zhang *et al.* 2015). In this study, we successfully created a mutant with increased ferulic acid tolerance. The improved tolerance to ferulic acid also resulted in improved uptake and conversion of ferulic acid and coniferyl alcohol. The conversion of coniferyl alcohol to ferulic acid was observed in many bacteria; however, no experimental proof exists for this pathway in fungi (Lubbers *et al.* 2019a). Here, we showed that coniferyl alcohol is converted to ferulic acid and further to vanillic acid. Improved tolerance to aromatic compounds could be due to an increased metabolic conversion rate or increased export of the aromatic compound out of the cells, possibly through increased

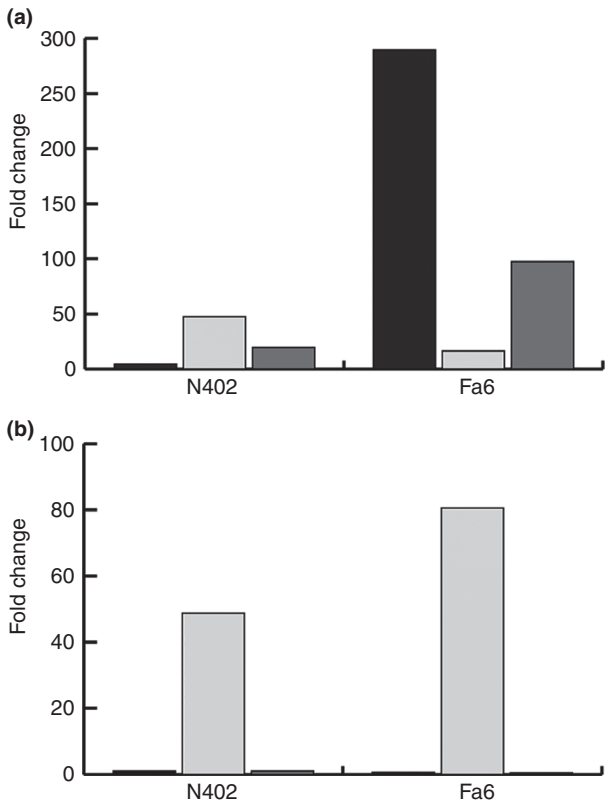

Figure 6 Expression of the feruloyl esterase encoding genes *faeA*, *faeB* and *faeC* on ferulic acid (b) and *p*-coumaric acid (a) compared to the no carbon source control. Bars highlighted in black are *faeA*, in light grey are *faeB* and in dark grey are *faeC*.

expression of the genes involved in these processes. Several putative transporters were upregulated and could be involved in the increase uptake of ferulic acid (data not shown). Principal component analysis of the complete transcriptome revealed that the transcriptional profile of Fa6 was changed in all tested conditions, suggesting a change in the transcriptional regulation of Fa6 compared to N402. The transcriptome data also revealed that genes encoding xylanolytic enzymes, including xylanases, LPMOs and FAEs, were induced in Fa6 compared to N402. Xylanases and FAEs are known to degrade feruloylated xylan and release ferulic acid from plant biomass (Faulds and Williamson 1995; de Vries *et al.* 1997; Kroon *et al.* 1999; Yu *et al.* 2002). The function of the induced LPMOs have not been characterized, however, it has been shown that LPMOs from AA9 catalyze oxidative cleavage of β-(1-4)-xylosyl bonds in xylan and β-(1-4)-glucosyl bonds in cellulose (Frommhagen *et al.* 2015, 2017; Brenelli *et al.* 2018). Both induced LPMOs have been predicted to act on cellulose (Borin *et al.* 2017) and one of them (NRRL3_3929) has been suggested to be regulated by AraR (Gruben *et al.* 2017). The transcriptional profile of the FAE encoding genes correlated with previous observations (de Vries *et al.* 2002; Dilokpimol *et al.* 2017). Ferulic acid can be linked to L-arabinose in xylan, which could explain why the other xylanolytic genes were also induced by ferulic acid. However, in N402 these xylanolytic genes were not induced, while *faeA* and *faeC* were lowly induced compared to Fa6. The gene *faeA* is regulated by XlnR and *faeB* is regulated by AraR, GaaR and RhaR (de Vries and Visser 1999; Gruben *et al.* 2017), however the genes encoding for these regulators, were not induced by Fa6 on ferulic acid. It has been shown that *faeA* was still induced in a XlnR mutant by ferulic acid (de Vries and Visser 1999), combined with our observations, we speculate that *A. niger* contains an additional transcription factor involved in the regulation of these xylanolytic genes. The increased conversion rate of ferulic acid and coniferyl alcohol by Fa6 indicates that genes encoding aromatic metabolic enzymes may be higher expressed in Fa6 than in N402. At this moment, only one enzyme has been identified (AlPad) that is involved in the ferulic acid metabolic pathway of *Aspergillus luchuensis* (Taira *et al.* 2018). A close homolog of AlPad in *A. niger* (NRRL3_8440) was induced by ferulic acid and *p*-coumaric acid in the wild type, but not in Fa6. A similar pattern was observed for a gene encoding an enzyme that was suggested to be involved in the CoA-dependent non-β-oxidative pathway (Srivastava *et al.* 2010), as this gene was induced by ferulic acid in N402 but not in Fa6. In addition, we recently demonstrated that the previously described ferulic acid decarboxylase is in fact a cinnamic acid decarboxylase (CdcA) (Lubbers *et al.* 2019b). The gene encoding this enzyme was also not induced by ferulic acid in our study. Based on their expression profiles, these genes are unlikely to be responsible for the improved conversion rate or the increased tolerance to ferulic acid by Fa6. Other enzymes that are known to be involved in aromatic conversions are cytochrome P450s (Matsuzaki and Wariishi 2004). Transcriptome data showed that four genes encoding cytochromes P450 were upregulated in Fa6. One of these (NRRL3_06869) was also induced at a lower level in N402 and is therefore the best candidate to be involved in the improved tolerance of the Fa6 mutant. Recently, we identified three new enzymes of the benzoic acid metabolic pathway (R.J.M. Lubbers *et al.* unpublished data), in addition to the previously described benzoate-*p*-hydroxylase (van Gorcom *et al.* 1990). While *bphA* and *phhA* were not induced by ferulic acid, the intradiol ring dioxygenases *prcA* and *hqdA* were both induced by ferulic acid, and at higher levels in Fa6. The benzoic acid metabolic genes were highly expressed on *p*-coumaric acid, but despite their increased expression levels in Fa6, no growth improvement was observed for Fa6 compared to

Table 2 Expression of genes encoding feruloyl esterases, xylanolytic enzymes and related regulators in N402 and Fa6. The *P*-values were calculated using Deseq2 software (Love *et al.* 2014)

		N402				Fa6			
		FPKM		Deseq2 fold change		FPKM		Deseq2 fold change	
Transcript_id(s)	Description	FA	NC	FA/NC	*P*-value	FA	NC	FA/NC	*P*-value
NRRL3_00007	Feruloyl esterase *faeA*	18·8	4·7	4·5	0·00	1211·6	3·3	289·5	0·00
NRRL3_02931	Feruloyl esterase *faeB*	1878·3	42·89	47·51	0·00	433·8	21·4	16·4	0·00
NRRL3_08993	Feruloyl esterase *faeC*	9·1	0·2	19·7	0·00	141·7	1·1	97·5	0·00
NRRL3_03339	Acetylxylan esterase CE1	1·6	17·6	0·1	0·00	42·1	7·6	4·6	0·00
NRRL3_08708	Xylanase *Xyn10B*	4·3	3·3	1·5	0·33	173·8	1·3	102·6	0·00
NRRL3_08707	Arabinoxylan arabinofuranohydrolase *Axh62A*	41·0	27·8	1·7	0·03	814·5	6·8	94·5	0·00
NRRL3_03928	Xylanase GH11	4·1	6·2	0·8	0·70	54·5	2·2	17·3	0·00
NRRL3_03929	Lytic polysaccharide monooxygenase AA9	50·3	124·9	0·5	0·00	473·2	24·0	16·7	0·00
NRRL3_01648	Xylanase *Xyn11B*	21·6	42·5	0·6	0·18	162·8	18·5	7·2	0·00
NRRL3_08994	Lytic polysaccharide monooxygenase AA9	1·2	0·0	4·9	0·04	69·0	1·1	42·6	0·00
NRRL3_04034	Xylanolytic transcriptional activator *xlnR*	72·7	70·1	1·2	0·23	114·0	107·8	0·93	0·76
NRRL3_07564	Arabinanolytic transcriptional activator *araR*	7·4	13·4	0·7	0·15	14·3	15·4	0·82	0·52

Abbreviations: FA, ferulic acid; NC, no carbon source.

Table 3 Expression of putative aromatic metabolic genes *A. niger* N402 and Fa6

		N402				Fa6			
Transcript_id(s)	Description	FA/NC	*P*-value	pCA/NC	*P*-value	FA/NC	*P*-value	pCA/NC	*P*-value
NRRL3_08296	Cinnamic acid decarboxylase *cdcA*	1·9	0·38	2·5	0·20	1·2	0·86	1·7	0·59
NRRL3_08297	Sorbic acid decarboxylase regulator *sdrA*	0·7	0·75	1·3	0·80	0·8	0·79	1·5	0·61
NRRL3_08298	Flavin prenyltransferase *padA*	2·2	0·10	2·9	0·02	2·0	0·23	1·8	0·30
NRRL3_00283	Benzoate 4-monooxygenase *bhpA*	0·7	0·03	9·5	0·00	1·2	0·29	13·4	0·00
NRRL3_08551	*p*-hydroxybenzoate-*m*-hydroxylase *phhA*	0·7	0·27	93·8	0·00	0·9	0·89	148·1	0·00
NRRL3_01405	Protocatechuic acid 3,4-dioxygenase *prcA*	2·8	0·00	57·0	0·00	4·8	0·00	234·8	0·00
NRRL3_02644	Hydroxyquinol 1,2-dioxygenase *hqdA*	4·3	0·00	1·2	0·40	8·1	0·00	3·2	0·00
NRRL3_11348	NADPH-cytochrome P450 reductase *cprA*	1·8	0·00	3·3	0·00	1·2	0·39	3·9	0·00
NRRL3_01060	AMP-dependent synthetase/ligase	12·54	0·00	8·44	0·00	0·90	0·57	1·56	0·00
NRRL3_06035	Enoyl-CoA hydratase	1·70	0·00	1·47	0·00	0·51	0·00	0·74	0·02
NRRL3_08440	Phenolic acid decarboxylase	2·3	0·00	8·5	0·00	0·5	0·00	1·9	0·00

Abbreviations: FA, ferulic acid; pCA, *p*-coumaric acid; NC, no carbon source.
The *P*-values were calculated using Deseq2 software (Love *et al.* 2014).

N402 on *p*-coumaric acid. Genome variant analysis was performed on the transcriptome data and resulted in the identification of 41 SNPs in 17 proteins. In Fa6, the putative transcriptional regulator NRRL3_6646, was mutated and results in a premature stop codon. In addition, the expression of this gene was reduced in both ferulic acid and *p*-coumaric acid. However, this analysis remains inconclusive since only low amount of fragments contained the SNP mutation and were not detected in all conditions (Table S1). In addition, mutations could be present within the noncoding regions of the genome which is not possible to detect with this analysis, nor can we exclude that the phenotype is based on an epigenetic effect. Therefore, it remains unknown which gene(s) are responsible for the improved tolerance to ferulic acid.

Future research will include analysis of the genome of Fa6 to identify the genetic modification responsible for its phenotype. This study demonstrates that the adaptive evolution can be used to obtain filamentous fungal strains with increased tolerance to unfavourable environments. The obtained mutant resulted in increased tolerance, metabolite uptake and conversion rate, which are all beneficial characteristics for strains used in industrial applications.

Acknowledgements

R.J.M.L. and A.D. were supported by the European Union's Horizon 2020 research and innovation program under grant agreement no. 720918 (FALCON). A.J.L. was

supported by Marie Curie ITN network SuBiCat FP7 607044. I.B. was supported by NWO/STW VICI grant 016.130.609 to R.P.d.V.

Conflict of Interest

No conflict of interest is declared.

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Supporting Information

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

Figure S1. Consumption of vanillic acid by N402 and Fa6. Line with '●' represents N402 and line with '▲' represents Fa6. Error bars represent the SD between two biological replicates.

Table S1. Genomic variant analysis of Fa6 transcriptome data.

Table S2. Expression of genes with SNPs identified by genome variant analysis.