L-Methionine repressible promoters for tuneable gene expression in *Trichoderma reesei*

Robert H. Bischof¹, Jennifer Horejs¹,³, Benjamin Metz¹,⁴, Christian Gamauf², Christian P Kubicek¹,³ and Bernhard Seiboth¹,³*

**Abstract**

**Background:** *Trichoderma reesei* is the main producer of lignocellulolytic enzymes that are required for plant biomass hydrolysis in the biorefinery industry. Although the molecular toolbox for *T. reesei* is already well developed, repressible promoters for strain engineering and functional genomics studies are still lacking. One such promoter that is widely employed for yeasts is that of the l-methionine repressible MET3 gene, encoding ATP sulphurylase.

**Results:** We show that the MET3 system can only be applied for *T. reesei* when the cellulase inducing carbon source lactose is used but not when wheat straw, a relevant lignocellulosic substrate for enzyme production, is employed. We therefore performed a transcriptomic screen for genes that are l-methionine repressible in a wheat straw culture. This analysis retrieved 50 differentially regulated genes of which 33 were downregulated. Among these, genes encoding transport proteins as well as iron containing DszA like monooxygenases and TauD like dioxygenases were strongly overrepresented. We show that the promoter region of one of these dioxygenases can be used for the strongly repressible expression of the *Aspergillus niger sucA* encoded extracellular invertase in *T. reesei* wheat straw cultures. This system is also portable to other carbon sources including d-glucose and glycerol as demonstrated by the repressible expression of the *Escherichia coli lacZ* encoded β-galactosidase in *T. reesei*.

**Conclusion:** We describe a novel, versatile set of promoters for *T. reesei* that can be used to drive recombinant gene expression in wheat straw cultures at different expression strengths and in an l-methionine repressible manner. The dioxygenase promoter that we studied in detail is furthermore compatible with different carbon sources and therefore applicable for manipulating protein production as well as functional genomics with *T. reesei*.

**Keywords:** *Trichoderma reesei*, Promoter, Repressible promoter, Tuneable promoter, TauD/TfdA like dioxygenase, l-Methionine, Wheat straw
of an inexpensive repressing substance that is effective at low concentrations is mandatory. However, a repressible promoter meeting these specifications is currently not available for T. reesei, where tuneable expression is almost exclusively controlled by the promoters of the cellobiohydrolase cel7a or endoxylanase xyn1 [4]. In these two cases, the repression is mediated by α-glucose [6, 7], which in turn leads not only to the repression of cellulases and xylanases but also to the repression of most of the other carbohydrate active enzymes (CAZYmes) [8, 9] which is usually undesirable. Repressible expression systems that have been proven to work in filamentous fungi and yeast include the thiamine repressible thiA system of Aspergillus fumigatus and Aspergillus oryzae [10, 11] and the copper dependent Saccharomyces cerevisiae CTR4 system in Schizosaccharomyces pombe and Cryptococcus neoformans [12–14]. Both systems have the advantage of responding to μM concentrations of the respective modulating substances, but suffer from the fact that these compounds may already be present in industrially used complex carbon sources such as wheat straw. Another widely employed system for different yeasts including S. cerevisiae, Ashbya gossypii, Candida albicans or Pichia pastoris is the L-methionine repressible MET3 system [15–18], which however has not been tested in filamentous fungi so far. We regard L-methionine as a promising repressor because its addition to T. reesei cultures was previously shown to have no effect on growth and CAZyme expression [19]. Owing to its use as a food and feed additive [20] it is also modestly prized and abundantly available. However, we show here that the expression of the T. reesei met3 orthologue responds to L-methionine in the same way as the S. cerevisiae gene only when lactose, but not when a complex carbon source such as wheat straw is used as the carbon source. Since wheat straw is one of the most relevant feedstocks for biorefineries [9, 21], we developed a repressible expression system for this carbon source. Using a transcriptomic approach, we identified a novel set of L-methionine repressible genes that feature a diverse range of basal expression strengths and degrees of achievable repression. We also demonstrate the applicability of the promotor region of the most strongly expressed of said genes for tightly repressible expression of intra- and extracellular reporter genes on wheat straw as well as on α-glucose and glycerol containing media.

Results
Repression of the T. reesei met3 (ATP sulphurylase-encoding) gene by L-methionine
The MET3 encoded ATP sulphurylase is involved in the assimilation of sulphate as part of L-methionine biosynthesis and its promoter has been successfully used in several yeasts to drive tightly repressible expression [15–18]. The T. reesei orthologue of the S. cerevisiae MET3 gene as well as the orthologues of the other sulphate assimilation and L-methionine biosynthesis genes were analyzed and their encoded activities as well as the gene IDs are shown in the additional files (see Additional file 1: Table S1; Figure S1). When we mapped the individual genes of the pathway to the recently published reconstructed chromosomes of T. reesei [22] to test if the genes are clustered, we found a random distribution across all seven chromosomes (see Additional file 1: Table S1). A blastp search with the S. cerevisiae Met3 protein against the T. reesei filtered protein models retrieved only a single protein with very high sequence similarity (Trirc2:47066, E-value of 2.88 × 10^-156). We therefore tested the expression of the T. reesei met3 gene in response to the addition of different concentrations of L-methionine under two different cellulase inducing conditions. Quantitative PCR (qPCR) analysis of met3 gene expression showed, that it was only partially downregulated by L-methionine when pretreated wheat straw was used as carbon source even at 10 mM of added L-methionine. A stronger repression was monitored when lactose was used as cellulase inducing carbon source. Here, the amount of the met3 transcript was reduced to about 10 % of the unrepressed control (Fig. 1). We therefore conclude that the met3 promoter of T. reesei cannot be used to tightly shut off gene expression when pretreated wheat straw is used as carbon source.

Fig. 1 Transcriptional regulation of the T. reesei met3 following L-methionine addition. L-methionine (0–10 mM) was added to 48 h old wheat straw (blue bars) or 40 h old lactose (red bars) grown cultures of T. reesei and incubated for further 2.5 h before transcript levels were determined. The met3 transcript levels are related to the expression before addition of L-methionine on wheat straw and lactose, respectively and normalised on tef1. Values shown are the mean and standard deviation of three biological replicates from independent cultivations.
Genome-wide identification of l-methionine repressible genes

In order to identify T. reesei genes that are differentially regulated by the addition of l-methionine to the fermentation medium, we compared the transcriptome of T. reesei growing on wheat straw in the absence and in the presence of exogenously added 0.1 mM of the amino acid (see “Methods”). As previously shown, these conditions represent a mycelium in the late growth phase [9]. This comparison retrieved 50 genes that were >twofold differentially expressed 2.5 h after the addition of 0.1 mM l-methionine to a 48 h old wheat straw culture (Table 1). 33 of them were downregulated and are thus either directly or indirectly repressed by l-methionine. Four of these genes (met5, met16, sul1 and met2) encode proteins that are involved in sulfur assimilation and l-methionine biosynthesis. The expression of other genes of these pathways was only slightly reduced in the presence of 0.1 mM l-methionine, and that of met6 (encoding l-methionine synthase) even slightly enhanced (Fig. 2). The transcription of the lim1 gene, which encodes the T. reesei orthologue of the S. cerevisiae/N. crassa F-box protein Met30/SCON-2 [19] was not regulated under these conditions. Other known regulators of l-methionine biosynthesis in S. cerevisiae (MET31, MET32, MET4, MET28 and CBF1) either had no or just a very weakly conserved orthologue in T. reesei (see Additional file 1: Table S1) and in the latter case they were also not regulated (see Fig. 2). Interestingly, 7 of the 10 l-methionine biosynthesis genes of T. reesei also lacked a CACGTG consensus sequence that is found in S. cerevisiae by the Cbi1 protein and involved in the regulation of MET genes [23, 24]. When balancing the genes repressed by the addition of 0.1 mM l-methionine to a wheat straw culture, it is intriguing to note that more than a third (13 of 37) of them encode proteins either involved in iron acquisition (iron transporter, ferrooxido-reductase, siderophore biosynthesis, oxalate homeostasis) or contain iron as an essential part for catalysis (flavin- and DszA-type monoxygenases, α-ketoglutarate dependent TauD like dioxygenases; Table 1). Also, the l-methionine repressible transcriptome was overrepresented (p = 0.007) by nine genes encoding transporters for amino acids and other nitrogen-containing compounds. These included three amino acid transporters of which Trire2:60144 shows a high similarity to high affinity l-methionine transporters, as well as allantoin-like transporters that could be involved in transport of pyrimidine bases or oligopeptides [25, 26] and Na⁺-coupled amino acid transporters. Unlike in other transcriptomic studies with T. reesei [9, 27, 28], only four of the downregulated genes (=8 % of the regulated transcriptome) comprised unknown genes, and no orphan genes were detected. As expected, none of the major plant biomass degradation related CAZymes were differentially regulated by the addition of 0.1 mM l-methionine which is in perfect agreement with the findings reported by Gremel et al. [19].

The expression of the five most strongly repressed genes was then tested by qPCR over a longer time period and with different concentrations of the repressor. As shown in Fig. 3 repression of these genes by l-methionine was strong 2 and 5 h after its addition at concentrations ≥0.1 mM but not detectable ≤10 µM. 8 h after l-methionine addition, the cultures supplemented with 0.1 mM of the amino acid showed transcript levels of the tested genes similar to the non-treated controls, suggesting that the repression is of a transient nature. In fact, the repression by 0.1 mM l-methionine declined already 5 h after its addition. When a final concentration of 1 mM l-methionine was added, repression of all tested genes was tight at all tested time points.

Construction of T. reesei strains with repressible lacZ and sucA reporters

We then selected the promoter (subsequently termed P_{123979} of the TauD like dioxygenase-encoding gene trire2:123979 for further testing, because it displayed the highest level of transcription of this gene family in the absence of exogenous l-methionine (log2 fluorescence of 12.75) and strong repression in the presence of 0.1 mM added l-methionine (see Table 1). As reporter genes, we chose a modified version of Aspergillus niger invertase encoding sucA (β-d-fructofuranosidase, EC 3.2.1.26; [29]) and the E. coli β-galactosidase encoding lacZ, respectively. As a comparison to the expression strength of P_{123979} we used the strong constitutive promoter of tef1 (P_{tef1}) as previously described [30, 31]. Both promoter regions were inserted into sucA and lacZ expression plasmids that also contained the hphB resistance cassette for selection. After transformation of the sucA expression cassettes into T. reesei QM9414 and purification of the transformants, we isolated at least three positive strains for the repressible promoter and two P_{tef1} reference strains having a single integration of the construct as verified by qPCR (see Additional file 1: Figure S2). In the same way, we also constructed strains expressing E. coli lacZ under the control of the promoters of P_{123979} and P_{tef1} and again isolated strains having a single copy integration of the expression cassette (see Additional file 1: Figure S3).

l-Methionine dependent repression of sucA expression on wheat straw medium

We then cultured the different sucA expressing strains on wheat straw containing medium for 48 h and added
Table 1  

| Gene ID   | Fold change | Log2 gene expression from array data | Functional annotation |
|-----------|-------------|-------------------------------------|-----------------------|
|           |             | Met− | Met+ |                                     |
| 112567    | 20.02       | Down | 12.49 | 8.16 | Taurine catabolism dioxygenase TauD/TfdAα |
| 6005      | 11.61       | Down | 11.55 | 8.01 | MSF permease, allantoin-permease likeβ |
| 54461     | 8.98        | Down | 10.09 | 6.92 | MSF permeaseβ |
| 73250     | 8.76        | Down | 10.30 | 7.16 | Urea transporter/Na⁺-amino acid transporterγ |
| 59876     | 8.61        | Down | 12.02 | 8.91 | DszA-type family xenobiotic monoxygenasesα |
| 104081    | 8.55        | Down | 10.63 | 7.53 | DszA-type family xenobiotic monoxygenasesα |
| 60144     | 8.54        | Down | 10.54 | 7.45 | Amino acid transporter, high affinity methionine transporterβ |
| 69563     | 8.08        | Down | 9.79  | 6.77 | MSF permeaseβ |
| 123979    | 6.61        | Down | 12.75 | 10.03 | Taurine catabolism dioxygenase TauD/TfdAα |
| 5369      | 5.83        | Down | 9.40  | 6.86 | Metallocarboxypeptidase, putative |
| 59333     | 5.61        | Down | 9.12  | 6.63 | MSF permease, allantoin permease likeβ |
| 56911     | 5.59        | Down | 9.89  | 7.41 | Urea transporter/Na⁺ amino acid transporterγ |
| 103039    | 5.06        | Down | 11.34 | 9.00 | Peptidase S41 |
| 81576     | 5.04        | Down | 13.64 | 11.31 | Assimilatory sulfite reductase, alpha subunitβ |
| 103012    | 4.75        | Down | 12.35 | 10.10 | Taurine catabolism dioxygenase TauD/TfdAα |
| 64049     | 4.48        | Down | 10.47 | 8.31 | Unknown protein |
| 62872     | 3.88        | Down | 14.05 | 12.10 | Unknown protein GPR1/FUN34/yaah-like |
| 68831     | 3.81        | Down | 10.63 | 8.70 | Amino acid transporterβ |
| 65410     | 2.95        | Down | 13.51 | 11.95 | Phosphoadenosine phosphosulfate reductaseβ |
| 124115    | 2.93        | Down | 13.34 | 11.79 | Phosphoenolpyruvate carboxykinase AcuF |
| 22110     | 2.79        | Down | 11.54 | 10.06 | Flavin-containing monoxygenaseα |
| 53964     | 2.56        | Down | 8.68  | 7.33 | Oxalate decarboxylaseβ |
| 109239    | 2.56        | Down | 9.31  | 7.95 | Unknown protein |
| 108781    | 2.48        | Down | 10.93 | 9.62 | DszA-type family xenobiotic monoxygenases |
| 121139    | 2.39        | Down | 11.10 | 9.84 | Amino acid transporter, AAT-familyβ |
| 79741     | 2.38        | Down | 13.11 | 11.86 | Sulfat transporter SUL1β |
| 69696     | 2.36        | Down | 10.18 | 8.94 | DszA-type family xenobiotic monoxygenasesα |
| 111912    | 2.35        | Down | 9.36  | 8.14 | Carbonic anhydrase |
| 120473    | 2.32        | Down | 11.41 | 10.19 | Dihydrolipoamide transacylase |
| 54219     | 2.28        | Down | 14.81 | 13.61 | CES acetyl xylan esterase |
| 112590    | 2.26        | Down | 9.26  | 8.09 | Siderophore biosynthesis lipase/esteraseα |
| 102820    | 2.18        | Down | 11.17 | 10.04 | Ferrooxidoreductaseα |
| 71005     | 2.16        | Down | 8.35  | 7.24 | NRPS siderophore synthase SID1α |
| 66517     | 2.11        | Down | 12.63 | 11.55 | Homoserine O-acetyltransferaseβ |
| 120864    | 2.16        | Up   | 10.76 | 11.88 | DIP2, encoding a nucleolar protein |
| 4109      | 2.26        | Up   | 10.68 | 11.85 | Aspartate/tyrosine/aromatic aminotransferase |
| 78650     | 2.21        | Up   | 11.92 | 13.06 | C2H2 transcriptional regulator |
| 56587     | 2.44        | Up   | 8.81  | 10.10 | GCNS-related N-acetyltransferase |
| 41590     | 2.09        | Up   | 11.58 | 12.64 | Iron transporterα |
| 3049      | 3.46        | Up   | 11.76 | 13.55 | Methionine aminopeptidase |
| 66854     | 3.14        | Up   | 7.57  | 9.22 | Monocarboxylate transporter |
| 60052     | 2.25        | Up   | 8.15  | 9.32 | Short chain dehydrogenase/reductase |
| 67840     | 2.06        | Up   | 10.62 | 11.66 | Shows similarity to S. cerevisiae Utp11 |
| 110767    | 2.31        | Up   | 9.70  | 10.91 | Unique protein |
| 120975    | 3.17        | Up   | 9.57  | 11.23 | Unknown protein |
| 110768    | 2.44        | Up   | 10.77 | 12.06 | Unknown protein |
| 77593     | 2.15        | Up   | 9.11  | 10.21 | Unknown protein |
l-methionine to a final concentration of 1 mM to the medium. We found that prior to the l-methionine addition the three P_{123979} strains accumulated 144 ± 10 % of the sucA mRNA as compared to P_{tef1} strains, while already 2 and 5 h after the l-methionine addition below 1 % of that amount was detected indicating a strong repression (Fig. 4, left side). The expression level in both P_{tef1}–sucA strains was furthermore indiscernible at a 95 % confidence interval. We then also compared the specific invertase activity in the supernatant of the P_{123979} expression strains with that of the P_{tef1} reference strains grown on wheat straw containing medium as shown in Fig. 5. When we added l-methionine to a final concentration of 1 mM to the fermentation medium of the P_{123979} strains at 18.5 h and another 5 mM at 27.5 h, we found only a drastically reduced accumulation of invertase activity in the supernatant at later time points, indicating that the promoter was tightly shut during that period. When we added the same amount of l-methionine to the P_{tef1} strains, we found no difference in the rate of invertase production during the next 18 h (2.46 ± 0.09 units per hour without l-methionine, 2.41 ± 0.09 units per hour with l-methionine). We also monitored the invertase activity in the supernatant of untransformed T. reesei QM9414 and found that it was below one unit per mg extracellular protein after 24, 48 and 72 h of fermentation and declined over the course of the fermentation.

**Portability of the l-methionine repressible expression system to other carbon sources and alternative repressors**

We then wanted to learn whether the P_{123979} promoter region could be used for the repressible expression on a broader range of carbon sources including the cellulase repressing carbon source d-glucose or the cellulase neutral carbon source glycerol, both of which are also more relevant in terms of functional genetics than wheat straw is. To this end, we tested the repression of the intracellularly produced β-galactosidase LacZ in two of the P_{123979}–lacZ strains during growth on minimal medium containing d-glucose or glycerol as carbon sources. To account for the different growth rates on both media the strains were cultured for 16 or 30 h, respectively and l-methionine added to a final concentration of 1 or 5 mM. 10 h later, mycelia were harvested and the intracellular LacZ activity determined. As shown in Table 2, both l-methionine concentrations (1 and 5 mM) led to
a tight repression of \( P_{123979} \) as compared to the control experiment.

\( l \)-cysteine is often used as an alternative repressor for \( MET3 \) orthologues in different yeasts and is linked to \( l \)-methionine metabolism via homocysteine and cystathionine (Additional file 1: Figure S1). When we tested the effect of \( l \)-cysteine on \( P_{123979} \) repression, only the 5 mM concentration had an equally pronounced effect, whereas concentrations of 1 mM resulted only in a minor reduction of \( \beta \)-galactosidase activity. Since repression by lower concentrations of \( l \)-methionine appeared as a transient phenomenon in this study, we also tested the effect of the non-metabolizable substitute \( l \)-ethionine on two
Table 2 Repression of LacZ activity in T. reesei α-glucose and glycerol cultures

|                     | Basal  | L-Methionine | L-Cysteine |
|---------------------|--------|--------------|------------|
|                     | 1 mM   | 5 mM         | 1 mM       | 5 mM       |
| Glucose             |        |              |            |
| P_{123979}-lacZ     | 1.27 ± 0.15 | 0.04 ± 0.01 | 1.02 ± 0.07 | 0.00 |
| P_{123979}-lacZ     | 1.46 ± 0.12 | 0.05 ± 0.00 | 0.81 ± 0.31 | 0.00 |
| Glycerol            |        |              |            |
| P_{123979}-lacZ     | 1.09 ± 0.08 | 0.09 ± 0.02 | n.d        | n.d        |
| P_{123979}-lacZ     | 1.02 ± 0.59 | 0.10 ± 0.00 | n.d        | n.d        |
| QM9414              | 0.00   |              |            |

|                     |        |              |            |
|                     | P_{123979}-lacZ | 0.05 ± 0.00 |            |

Intracellular LacZ activity relative to an equally treated constitutively expressing lacZ strain (P_{lacZ}).

Discussion

L-methionine as a repressor of gene expression in yeasts is well established. In S. cerevisiae, repression of the L-methionine biosynthesis genes MET2, MET3, MET5, MET6, MET14 and MET25 has been shown [17, 32] and the promoters of the MET3 orthologues of C. albicans, A. gossypii and P. pastoris have been used for repressible gene expression [15, 16, 18]. Our results show that when a complex carbon source such as pretreated wheat straw is used, only the phosphoadenylyl-sulfate reductase encoding met16 (trire2:65410), sulfite reductase encoding met5/ecm17 (trire2:81576), homoserine O-acetyltransferase encoding met2 (trire2:66517) and sulfate permease encoding sul1 (trire2:79741) are more than twofold repressed by 0.1 mM L-methionine in T. reesei. In contrast, the orthologue of sulfate adenyltransferase encoding met3 (trire2:47066) was strongly repressed by L-methionine at concentrations higher than 0.1 mM only when lactose was used as the carbon source and the degree of repression was comparable to that observed in S. cerevisiae [17, 32].

To identify novel repressible promoters we used therefore a genome-wide screen under L-methionine repressing conditions. We found genes for transport proteins to be a highly enriched group in the downregulated transcriptome of T. reesei confronted with L-methionine. Two of the transporters that were downregulated in the presence of L-methionine appear to be high affinity amino acid transporters and one of them indeed shows moderate sequence similarity to the characterized S. cerevisiae L-methionine and L-cysteine transporter Mup1 ([33], blastp score 4.7 E−78). It is therefore tempting to speculate that the uptake of L-methionine itself represents another control circuit for the maintenance of L-methionine homeostasis in T. reesei. Repression of L-methionine transport by L-methionine at the transcriptional level also occurs in Penicillium chrysogenum and S. cerevisiae [34, 35] as well as in E. coli [36]. Our findings that a third of the genes that were repressed by 0.1 mM L-methionine encoded proteins involved in iron acquisition or contained iron suggested a relationship between iron metabolism and L-methionine availability.

Recently, Amich et al. [37] have demonstrated a cross-talk between iron homeostasis and sulphur metabolism: starving an Aspergillus fumigatus mutant defective in the sulfur metabolite transcriptional repressor MetR for sulphur results in increased expression of the genes of the iron regulon. The molecular basis for this cross-talk is the upregulation of iron acquiring proteins when the biosynthesis of Fe-S cluster enzymes is impaired because of a shortage of L-cysteine [38]. Consequently, loss-of-function of MetR—and thus sulfur derepression—results in increased cellular iron as well as ferriciricin contents accompanied by transcriptional derepression of genes involved in iron acquisition such as siderophore biosynthesis and uptake as well as reductive iron assimilation [37]. Our transcriptomic results under L-methionine addition (=sulfur repression) are essentially the opposite of those with the A. fumigatus MetR mutant and would therefore be in perfect agreement with the operation of an iron-sulfur crosstalk also in T. reesei. However, the strong repression of the iron containing oxygenases may also have an alternative explanation: in bacteria, Fe²⁺/α-ketoglutarate dependent TaeU/TfdA like dioxygenases are involved in the release of sulfur from the aminosulfonic acid taurine and other organic sources of sulfur [39]. In E. coli, their transcription as well as that of other sulfur starvation induced genes is positively influenced by the transcription factor Cb1 [40–42]. A negatively regulating element in this system is the sulfur assimilation metabolite adenosine 5′-phosphosulfonate, which binds Cb1 and abolishes its activating function [43, 44]. Similarly, dibenzo thiophene sulfone monooxygenase (DszA) is involved in biosulfurization in Rhodococcus erythropolis [45, 46] and repressed by both L-methionine and L-cysteine. If a similar regulation occurs in fungi, it
would offer an alternative explanation for the negative regulation of these oxygenases by L-methionine. This in turn would mean that this system can likely also be transferred to other species, because TauD/TfdA like dioxygenases are in fact quite common in other Pezizomycotina and particularly also among industrially relevant Aspergillus spp. and the plant pathogenic Fusarium spp. (Additional file 1: Figure S4). Although the transient nature of L-methionine repression of the P_{123979} promoter at low concentrations is advantageous to effectively deactivate and reactivate gene expression, it might also be useful to have a repression system that is tightly shut by low concentrations of a repressor during a longer period of time. Attempts to use the L-methionine non-metabolizable analogue L-ethionine for this purpose failed, which probably points to the fact that—like in S. cerevisiae and A. nidulans [47, 48]—S-adenosylmethionine, rather than L-methionine itself, is the repressing metabolite. L-Ethionine cannot be adenylated and inhibits the SAM-dependent methyltransferases [49]. An alternative to overcome this bottleneck would be to knock out the gene further metabolizing S-adenosylmethionine (i.e. adenosyl-homocysteine hydrolase) or placing it under the control of an L-methionine repressible promoter.

Conclusions

In the present manuscript we describe novel L-methionine repressible genes that serve as an alternative to the well-known S. cerevisiae MET3 system under conditions where its T. reesei orthologue met3 is non-repressible, i.e. when the industrially relevant carbon source wheat straw is used. The described set of repressible genes covers a wide array of expression strengths and levels of achievable repression. We demonstrate that we can use the promoter region of a TauD/TfdA-like dioxygenase to achieve repressible gene expression of two reporter genes in T. reesei and that this system can also be used on less complex carbon sources such as D-glucose or glycerol and is likely transferable also to other fungi. Taken together these findings show that the present system offers a versatile means to realize repressible gene expression and therefore represents a valuable tool for industrial strain engineering and functional genetics of T. reesei.

Methods

Strains and culture conditions

Trichoderma reesei QM9414 (ATCC 26921) was used throughout the study and maintained on potato dextrose agar (Difco). Cultivation of T. reesei with pretreated wheat straw, D-glucose, glycerol or lactose as the sole carbon source was routinely performed as previously reported [9]. In brief, pretreated wheat straw up to a final concentration of 10 g L⁻¹ dry weight was added to unbuffered mineral medium, the pH set to 4.8 with 1 M KOH and then autoclaved. CaCl₂ was added separately after autoclaving as were the other carbon sources. 1 x 10⁶ spores ml⁻¹ were then aseptically added to 200 ml of the medium in a 1 L Erlenmeyer flask. The culturing conditions were 28 °C and 250 rpm in a rotary shaker. For the cultivations where extracellular invertase activity was measured, a slightly modified medium that contained per liter: 5 g (NH₄)₂SO₄, 2 g KH₂PO₄, 0.3 g MgSO₄, 0.4 g CaCl₂, 1.7 g yeast nitrogen base without amino acids and (NH₄)₂SO₄, 10.22 g (i.e. 50 mM) potassium phthalate, 20 g pretreated wheat straw, 5 mg FeSO₄ × 7H₂O, 1.6 mg MnSO₄ × H₂O, 1.4 mg ZnSO₄ × 7 H₂O, 1.61 mg CoCl₂ and 0.1 mg CuSO₄ × 5 H₂O was used.

Transcriptomic analysis

Trichoderma reesei QM9414 cultures were pregrown for 48 h on wheat straw medium after which 0.1 mM of L-methionine were added to the cultures. Mycelia were harvested after 2.5 h, washed and instantly frozen in liquid nitrogen. Genome wide gene expression analysis was performed as previously described [9]. The micro-array data and the related protocols are available at the GEO web site (http://www.ncbi.nlm.nih.gov/geo/) under accession number: GSE69745. Groups of eukaryotic orthologous genes (KOG) were retrieved from the T. reesei genome website (http://genome.jgi-psf.org/cgi-bin/kogBrowser?db=Trire2). When no KOG assignment was available there, sequences were manually assigned after sequence analysis using Interpro (http://www.ebi.ac.uk/interpro/) and OrthoDB (http://orthodb.org/orthodb7). In ambiguous cases, KOG category S for “unknown function” was assigned. Enrichment of KOG groups was determined by Fisher’s exact test.

Analysis of the L-methionine biosynthesis pathway and related pathways in T. reesei

Protein sequences were retrieved from the KEGG catalogue of the JGI T. reesei portal (http://genome.jgi-psf.org/cgi-bin/metapathways?db=Trire2) and verified by comparison to the NCBI database using blastp.

Reverse transcription qPCR

Culture conditions were identical to the cultures from the transcriptome analysis if not stated otherwise and methods for total RNA extraction and qPCR measurements are described in detail elsewhere [9]. Primers sequences and amplification efficiencies are given in the supplementary material (Additional file 1: Table S2). Determination of the PCR efficiency was performed using triplicate reactions from a decadic dilution series of cDNA, and the amplification efficiency then calculated from the given
slopes using the realplex v2.2 software. Expression ratios were calculated using REST® software [50].

Construction of sucA and lacZ reporter vectors

The sucA reporter gene was de novo synthesized by MWG Operon (Ebersberg, Germany), whereby a 6x His-tag was introduced C-terminally and the native SucA signal peptide was replaced by the signal peptide from T. reesei CEL7A (MYRKLAVISALATARA). Also, two restriction sites (SalI and ClaI) of the sucA ORF were removed by silent mutations (Codon526:CAC⇒GAC; Codon541:AAT⇒GAT). Constructions were made in pBS_gfp_ThrH3_hph, an egfp expression vector consisting of the egfp gene, a 369 bp terminator fragment of the histone 3 (hH3) terminator and the hph expression cassette [51] as selection marker (Robert Bischof and Bernhard Seiboth, unpublished data). The sucA gene was inserted into the ClaI/SalI fragment of pBS_gfp_ThrH3_hph replacing egfp and resulting in pRB-X. Fragments of the candidate l-methionine repressible promoter P123979 and the tef1 promoter region were amplified from chromosomal DNA of T. reesei QM9414 using primers Inf_pRB-XVI_fw and Inf_pRB-XVI_rv and Inf_pRB-XVIII_fw and Inf_pRB-XVIII_rv, respectively and subsequently introduced into the SalI site of pRB-X by recombinational cloning with the Infusion® HD Cloning Kit (Clontech, CA, USA) resulting in plasmids pRB-XVI (P123979 sucA) and pRB-XVIII (Ptef1 sucA), respectively. Similarly, the lacZ gene (GenBank:NC_000913) was de novo synthesized, the ClaI site removed (Codon 280 ATC⇒ATT) and inserted into the SalI/ClaI site of pBS_gfp_ThrH3_hph resulting in pRB-XI. For the construction of lacZ reporter vectors, the lacZ β-complementation region of pLHhph [51] was eliminated by PCR amplification of the vector using primers pHf and pHlHp-lacZ_rv. The PCR mix was then digested with DpnI to remove the template DNA by methylation specific restriction. The promoter fragments of trire2:123979 (i.e. P123979) and tef1 (Ptef1) were amplified from chromosomal T. reesei DNA using primers XVII_inf_P_fw and XVII_inf_P_rv and XIX_inf_P_fw and XIX_inf_P_rv, respectively. The lacZ gene was amplified from pRB-XI using primers XVII_inf_L_fw and XVII_inf_L_rv (for P123979) and both fragments simultaneously inserted into the pHhph-lacZ DNA fragment via Infusion® HD Cloning Kit resulting in pH-III (P123979 lacZ) and pH-IV (Ptef1-lacZ). Oligonucleotides are given in the supplementary material (Additional file 1: Table S2).

Fungal transformation and strain characterization

Recombinant T. reesei strains were generated by protoplast mediated transformation as described earlier [52] using MEX plates containing 100 µg ml⁻¹ hygromycin for selection. Transformants were then passaged to PDA plates containing the same amount of hygromycin and subsequently purified twice via single spore isolation on PDA plates containing 0.1 % (w/v) Triton X-100 as colony restrictor. DNA extraction, PCR screening and gene copy number estimation by qPCR was done essentially as described by Tisch et al. [53]. Primer sequences for the PCR screening are given in the supplementary material (Additional file 1: Table S2) and positive (plasmid DNA) as well as negative controls (genomic DNA of T. reesei QM9414) were included in the PCR screening.

Measurement of extracellular invertase activity

5 ml culture broth samples were prefiltered through Miracloth tissue and centrifuged. Supernatants were immediately snap-frozen in liquid nitrogen and kept on −20 °C until they were analyzed. Invertase activity towards 12.5 mM saccharose in the supernatant was assayed in 50 mM sodium citrate buffer (pH = 4.6) at 25 °C for 20 min and the reaction stopped by the addition of 50 mM Trizma base. Each sample was assayed as a technical triplicate. The released d-glucose was assayed using a commercially available d-glucose assay reagent (Sigma, St. Louis, MO, USA). Protein concentrations were determined with the Bio-Rad protein assay using BSA as standard (Biorad, Hercules, CA, USA). Non-inoculated substrate controls were included in both cases and the absorbances read out using a Spectra Max Plus 384 microplate reader (Molecular Devices, Sunny Vale, CA, USA). Coefficients of variation were typically at or below 5 %. One unit was defined as the amount of enzyme, which hydrolyses 1 µmol of sucrose to d-glucose and d-fructose per minute.

Measurement of intracellular β-galactosidase activity

The mycelium from 10 ml of culture broth was harvested by filtration with Miracloth tissue, washed with 50 ml ice cold tap water and snap-frozen in liquid nitrogen. Mycelia were stored at −80 °C until analyzed. Cell extracts were prepared as principally described elsewhere [54] but using EDTA free cOmplete ULTRA Tablets (Roche, Dubai, UAE) instead of phenylmethylsulfonylfluorid and pepstatin A only. β-galactosidase activity towards 20 mM ortho-nitrophenyl-β-d-galactosid (ONPG) was assayed in 10 mM phosphate buffer (pH = 7.4) at 40 °C for 20 min. Each sample was assayed as a biological and technical triplicate. The nitrophenol content of the hydrolysis reaction was directly read out using a Spectra Max Plus 384 microplate reader and the activity calculated using Lambert–Beer’s law and a molar extinction coefficient of 18.5 ml mol⁻¹ cm⁻¹ for nitrophenol. The intracellular
protein content was assayed as described above. One unit was defined as the amount of enzyme, which hydrolyses 1 µmol of ONPG to β-D-galactose and nitrophenol per minute.

**Additional file**

**Additional file 1**: Table S1. T. reesei orthologues of -methylthione biosynthesis related proteins and genomic location of the corresponding gene; Table S2. Oligonucleotides used in this study; Figure S1. Metabolic pathway of sulphate assimilation and -methylthione biosynthesis; Figure S2. Gene copy number estimation of T. reesei sucA transformants by qPCR; Figure S3. Gene copy number estimation of T. reesei lacZ transformants by qPCR; Figure S4. Phyllogenetic analysis of TauD-like dioxygenases.

**Authors' contributions**

RS planned and carried out experiments, analysed and interpreted data and wrote the manuscript; JH carried out experiments, analysed and interpreted data; BM planned and carried out experiments, analysed and interpreted data; CG initiated the study, interpreted and discussed experimental data and was involved in the design of the study; BS and CPK initiated and designed the study, planned experiments, interpreted and discussed data and edited the manuscript. All authors read and approved the final manuscript.

**Author details**

1. Austrian Centre of Industrial Biotechnology (ACIB) GmbH c/o Institute of Chemical Engineering, Technische Universität Wien, Gumpendorferstraße 1a, 1080 Vienna, Austria. 2. Biotech and Renewables Center, Clariant GmbH, 1060 Vienna, Austria. 3. Kunamneni A, Plou FJ, Alcalde M, Ballesteros A (2014) Use of cellulases from Trichoderma reesei. Biotechnol Biofuels 6:127. doi:10.1186/1754-6834-6-127

**Acknowledgements**

This work has been supported by the Federal Ministry of Science, Research and Economy (BMWFV), the Federal Ministry of Traffic, Innovation and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standorta-Tirol, the Government of Lower Austria and ZIT—Technology Agency of the City of Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG. We also want to thank Claudia Koger, Alexander Beinhauer and Teresa Deckardt for their technical assistance.

**Compliance with ethical guidelines**

**Competing interests**

The authors declare that they have no competing interests.

**Received:** 18 June 2015  **Accepted:** 30 July 2015  **Published online:** 14 August 2015

**References**

1. Ferreira NL, Margeot A, Blanquet S, Berrin JG (2014) Use of cellulases from Trichoderma reesei in the twenty-first century part I: current industrial uses and future applications in the production of second ethanol generation. In: Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy MG (eds) Biotechnology and biology of Trichoderma. Elsevier, Oxford, pp 351–362

2. Bischof R, Seiboth B (2014) Molecular tools for strain improvement of Trichoderma spp. In: Gupta VK, Schmoll M, Herrera-Estrella A, Upadhyay RS, Druzhinina I, Tuohy MG (eds) Biotechnology and biology of Trichoderma. Elsevier, Oxford, pp 179–191

3. Dehli T, Sorensen JS, Jensen PR (2012) Tunable promoters in synthetic and systems biology. Subcell Biochem 64:181–201

4. Strauss J, Mach RL, Zeilinger S, Hartler G, Stoffler G, Wolschek M et al (1995) Cre1, the carbon catabolite repressor protein from Trichoderma reesei. FEBS Lett 376(1–2):103–107

5. Ilmén M, Thrane C, Penntilä M (1996) The glucose repressor gene cre1 of Trichoderma isolation and expression of a full length and a truncated mutant form. Mol Gen Genet 251(4):451–460

6. Ries L, Pullan ST, Delmas S, Malla S, Blythe MJ, Archer DB (2013) Genome-wide transcriptional response of Trichoderma reesei to lignocellulose using RNA sequencing and comparison with Aspergillus niger. BMC Genomics 14:541. doi:10.1186/1471-2164-14-541

7. Bischof R, Fourtis L, Limbeck A, Gamauf C, Seiboth B, Kückeck CP (2013) Comparative analysis of the Trichoderma reesei transcriptome during growth on the cellulase inducing substrates wheat straw and lactose. Biotechnol Biofuels 6:127. doi:10.1186/1754-6834-6-127

8. Shoji JI, Maruyama J, Arika M, Kitamoto K (2005) Development of Aspergillus oryzae thiaz promoter as a tool for molecular biological studies. FEBS Microbiol Lett 240(1):41–46

9. Cary JW, Bhatnagar D (1995) Nucleotide sequence of the transporter protein encoding gene of Trichoderma reesei. Biochim Biophys Acta 1289(2):169–176

10. Shoji JI, Maruyama J, Arika M, Kitamoto K (2005) Development of Aspergillus oryzae thiaz promoter as a tool for molecular biological studies. FEBS Microbiol Lett 240(1):41–46

11. Cary JW, Bhatnagar D (1995) Nucleotide sequence of the transporter protein encoding gene of Trichoderma reesei. Biochim Biophys Acta 1289(2):169–176

12. Bellemare DR, Sanschagrin M, Beaudoin J, Labbé S (2001) A novel copper-regulated promoter system for expression of heterologous proteins in Schizosaccharomyces pombe. Gene 273(2):191–198

13. Orly JJ, Griffith CL, Doering TL (2004) An efficiently regulated promoter system for Cryptococcus neoformans utilizing the CTR4 promoter. Yeast 21(11):919–926

14. Lv X, Zheng F, Li C, Zhang W, Chen G, Liu W (2015) Characterization of a copper responsive promoter and its mediated overexpression of the xylanase regulator 1 results in an induction-independent production of cellulases in Trichoderma reesei. Biotechnol Biofuels 8(1):67

15. Dunkler A, Wendland J (2007) Use of MET3 promoters for regulated gene expression in Asbyhia gossypii. Curr Genet 52(1):1–10

16. Care RS, Trehvert J, Birley KM, Sudbery PE (1999) The MET3 promoter: a new tool for Candida albicans molecular genetics. Mol Microbiol 34(4):792–798

17. Cherest H, Thao NN, Sudrin-Kerjan Y (1985) Transcriptional regulations of the MET3 gene of Saccharomyces cerevisiae. Gene 34(2–3):269–281

18. Delic M, Mattanovich D, Gasser B (2013) Repressible promoters—a novel tool to generate conditional mutants in Pichia pastoris. Microb Cell Fact. 12:6. doi:10.1186/1475-2859-12-6

19. Gremel G, Dorrer M, Schmoll M (2008) Sulphur metabolism and cellulase gene expression are controlled processes in the filamentous fungus Hypocrea jecorina (anamorph Trichoderma reesei). BMC Microbiol 8:174. doi:10.1186/1471-2180-8-174

20. Ikeda M (2003) Amino acid production processes. In: Themmel J, Faurie R (eds) Microbial production of l-amino-acids. Advances in biochemical engineering/biotechnology. Springer Verlag Berlin Heidelberg, Germany

21. Talebiania F, Karakashiev D, Angelidaki I (2010) Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation. Bioresour Technol 101(13):4744–4753

22. Marie-Nelly H, Marbouty M, Cournauc A, Flot J-F, Liti G, Parodi DP et al (2014) High-quality genome (re)assembly using chromosomal contact data. Nat Commun 5:5695. doi:10.1038/ncomms6695

23. Kent NA, Ebert SM, Mellor J (2004) CBF1p is required for chromatin remodeling at promoterproximal CACGTG motifs in yeast. J Biol Chem 279(26):2716–2713

24. Eck R, Stoyan T, Knkel W (2001) The centromere-binding factor CBF1 from Candida albicans complements the methionine auxotrophic phenotype of Saccharomyces cerevisiae. Yeast 18(11):1047–1052

25. Zhang J, Smith KM, Taekaberry T, Sun X, Carpenter P, Slusgski MD et al (2016) Characterization of the transport mechanism and permeant binding profile of the uridine permease Fup1p of Saccharomyces cerevisiae. J. Biol Chem 281(38):28210–28221
expression of the tauABC2D operon and other sulfur starvation-inducible genes in \textit{Escherichia coli}. J Bacteriol 179(24):7651–7678.

42. Van der Ploue JR, Weiss MA, Saller E, Nashimoto H, Saito N, Kertesz MA et al (1996) Identification of sulfate starvation-regulated genes in \textit{Escherichia coli}: a gene cluster involved in the utilization of taurine as a sulfur source. J Bacteriol 178(18):5435–5446.

43. Bykowski T, Van der Ploue JR, Iwanicka-Nowicka R, Hryniewicz MM (2002) The switch from inorganic to organic sulfur assimilation in \textit{Escherichia coli}: adenosine 5′-phosphosulfate (APS) as a signalling molecule for sulphate excess. Mol Microbiol 43(5):1347–1358.

44. Stec E, Wikowska-Zimny M, Hryniewicz MM, Neumann P, Wilkinson AJ, Brzozowski AM et al (2006) Structural basis of the sulphate starvation response in \textit{E. coli}: crystal structure and mutational analysis of the cofactor-binding domain of the Cbl transcriptional regulator. J Mol Biol 364(3):309–322.

45. Oldfield C, Pogrebinsky O, Simmonds J, Olsson ES, Kulpa CF (1997) Elucidation of the metabolic pathway for dibenzothiophene desulphurization by \textit{Rhodococcus sp. strain IGT58}. (ATCC 53968). Microbiology 143(9):2961–2973.

46. Kiblkane JJ 2nd, Jackowski K (1992) Biodesulfurization of water-soluble coal-derived material by \textit{Rhodococcus rhodochrous} IGT58. Biotechnol Bioeng 40(9):1107–1114.

47. Thomas D, Kuras L, Barbery R, Cherest H, Blaiseau PL, Surdin-Kerjan Y (1995) Met30p, a yeast transcriptional inhibitor that responds to S-adenosylmethionine, is an essential protein with WD40 repeats. Mol Cell Biol 15(12):6526–6534.

48. Natorff R, Balinski M, Palszewski A (1993) At least four regulatory genes control sulphur metabiltione repression in \textit{Aspergillus nidulans}. MGG 238(1–2):185–192.

49. Fischer JA, McCarrn MP, Snetselaar KM (2001) Methylation is involved in the \textit{Ustilago maydis} mating response. Fungal Genet Biol 34(1):21–31.

50. Pfaffli MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30(9):e36.

51. Hartl L, Kubicek CP, Seiboth B (2007) Induction of the gal pathway and cellulase genes involves no transcriptional inducer function of the galactose pathway and \textit{tauABCD} expression of the \textit{tauABCD} operon. Cellulose 14(1):23–31.

52. Gruber F, Visser J, Kubicek CP, De Graaff LH (1990) The development of a heterologous transformation system for the cellulolytic fungus \textit{Trichoderma reesei} based on a \textit{pyrG} vector. Mol Cell Biol 10(1):1805–1813.

53. Tisch D, Kubicek CP, Schmoll M (2011) The phoducin-like protein PhlP1 impacts regulation of glycoside hydrolases and light response in \textit{Trichoderma reesei}. BMC Genomics 12:613. doi:10.1186/1471-2164-12-613.

54. Mustalahti E, Saloheimo M, Joensuu JJ (2011) Intracellular protein production in \textit{Trichoderma reesei} (\textit{Hypocrea jecorina}) with hydrophobin fusion technology. New Biotechnol 30(2):262–268.