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DIPLOMARBEIT

The impact of the unfolded protein response on enzyme secretion in *Trichoderma reesei*

Der Einfluss der ungefalteten Proteinantwort auf Enzymsekretion in *Trichoderma reesei*

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Contents

1 Abstract

Understanding and optimizing the protein secretion in the industrial workhorse *Trichoderma reesei* is of major interest. In the filamentous fungus, the secretion of heterologous proteins or high production rates of native enzymes in the strain RUT-C30 were observed to induce the 'unfolded protein response' (UPR). The UPR is triggered by an accumulation of un- or misfolded proteins in the endoplasmic reticulum (ER) when the protein processing capacity of the ER is surpassed. To reduce the ER stress, the UPR up-regulates the secretion machinery, but the expression of several secreted enzymes is repressed as well. More research is required regarding this so-called 'repression under secretion stress' (RESS) and the UPR in *T. reesei*.

Here, the UPR and RESS were investigated on the transcriptional level by reverse transcription quantitative PCR (RT-qPCR). Transcript levels of genes encoding (hemi-)cellulases, UPR indicators and (hemi-)cellulase regulators were monitored in a wild-type strain and two Xyr1 overexpression strains. One overexpression strain produces the wild-type Xyr1 and the other a modified Xyr1 version. The enhanced enzyme secretion in these strains was speculated to induce the UPR. However, the present thesis suggests that the elevated enzyme secretion does not activate the UPR. This was indicated by similarly low mRNA levels of the UPR indicators (*hac1, bip1)* in the wild-type strain and the Xyr1 overexpression strains. To reveal the mediators and regulatory mechanisms of RESS, we investigated the involvement of the (hemi-)cellulase activators Xyr1, Rxe1 and Ace3 in an indirect induction of RESS. To this end, ER stress was induced by the addition of dithiothreitol (DTT). The transcription factor Ace3 seems to be neither a RESS-target nor -mediator, as no significant impact of ER stress on *ace3* transcription was observed. The involvement of Rxe1 in RESS as a target or mediator remains unclear due to inconsistent *rxe1* transcript levels in the three strains investigated. Xyr1 appears also not to be indirectly mediating RESS: the transcription of (hemi-)cellulase encoding genes was down-regulated during UPR even when the (hemi-)cellulase activator Xyr1 was overexpressed. Whether the *xyr1* gene is a UPR- or RESS-target might depend on the carbon source, as a clear decline of *xyr1* mRNA levels under ER stress was solely observed on lactose.

Different mechanisms for the down-regulation of secreted enzymes depending on the cultivation condition might exist in *T. reesei.* On the one hand, transcript levels of hemicellulose- and cellulose-degrading enzymes showed a different temporal decrease during ER stress. On the other hand, the absolute change of transcript levels of the genes encoding UPR indicators and transcriptions factors was different for xylan- and lactose-grown samples.

In addition, this thesis suggests a mutual regulatory influence between Xyr1 and Rxe1 (regulator 1 of Xyr1 expression), because transcript levels of *rxe1* were enhanced by factor of 10 by the overexpression of wild-type Xyr1.

2 Scientific Background

2.1 *Trichoderma reesei* - an industrial workhorse for (hemi-)cellulase production

The filamentous ascomycete *Trichoderma reesei* (*Hypocrea jecorina* (Kuhls et al. 1996)) has served as one of the most important industrial fungi for enzyme production owing to its outstanding secretory capacities of up to over 100 g/L (Cherry and Fidantsef 2003; Bischof et al. 2016). Native enzymes, namely cellulases and hemicellulases, are the main industrial products of *T. reesei* (Bischof et al. 2016). These enzymes are applied in many industrial sectors including feed, pulp, paper, detergent, textile and food industries (Kuhad et al. 2011). For instance, cellulases are used for stonewashing of jeans or clarification of fruit juices (Kuhad et al. 2011). However, the far most intriguing utilization of cellulolytic enzymes in recent years has been the sustainable production of biofuel and other chemicals by the conversion of plant biomass (Kumar et al. 2008). *T. reesei* has been harnessed for recombinant protein production, even if yields do not reach levels of native enzymes (Saloheimo and Pakula 2012). Furthermore, *T. reesei* has been recognized as a model organism of fungal cellulose and hemicellulose degradation (Saloheimo and Pakula 2012).

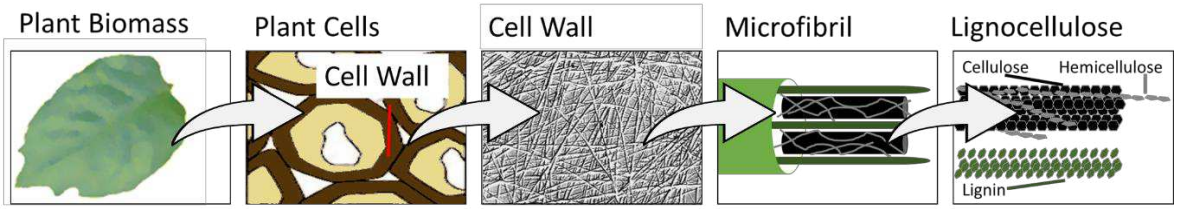

Figure 1: Localization of lignocellulose in plant biomass.

In nature, *T. reesei* thrives on dead plant mass using cellulases and hemicellulases to degrade polysaccharides present in lignocellulosic microfibrils of plant cell walls (**Figure 1**) (Nogueira et al. 2018). Lignocellulose is the most abundant renewable resource on Earth and consists of cellulose (40–50%), hemicellulose (20–40%) and lignin (20–30%) (Kobayashi et al. 2012; Nogueira et al. 2018). Cellulose is a polysaccharide composed of D-glucose linked by ß-1,4-glycosdic bonds. Inter- and intramolecular hydrogen bonds between glucose chains result in crystalline fibres that show high chemical and mechanical stability as well as water-insolubility (Kobayashi et al. 2012; Sehaqui et al. 2011). Hemicellulose is a collective term for polysaccharides present in plant cell walls that are formed by pentoses (xylan, arabinose) and to a smaller extent by hexoses (e.g. mannose). Within side chains, various saccharide derivates can be found (Saloheimo and Pakula 2012). Hemicelluloses are less homogenous than cellulose i.e. components vary between plants and even between their parts. Several forms of glycosidic bonds result in an amorphous, branched structure. In contrast to (hemi-)cellulose, lignin is a polymer based on

aromatic monomers, e.g. p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Kobayashi et al. 2012).

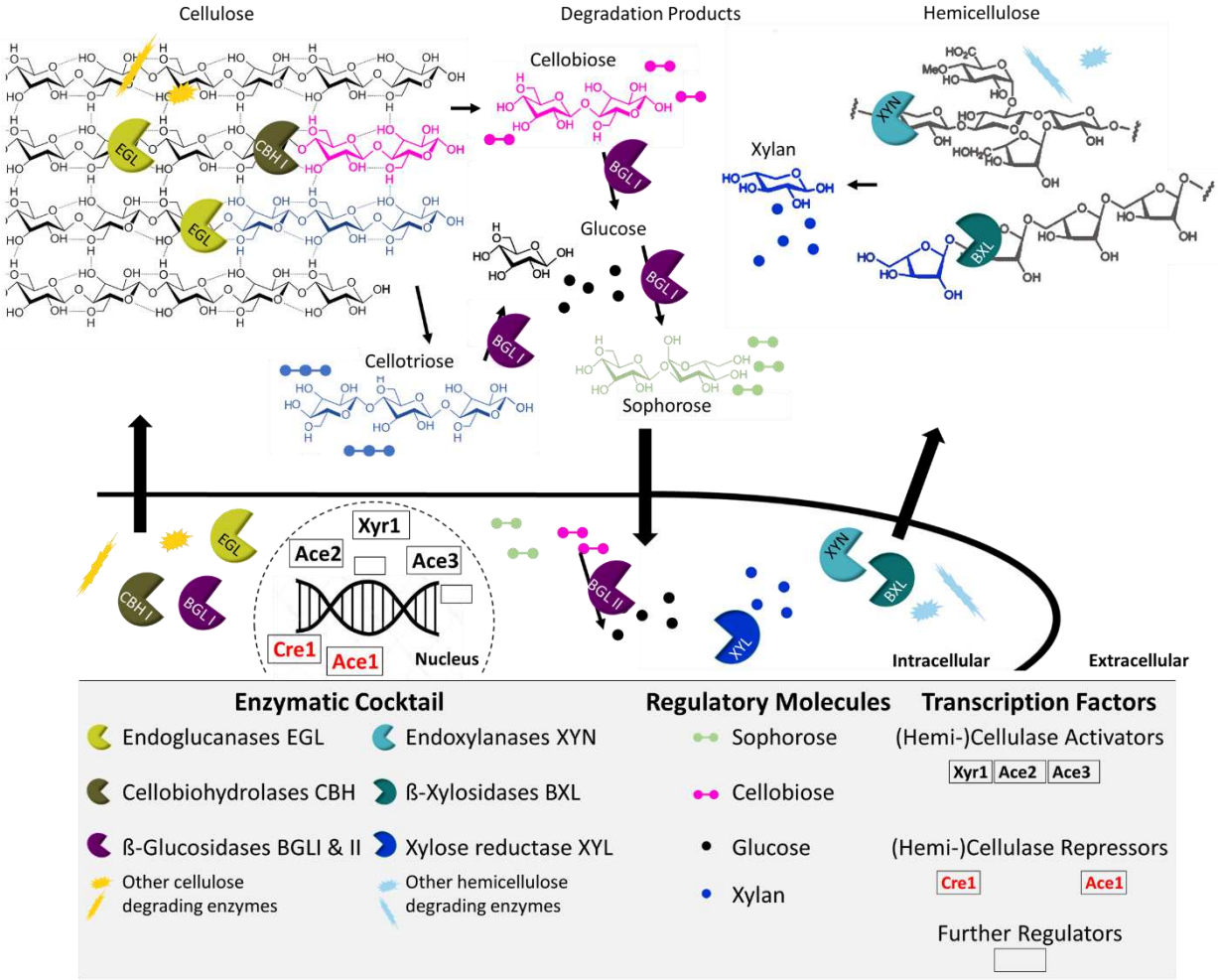

Figure 2: Scheme of the enzymatic degradation of cellulose and hemicellulose by T. reesei. The figure shows key molecules, enzymes, and regulators of (hemi-)cellulase expression. Enzymes required for cellulose break-down are endoglucanases (EGL), cellobiohydrolases (CBH), and β-glucosidases (BGLI, BGLII) next to other accessory enzymes. Enzymes required for degrading hemicelluloses are endoxylanases (XYN), ß-xylosidases (BXL), and a xylose reductase (XYL) in cooperation with further enzymes.

T. reesei secretes a cocktail of enzymes that act synergistically to degrade the insoluble polysaccharides present in lignocellulose. The degradation products consist of soluble sugars, i.e. small oligosaccharides and monosaccharides. These sugars are sufficiently small to be taken up inside fungal cells where they serve as a carbon energy source (Nogueira et al. 2018). **Figure 2** shows this process schematically including chemical structures and enzymes most important to this thesis. Depending on the substrate to be depolymerized, lignocellulolytic enzymes are classified as lignin-modifying enzymes, cellulases and hemicellulases - degrading lignin, cellulose and hemicelluloses respectively. The most prominent cellulases in *T. reesei* are the cellobiohydrolases (EC 3.2.1.91) CBHI and CBHII which mainly cleave off the dimer cellobiose

from the ends of the polysaccharide chain. The cellulase endoglucanase EGLI (EC 3.2.1.4) hydrolyses glycosidic bonds in the middle of the chain. This generates new chain ends, which can be subsequently attacked by cellobiohydrolases. Another cellulose category are ß-glucosidases (EC 3.2.1.21) which convert cellobiose and other small oligosaccharides (e.g. cellotriose) to glucose extracellularly (BGLI) or intracellularly (BGLII). In addition, ß-glucosidases exhibit a transglycosylation activity connecting two glucose molecules to sophorose (Xia et al. 2018). The main hemicellulases are the two endo-ß-1,4-xylanases XYNI and XYNII (EC 3.2.1.8) and ß-xylosidase BXLI (EC 3.2.1.37). XYNI and XYNII cleave the xylan backbone in the middle and generate new chain ends. BXLI depolymerizes chain ends and releases D-xylose. For introducing D-xylose into the catabolic pathways, D-xylose is reduced to D-xylitol by the xylose reductase Xyl1 (EC 1.1.1.307). Hemicellulases comprise also other backbone cleaving enzymes, e.g. endomannases cleaving mannan chains. Further hemicellulases are side chain- and substitution-cleaving enzymes including arabinofuranosidases, α-glucuronidases, α-galactosidases and acetyl xylan esterases. Next to cellulases and hemicellulases, which are hydrolytic enzymes, accessory non-hydrolytic enzymes are involved in breaking down polysaccharides, e.g. swollenins and lytic polysaccharide monooxygenases (EC 1.14.99.56) (Nogueira et al. 2018).

2.2 Protein expression and secretion in *T. reesei*

The expression of (hemi-)cellulose-degrading enzymes in *Trichoderma reesei* is regulated by complex mechanisms adapting the enzymatic pedigree to the available carbon source. This means, the energy-consuming production of high levels of extracellular enzymes only takes place when the fungus requires polysaccharides as an energy or carbon source (Aro et al. 2005). The regulation of the production of biomass-degrading enzymes relies on a positive feedback mechanism: degradation products, i.e. soluble oligo- and monosaccharides, regulate the enzymes required for degrading corresponding polysaccharidic substrates. For example, xylan-depolymerizing enzymes are induced by D-xylose and xylobiose, the degradation products of xylan. Xylanases can be induced by sophorose as well (Mach-Aigner et al. 2010). Cellobiose and sophorose, products of cellulose degradation, promote expression of cellulose-degrading enzymes (**Figure 2**) (Verbeke et al. 2009; Ilmén et al. 1997). In addition, lactose, a saccharide absent in the natural habitat of *T. reesei,* was found to induce cellulase and xylanase production. Lactose is a disaccharide of ß-1,4-glycosidic bonded D-galactose and D-glucose. Lactose presumably possesses inducing properties as it resembles the ß-galactoside side chains of xyloglucans (Ivanova et al. 2013). Even though cellobiose, sophorose, and lactose all act as inducer of cellulase expression, they have varying induction effects, i.e. sophorose is the strongest inducer (Verbeke et al. 2009). When large amounts of absorbable and easily metabolizable

monosaccharides are present, e.g. due to proceeded lignocellulose degradation, lignocellulolytic enzymes are not needed anymore. Thus, glucose and high concentration of D-xylose, for example, repress (hemi-)cellulase expression as part of the carbon catabolite repression (Antoniêto et al. 2014; Mach-Aigner et al. 2010).

The genes of the main (hemi-)cellulose-degrading enzymes are regulated in a coordinate manner in *T. reesei.* This makes sense considering that lignocellulosic polysaccharides are degraded synergistically by different enzymes (Novy et al. 2019). The main activator of cellulase and hemicellulase expression is Xyr1 (xylanase regulator 1), a Gal4-like transcription factor. Xyr1 influences the expression of nearly all plant cell wall-degrading enzymes (Stricker et al. 2006) and of the aldose reductase Xyl1 (Stricker et al. 2007). The expression of Xyr1 itself is up-regulated under cellulase inducing conditions (e.g. sophorose, lactose) and repressed under conditions that cause carbon catabolite repression (glucose, high D-xylose-concentrations) (Mach-Aigner et al. 2008; Portnoy et al. 2011; Derntl et al. 2013). Deletion of Xyr1 caused a complete abolishment of the expression of most lignocellulolytic enzymes and affected the catabolism of lactose and different hemicellulose monomers, including D-xylose and L-arabinose (Stricker et al. 2006; Akel et al. 2009; Seiboth et al. 2007). In contrast, overexpression of Xyr1 increased cellulase and xylanase transcript levels depending on the carbon source (Derntl et al. 2019). Besides Xyr1, there are some other activating transcription factors, e.g. Ace2 (Aro et al. 2001) and Ace3 (Häkkinen et al. 2014).

The main repressor of cellulases and hemicellulases is Cre1, a Cys2His2 zinc finger protein and mediator of the carbon catabolite repression (Ilmén et al. 1996; Strauss et al. 1995). Cre1 affects (hemi-)cellulase expression either indirectly, by down-regulating the expression of proteins required for the uptake of inducers into the cell, or directly, by binding to cellulase and xylanase genes (Antoniêto et al. 2014). Cellulase and hemicellulase encoding genes can also be down-regulated by further transcription factors (Novy et al. 2019), e.g. Ace1.

Proteins to be secreted into the cell exterior for biomass degradation are trafficked through the endomembrane system, what is known as the secretory pathway (**Figure 3**) (Conesa et al. 2001; Saloheimo and Pakula 2012). A signal sequence at the N-terminus of proteins introduces proteins to the secretory pathway by directing the polypeptide chains into the endoplasmic reticulum (ER), either co- or post-translationally. In the ER, chaperones assist to fold proteins into their final conformation by binding to hydrophobic regions of the protein and preventing unfavorable interactions between these regions. The most important chaperone is BiP1 (binding protein 1) belonging to the heat shock protein (HSP70) family. Furthermore, the formation of disulphide bridges within the polypeptide chain is catalyzed by foldases in the ER, e.g. by protein disulphide isomerase (Pdi1). Besides folding, proteins can be matured by glycosylation, phosphorylation or

subunit assembly in the ER. Proteins to be secreted are then transferred via vesicle transport from the ER to the Golgi apparatus and later from the Golgi apparatus to the plasma membrane. These vesicles reach their destination by the guidance of a special protein coat and assistance of the cytoskeleton. Eventually, vesicles dock and fuse with the target membrane to release their content into the Golgi matrix or the extracellular space (Saloheimo and Pakula 2012; Conesa et al. 2001). Next to the conventional secretory pathway, unconventional pathways for protein secretion in fungi were found. Unconventional pathways involve extracellular vesicle secretion (Oliveira et al. 2013), are independent of the Golgi apparatus (Giraldo et al. 2013) or independent of both the ER and Golgi apparatus (Stock et al. 2012). This topic has yet to be studied in *T. reesei*.

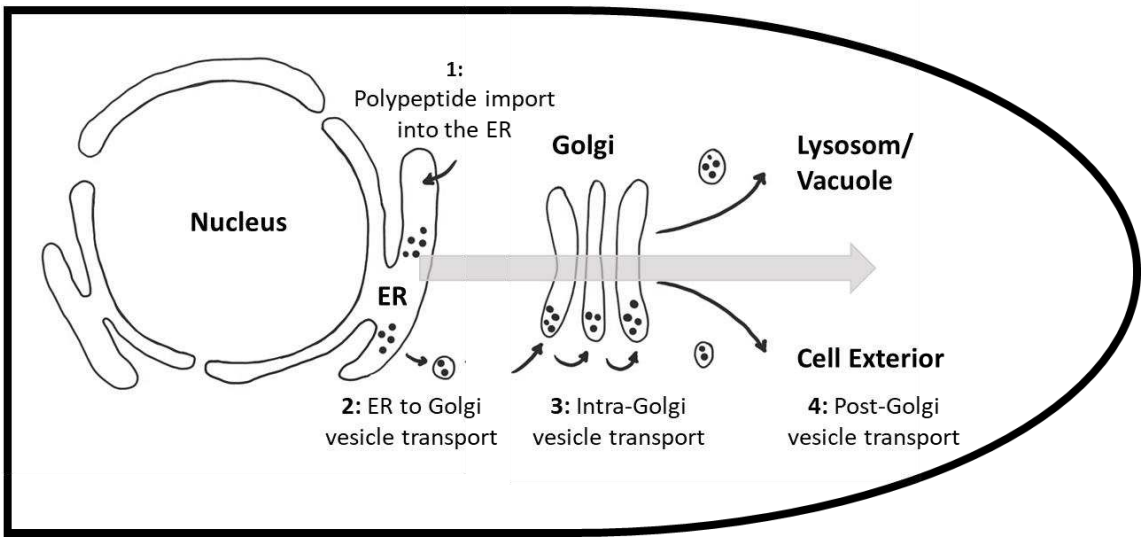

Figure 3: Steps of the secretory pathway.

Protein secretion mainly occurs at growing hyphal tips but can also happen in other fungal parts (Shoji et al. 2014). It has been observed that a foreign protein, barley cysteine endopeptidase, was localized in apical compartments of young hyphae whereas the native cellulase CBHI was secreted all over the mycelium (Nykänen et al. 2002; Nykänen et al. 1997). Thus, there might be spatial restrictions in secretion of foreign proteins whereas native proteins are secreted from all fungal parts.

2.3 Unfolded protein response (UPR)

Eukaryotes are able to sense an accumulation of un- or misfolded proteins in the endoplasmic reticulum (ER) that may arise due to surpassing the protein processing capacity of the ER. To alleviate this stress situation in the ER and to adapt to increased demands on the secretory pathway, a conserved signal pathway called the unfolded protein response (UPR) is induced (Kozutsumi et al. 1988; Kohno et al. 1993; Mori et al. 1993). The UPR has been intensively studied

in eukaryotes like mammalians (Chakrabarti et al. 2011), plants (Angelos et al. 2017) or yeast (Hernández-Elvira et al. 2018), and to some extent in filamentous fungi including *T. reesei*.

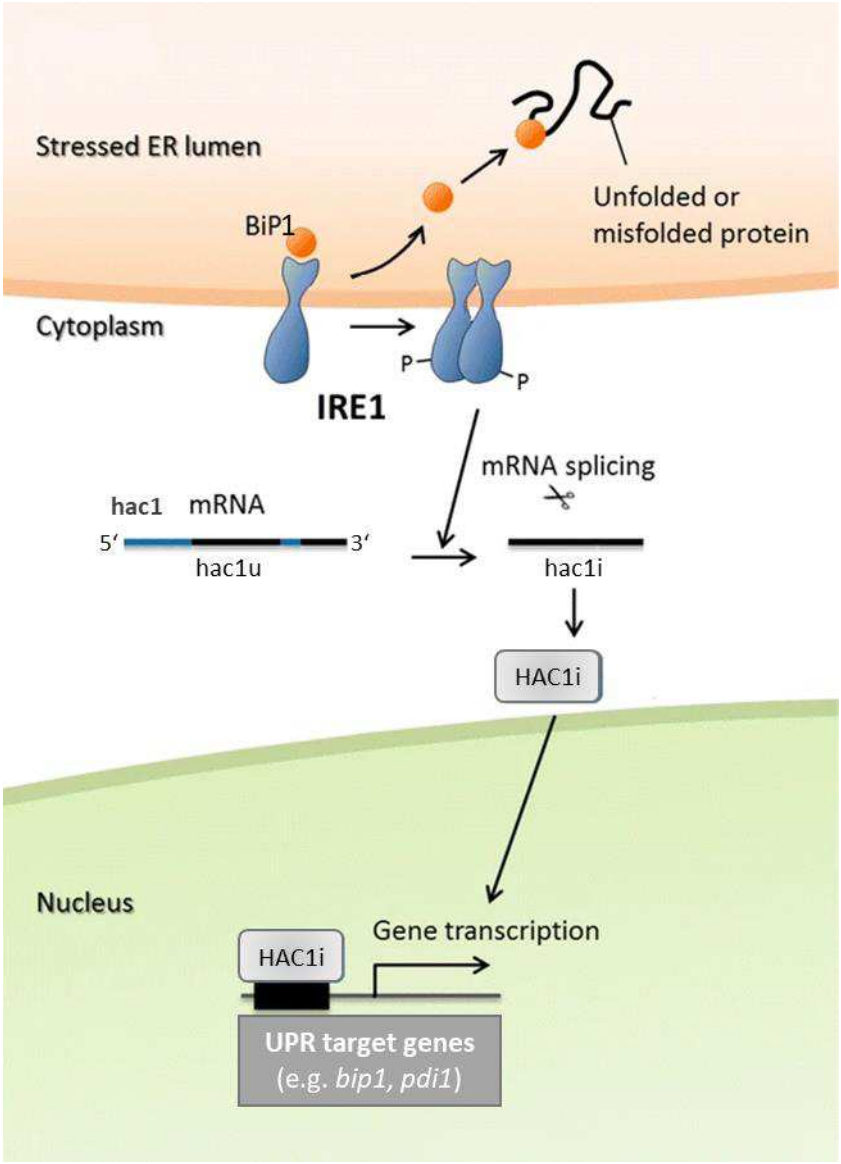

Figure 4: The unfolded protein response (UPR) in T. reesei. An accumulation of un- or misfolded proteins in the ER lumen hampers BiP1 to bind to the transmembrane protein Ire1. This results in a homo-dimerisation and autophosphorylation of Ire1. The mRNA encoding the transcription factor Hac1 is then spliced by the activated Ire1 and translated. The Hac1i protein enters the nucleus where it regulates the transcription of UPR target genes.

Figure 4 present the UPR schematically. The key regulatory elements in UPR in *T. reesei* are the transmembrane protein kinase Ire1 (inositol requiring enzyme 1) (Valkonen et al. 2004) and the basic leucine zipper transcription factor Hac1 (Saloheimo et al. 2003). Ire1 is the most upstream element of the UPR. Its N-terminal domain, facing into the ER lumen, binds to the chaperone BiP1 (binding protein 1). In case of ER stress, BiP1 binds to unfolded proteins instead of Ire1. This results in homo-dimerization of Ire1 and an activation of a cytoplasmic C-terminal RNase domain of Ire1 by trans-autophosphorylation at the C-terminus (Valkonen et al. 2004; Cox et al. 1993).

The uninduced mRNA *hac1u* of the transcription factor Hac1 is then activated post-transcriptionally in a non-conventional splicing reaction by Ire1: in *T. reesei*, a short intron (20 nt) and a long part (200 nt) at the 5'-end of the mRNA are spliced yielding induced *hac1i*. The removal of the intron changes the reading frame of the mRNA, whereas the truncation at the 5'-end leads to a more efficient translation (Saloheimo et al. 2003). After translation of the mature *hac1i* mRNA, the Hac1 protein enters the nucleus where it binds to a consensus region in the promoters of UPR target genes to (up-)regulate their transcription (Mulder et al. 2004). Genes regulated by Hac1 in filamentous fungi serve to re-establish ER homeostasis by increasing the protein folding capacity, expanding the ER surface, restructuring of cellular transport processes like ER to Golgi trafficking, as well as by degrading irreversibly misfolded proteins via the endoplasmic reticulum associated degradation (ERAD) pathway (Heimel 2015; Ng et al. 2000; Travers et al. 2000). Hence, predominant UPR target genes found in filamentous fungi are ER-resident chaperones and foldases (e.g. *bip1*, *pdi1* in *T. reesei* (Arvas et al. 2006)), genes involved in phospholipid metabolism, translocation, protein glycosylation, cell wall biosynthesis, vesicular transport and protein degradation (Fan et al. 2015; Zhou et al. 2016).

Several factors were found to trigger the UPR. For instance, the expression of heterologous proteins can cause un- or misfolded proteins and therefore the ER stress response (Punt et al. 1998; Heimel 2015). But high level of endogenous secretory proteins were found to evoke the UPR as well: for example, transcript levels of *bip1*, *pdi1* (both UPR targets) and *hac1* (UPR mediator) were elevated at high cellulase synthesis rates in the *T. reesei* mutant Rut-C30, the hyper-producer of cellulolytic enzymes (Pakula et al. 2005). The UPR can also be induced by chemicals including dithiothreitol (DTT), a reducing agent preventing the formation of disulphide bonds in the ER. Hence, DTT causes unfolded proteins (Pakula et al. 2003). Another chemical, brefeldin A, impairs the ER to Golgi apparatus transport or even disrupts the Golgi apparatus (Vogel et al. 1993). Tunicamycin activates the UPR by inhibiting N-linked glycosylation of proteins (Koizumi et al. 1999; Speake et al. 1981). The Ca^{2+}-ionophor A23187 is a chemical that was shown to hinder protein folding and to provoke the UPR in mammalians by disturbing the intracellular Ca^{2+}-homeostasis (Wileman et al. 1991). In contrast to the factors mentioned so far, the UPR can also be activated without an accumulation of misfolded proteins by a constitutive expression of UPR mediators like Hac1 or Ire1 (Valkonen et al. 2004; Valkonen et al. 2003).

2.4 Repression under secretion stress (RESS)

Another pathway to re-establish ER homeostasis upon high loads of unfolded proteins is a pathway termed repression under secretion stress (RESS). It exists in filamentous fungi (Pakula et al. 2003; Al-Sheikh et al. 2004; Sims et al. 2005) and plants (Martínez and Chrispeels 2003), but

not in the yeast *Saccharomyces cerevisiae*. The RESS pathway describes a down-regulation of several genes encoding secreted proteins in case of secretion stress. Thus, RESS aims at decreasing the workload of the ER and the secretory pathway. This approach contrasts other ER stress responses which aim at increasing the ER and secretion capacity as well as at removing misfolded proteins from the ER (Heimel 2015). In *T. reesei*, RESS has been shown to influence the expression of the secreted (hemi-)cellulases CBHII, EGLI, EGLII, XYNI and the hydrophobin Hfb2 (Pakula et al. 2003). It could be further demonstrated that RESS is a specific down-regulation, as not all (secreted) proteins are affected (Al-Sheikh et al. 2004; Sims et al. 2005; Guillemette et al. 2007; Carvalho et al. 2012; Pakula et al. 2003). Consequently, RESS provides sufficient resources for protein folding and secretion of those proteins that indispensable to the cells (Heimel 2015). Promotors of genes not regulated by RESS render RESS an intriguing tool in biotechnological approaches to increase secreted protein yields. So far, cis-acting elements involved in RESS have been identified e.g. in an *Aspergillus niger* gene (glaA) (Al-Sheikh et al. 2004). In *T. reesei*, RESS specific promoter sequences are still unknown.

RESS seems to be part of the UPR in *T. reesei* as UPR inducing agents (DTT, BFA and A23187) also induced RESS (Pakula et al. 2003). However, it is not known neither at what hierarchical level of the UPR signal transduction pathway in *T. reesei* RESS is triggered, nor what mediators are involved. So far, it has only been found that, in *T. reesei*, RESS is not mediated by the carbon catabolite repressor Cre1 (Pakula et al. 2003). HacA, an analogue of Hac1, has been suggested as mediator of RESS in *Aspergillus niger*. In the corresponding study, genes encoding for starch-degrading enzymes and a gene encoding an activating transcription factor (AmyR) of starch-degrading enzymes were found to be down-regulated upon expression of the constitutively active *hacA*-form (Carvalho et al. 2012). On the contrary, transcriptome profiling of an *hac1*-deletion strain of *Neurospora crassa* implied that the UPR pathway does not directly regulate the expression of cellulases and that RESS is independent of the UPR: In the Δ*hac1*-strain, expression of cellulase genes was induced to similar levels compared with its parental strain without ER stress, but was down-regulated under acute ER stress (Fan et al. 2015). Due to this study and the fact that RESS acted very quickly upon UPR induction in an earlier study in *T. reesei* (Pakula et al. 2003), RESS might also be triggered independently of the UPR mediator Hac1 in *T. reesei*. If the down-regulation of secreted enzymes were induced independently of Hac1, it could rely on a direct cleavage of mRNA of secreted enzymes by Ire1. Ire1 is the enzyme that also activates the UPR by *hac1*-cleavage (see above). This post-transcriptional strategy aims at minimizing protein synthesis until the ER is cleared of un- or misfolded proteins and is termed regulated Ire1-dependent decay (RIDD) (Wu et al. 2014). RIDD occurs in several organisms including mammals, fission yeast and plants (Mishiba et al. 2013; Hollien et al. 2009; Kimmig et al. 2012). However, it is not known whether RESS acts instead of RIDD or additionally to RIDD in *T. reesei*. Analogous to

a second signal transduction cascade for the UPR in mammals, RESS could be induced even further upstream by a transmembrane protein kinase. The mammalian protein kinase-like ER kinase (PERK) oligomerizes under ER stress and phosphorylates itself and eIF2α (eukaryotic initiation factor 2 α). This leads to a general attenuation of protein synthesis. PERK additionally activates the transcription factor ATF4 which induces UPR target genes (Wu et al. 2014). RESS could also be activated similarly to a third signal transduction mechanism in mammals: upon ER stress, a transmembrane transcription factor (ATF6) coming from the ER is first cleaved in the Golgi apparatus before a cytosolic fragment of ATF6 enters the nucleus to induce downstream UPR targets (Wu et al. 2014).

3 Motivation and Objectives

Studying the UPR has great potential to provide means for the optimization of industrial enzyme production. The activation of the UPR enhances the capacity of the secretory pathway. Elevated secretion capacities could help overcome the bottleneck of a limited protein folding capacity in the production of heterologous and native secreted proteins (Heimel 2015; Gouka et al. 1997). However, the repression of several secreted enzymes is provoked by UPR-inducing conditions as well (RESS, see above section 2.4). A better understanding of RESS is required to avoid this negative side-effect for industrial applications.

Filamentous fungi appear to activate the UPR when expressing certain heterologous proteins, but also naturally at high enzyme production levels, as mentioned above in section 2.3. Therefore, we wondered, if overexpression of the main (hemi-)cellulase activator Xyr1 causes the UPR due to enhanced enzyme secretion. To answer this question, mRNA levels of genes encoding UPR indicators and (hemi-)cellulases were analysed in a wild-type like strain, *Δtmus53,* and two Xyr1 overexpression strains, TX(WT) and TXY(1). TX(WT) overexpresses the native Xyr1, whereas TXY(1) overexpresses a modified Xyr1 version.

Another question of this thesis is how RESS is mediated during the UPR. More specifically: Are the (hemi-)cellulase activating transcription factors, Xyr1, Ace3 or Rxe1 RESS targets? If so, do Xyr1, Ace3 and Rxe1 indirectly mediate RESS? The corresponding experimental set-up included a chemical UPR induction and a time-dependent transcription analysis. Transcription of genes encoding (hemi-)cellulases, UPR indicators and the transcription factors was analysed in the strain *Δtmus53* and the two Xyr1 overexpression strains TX(WT) and TXY(1).

The method chosen for transcript analysis was reverse transcription quantitative polymerase chain reaction (RT-qPCR) preceded by RNA extraction and cDNA synthesis.

4 Results and Discussion

The following results and discussion part divides into four sections: In the first section, important factors, that influenced qPCR results, i.e. relative transcript levels, are discussed. In the second section, the inducibility of the UPR by the reducing agent DTT is presented for all three strains used. The third part elucidates the influence of Xyr1 overexpression on transcription of (hemi-)cellulase-encoding genes and on ER stress. The fourth part elaborates the involvement of the transcription factors Xyr1, Ace3 and Rxe1 in the UPR and RESS.

4.1 Important Factors influencing Relative Transcript Levels

A few particularly important factors were encountered when calculating the relative transcript levels of genes of interest. The method used was reverse transcription quantitative polymerase chain reaction (RT-qPCR). This method required liquid cultivation, sampling, RNA extraction, cDNA synthesis and the actual qPCR measurement. RNA amounts are calculated based on the threshold cycle (C_t) of the qPCR and the amplification efficiency in a qPCR cycle. The C_t-value negatively correlates to the number of cDNA copies of a gene. The amplification efficiency equals 2.0 for a theoretical duplication of cDNA copies per cycle. In practice, E differs from 2.0. To obtain the relative transcript level, the cDNA amount of a target gene is normalized by a reference gene present in the same sample. Moreover, the normalized cDNA amount is related to the normalized amount of target cDNA in a reference sample (**Formula 1**, section 6.2). Regarding these steps and factors mentioned, the biggest influence in the present experiments was exerted by:

- **The reference gene**
- **The qPCR efficiency of amplification**
- **The cDNA synthesis/ RNA concentration**

Reference gene. One of the two reference genes, *sar1,* was found to be unsuitable for the experimental conditions of interest. The cDNA amounts of the reference genes *sar1* and *act1* should account for the total cDNA amount analysed to enable inter-sample comparison of target genes. *Sar1* is a gene encoding a small GTPase that is involved in vesicle budding from the ER, i.e. Sar1 is involved in the secretion pathway. *Act1* encodes for actin which plays roles in exocytosis, endocytosis, organelle movement and cytokinesis in fungi (Berepiki et al. 2011). Both genes have served as suitable, i.e. stably expressed, reference genes for transcription analysis in *T. reesei,* e.g. (Derntl et al. 2019; Steiger et al. 2010). However, it must be ensured that transcription is indeed stable under chosen experimental conditions or in different growth phases. Even though *sar1* has been reported to be a very stable reference gene (Steiger et al. 2010), it can be up-regulated under

UPR induction (Saloheimo et al. 2004). The latter has been overlooked when performing the experiments. This meant that the RNA levels in DTT-related experiments were normalized by only one reference gene, *act1*. The effect of the *sar1* up-regulation is clearly visible in exemplary **Figure 5**: *sar1*-normalized transcript levels of *hac1* seemed to increase less after DTT addition than *act1*-normalized data. For lactose data, the difference between the DTT-treated and the control sample at 3 h after DTT addition became even so small, that this difference would not have been considered as significant anymore (**Figure 5A**). Hence, *sar1*-normalized transcript levels could have led to a misinterpretation.

The normalization with both reference genes, *act1* and *sar1*, was still helpful to recognize data variation based on the normalization step. An alternative reference gene for future research would be *gpd1* (glycerol-3-phosphate dehydrogenase gene, (Pakula et al. 2003; Valkonen et al. 2004; Hong et al. 2014).

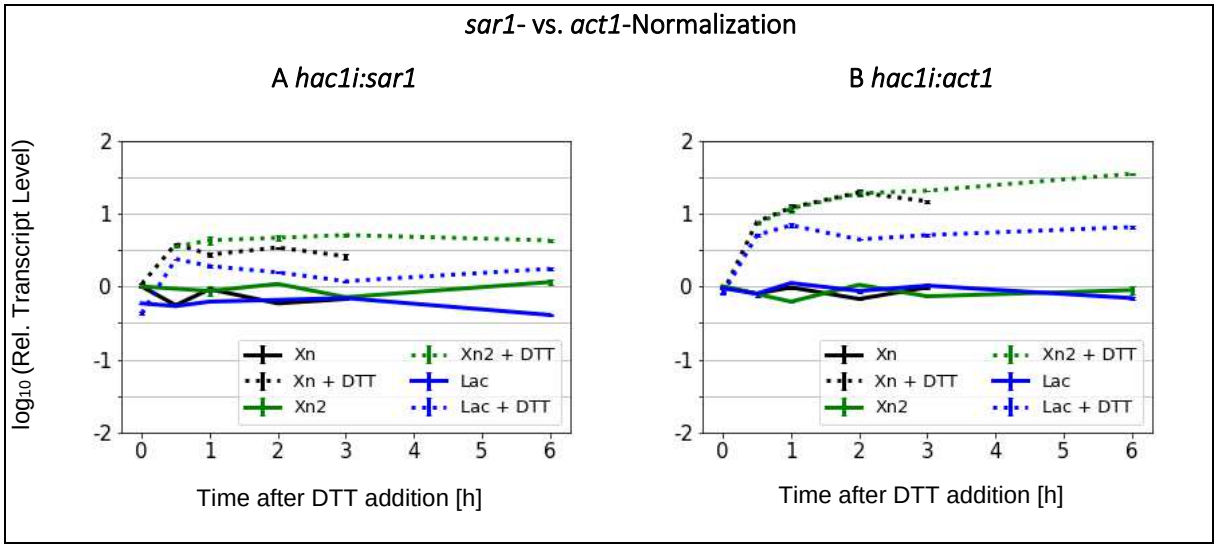

Figure 5: Influence of the reference gene on logarithmic relative transcript levels - exemplarily shown for hac1i in Δtmus53. A: Data normalized by sar1, B: Data normalized by act1. qPCR efficiencies were set to 1.76. Δtmus53 was grown on lactose (Lac) for 36.5h, and on xylan (Xn, Xn2) for 24 h in two independent experiments. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. The sample "Δtmus53 – Xn – 0 h" served as reference sample. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. Vertical bars indicate the difference between two technical replicates.

Although the expression of *act1* has been found to depend on the carbon source in yeast (*Saccharomyces cerevisiae* (Teste et al. 2009)), the difference between carbon sources appeared negligible in absence of the UPR here: similar levels were measured for a constitutively transcribed gene (fusion-*xyr1* under *tef1* promoter) on glucose, lactose, xylan and CMC in TXY(1) (see section 4.3, **Figure 11**). In contrast, differences between lactose and xylan data of DTT-treated samples might stem from a different *act1* transcription on these carbon sources during the UPR. This will be further discussed in section 4.4. The exact magnitude of the influence of *act1*-normalization on transcript levels must be determined in future experiments.

qPCR efficiency. The qPCR efficiency mainly influenced the precision of measurements. When the efficiency strongly varied between technical duplicates (e.g. 1.76 vs. 1.94) with similar C_t-values (19.1 and 19.3, respectively), differences of approx. 1 $\log_{10}$-unit were observed in the corresponding "relative transcript level"-values. These differences are reflected in the vertical bars in **Figure 6 & 7** (left column). Notably, the precision of the two technical replicates also strongly varied within a data set (see e.g. *hac1i* transcript levels of the "Lac + DTT" data set, **Figure 6B** (left graph)). Generally, the maximum of all measured efficiencies was 2.03 and the minimum 1.62.

The measured efficiencies were exchanged by the mean efficiency of all measurements (1.76) to reduce data processing caused imprecision. Consequently, an error was accepted as the efficiency depends on the sample type, sample treatment and the primers for a target gene (Pérez et al. 2013). This error seemed small because the means of efficiency varied by max. 0.02 units between cultivation conditions, strains, or genes. The corresponding change in relative transcript levels was about ± 0.1 – 0.2 $\log_{10}$-units which is small, compared to biological and/or sample preparation deviations (see below).

There were several arguments in favour of using a uniform efficiency. First, varying efficiencies proved to be the main reason for variation between technical duplicates. Therefore, the vertical bars, i.e. the difference between the duplicates, decreased to a barely visible length with a uniform efficiency (**Figures 6 & 7** (right column)). Second, the temporal developments of transcript levels of two independent experiments matched now well (see "Xn" and "Xn2", **Figure 6** (right column)). By using uniform efficiencies, the average differences between corresponding data pairs of the two experiments dropped from over 0.30 to -0.06 and -0.02 $\log_{10}$-units, for xylan and "xylan + DTT" data respectively. Third, the curves looked smoothed and made biologically more sense with uniform qPCR efficiencies. For example, with non-uniform efficiencies, the UPR induction by DTT in TXY(1) on lactose seemed to lower the transcript level of the UPR indicator *bip1* instead of rising it (**Figure 7**). In addition, the difference between the lactose samples seemed larger before than after DTT addition to one part of the samples. But with uniform efficiencies, "0 h-samples" showed similar *bip1* transcript levels and DTT increased *bip1* transcription, as expected. Also, *hac1i, hac1u* and *bip1* transcript levels of the DTT-free control samples remained constant during 6 h with uniform efficiencies and were similar for lactose and xylan (**Figures 6A, 6C & 7** (right graphs)).

Nevertheless, the efficiency should be calculated additionally via a standard curves for validation in the future (Svec et al. 2015).

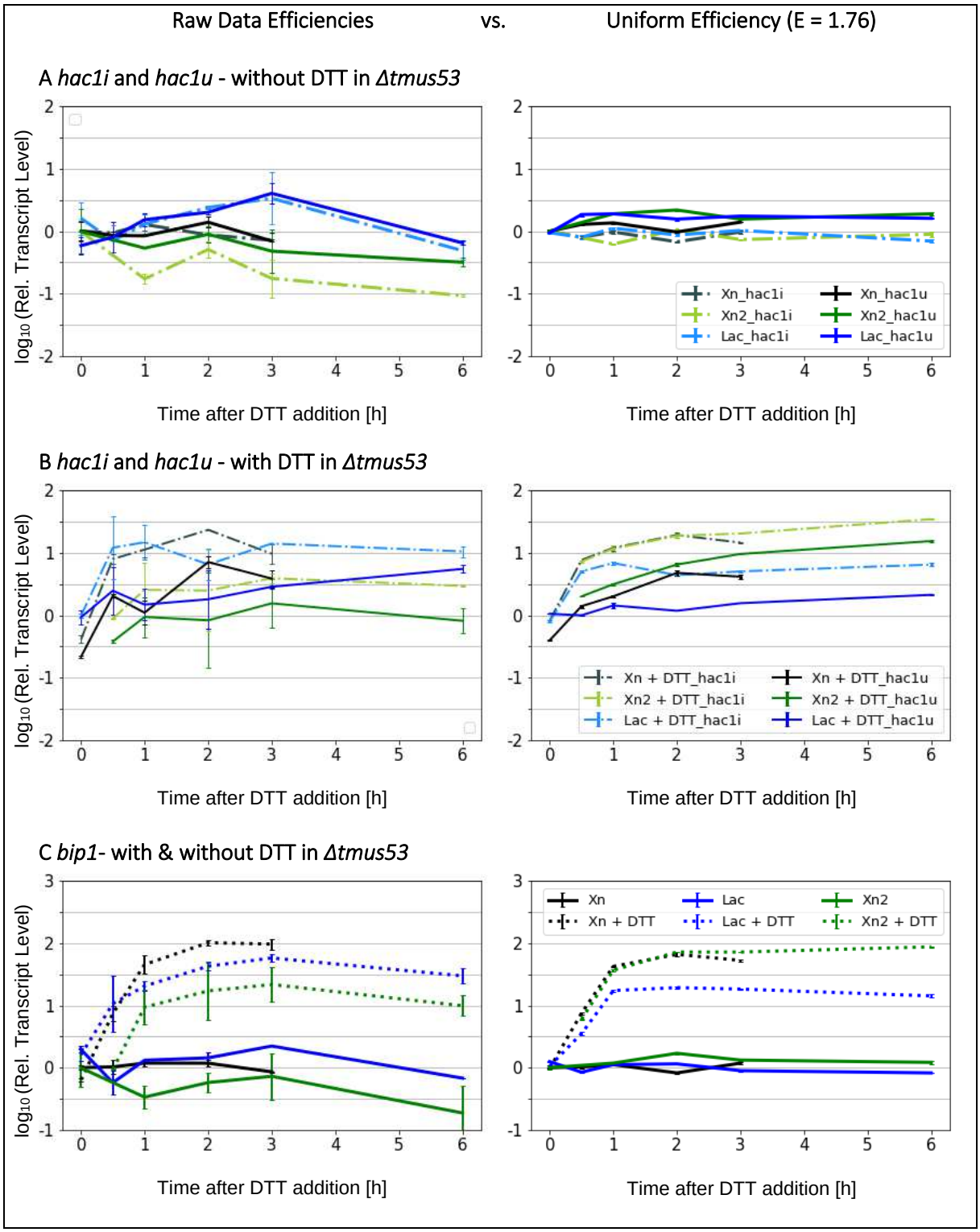

Figure 6: Influence of the qPCR efficiency on logarithmic relative transcript levels - exemplarily shown for hac1i and hac1u (A, B) and bip1(C) in the strain Δtmus53. Rel. transcript levels were normalized by act1 and calculated with raw data efficiencies (left column), or with a uniform, averaged efficiency (E = 1.76, right column). Δtmus53 was grown on lactose (Lac) for 36.5h, and on xylan (Xn, Xn2) for 24 h in two independent experiments. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample. The legend refers to the left and right graph. Vertical bars indicate the difference between two technical replicates.

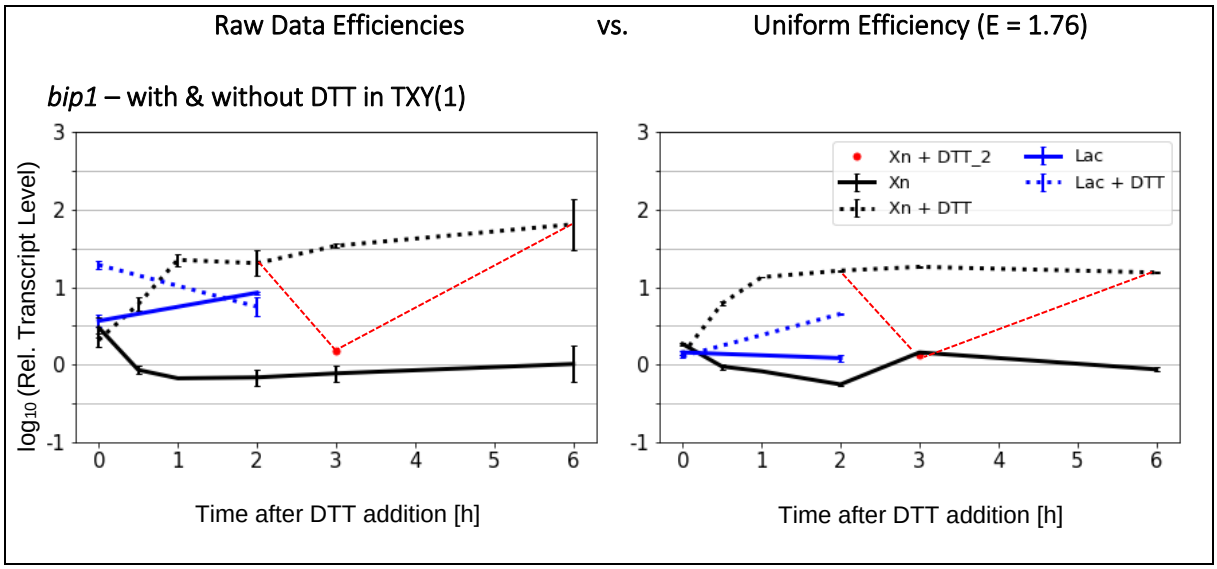

Figure 7: Influence of the qPCR efficiency and the cDNA synthesis on logarithmic relative transcript levels - exemplarily shown for bip1 in the strain TXY(1). Rel. transcript levels were normalized by act1 and calculated with raw data efficiencies (left column), or with a uniform, averaged efficiency (E = 1.76, right column). The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample (compare to Figure 6C). TXY(1) was grown on lactose (Lac) for 69.5 h, and on xylan (Xn) for 47 h. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. The red data point and lines show the effect of an erroneous cDNA synthesis. Vertical bars indicate the difference between two technical replicates.

cDNA synthesis/ RNA concentration. Due to an erroneous cDNA synthesis, the relative transcript level changed up to over 1 $\log_{10}$-unit for the sample "TXY(1) – Xn + DTT – 3 h". Hence, the corresponding data points would have changed the temporal trends completely (e.g. for *bip1*, see red point and lines in **Figure 7**). Besides an unlikely temporal development observed for all target genes, the error was indicated by unusual high C_t-values for the housekeeping genes *sar1* and *act1* (26.0 and 23.3 respectively). Therefore, the cDNA synthesis was repeated with a doubled amount of RNA template, resulting in reasonable C_t-values for the housekeeping genes (*sar1*: 21.9, *act1*: 20.7). The corresponding relative transcript level led to a more logical temporal development (**Figure 7**: "Xn + DTT – 3 h" (black point)).

The error was probably a pipetting error, or an erroneous RNA concentration distorted by a genomic DNA contamination.

Estimation of precision. After reducing the contributions of the efficiency and an erroneous sample preparation to the imprecision of the method, the standard error of the mean was estimated. It accounts for the deviation between biological samples and deviations based on the sample preparation - from sampling to mixing of qPCR reagents. The standard error of the mean was only estimated and not calculated because maximal two independent means were available. The whole experiment - from cultivation to qPCR analysis – was repeated twice for *Δtmus53* on xylan with and without DTT. This resulted in the two independent "xylan"-data sets "Xn" and "Xn2" (see green and black lines, e.g. in **Figure 6**). The difference between the "Xn" and "Xn2" and

between “Xn + DTT” and “Xn2 + DTT” was calculated for each time point for all genes. The averaged absolute differences between corresponding data pairs were 0.13 and 0.14 $\log_{10}$-units for all genes, for data sets without and with DTT respectively. The maximal absolute differences were 0.35 and 0.41, for data sets without and with DTT respectively. Independent means of comparable samples were also obtained for the samples before DTT addition (“0 h - Xn” & “0 h - Xn+DTT”, “0 h - Lac” & “0 h - Lac+DTT”). The absolute differences between these data pairs, averaged over all genes, were for all three strains 0.06-0.20 $\log_{10}$-units for xylan and lactose. And the maximal difference was 0.45 $\log_{10}$-units. Consequently, the standard error of the mean of relative transcript levels was estimated as ±0.5 $\log_{10}$-units. For interpretation, temporal changes or differences between data sets larger than 0.5 $\log_{10}$-units were considered significant. 0.5 $\log_{10}$-units equal a factor a 3.16.

4.2 DTT-induced UPR activation in strains *Δtmus53*, TX(WT) and TXY(1)

The inducibility of the unfolded protein response (UPR) by DTT was tested in three strains: in two strains overexpressing the main (hemi-)cellulase activator Xyr1, TX(WT) and TXY(1) (Derntl et al. 2019), and a wild-type-like strain, Δ*tmus53*. In both Xyr1-overexpressing strains, TX(WT) and TXY(1), the transcription factor is expressed constitutively and independently of the carbon source as the *xyr1* gene is under control of the strong constitutive promoter *tef1*. TX(WT) overexpresses wild-type Xyr1. TXY(1) synthesizes a Xyr1-fusion transcription factor that induces xylanases and cellulases nearly carbon source independently. The C-terminal transactivation domain of Xyr1, that is crucial for a carbon source-dependent (hemi-)cellulase regulation (Derntl et al. 2019), is exchanged in the Xyr1-fusion protein. The original C-terminus was replaced by the strong C-terminal transactivation domain of Ypr1. The yellow pigment regulator 1 Ypr1 is a Gal-4-like transcription factor from a secondary metabolite biosynthetic gene cluster (sorbicillinoids) (Derntl et al. 2016).

TX(WT), TXY(1) and Δ*tmus53* were cultivated on carbon sources that induce (hemi-)cellulases in these strains. The reducing agent DTT prevents the formation of disulphide bonds in the ER. DTT was added to one part of the biological replicates when enough mycelium for sampling had grown. Mycelium of the biological replicates without and with DTT was sampled and pooled before and up to 6h after to the addition of DTT. The RNA was isolated, and reverse transcribed into cDNA for qPCR analysis. Relative transcript levels of the UPR indicators *hac1u, hac1i,* and *bip1* were assessed. *Act1* and the sample “Δtmus53 – xylan – without DTT – 0 h” were chosen as reference gene and reference sample, respectively.

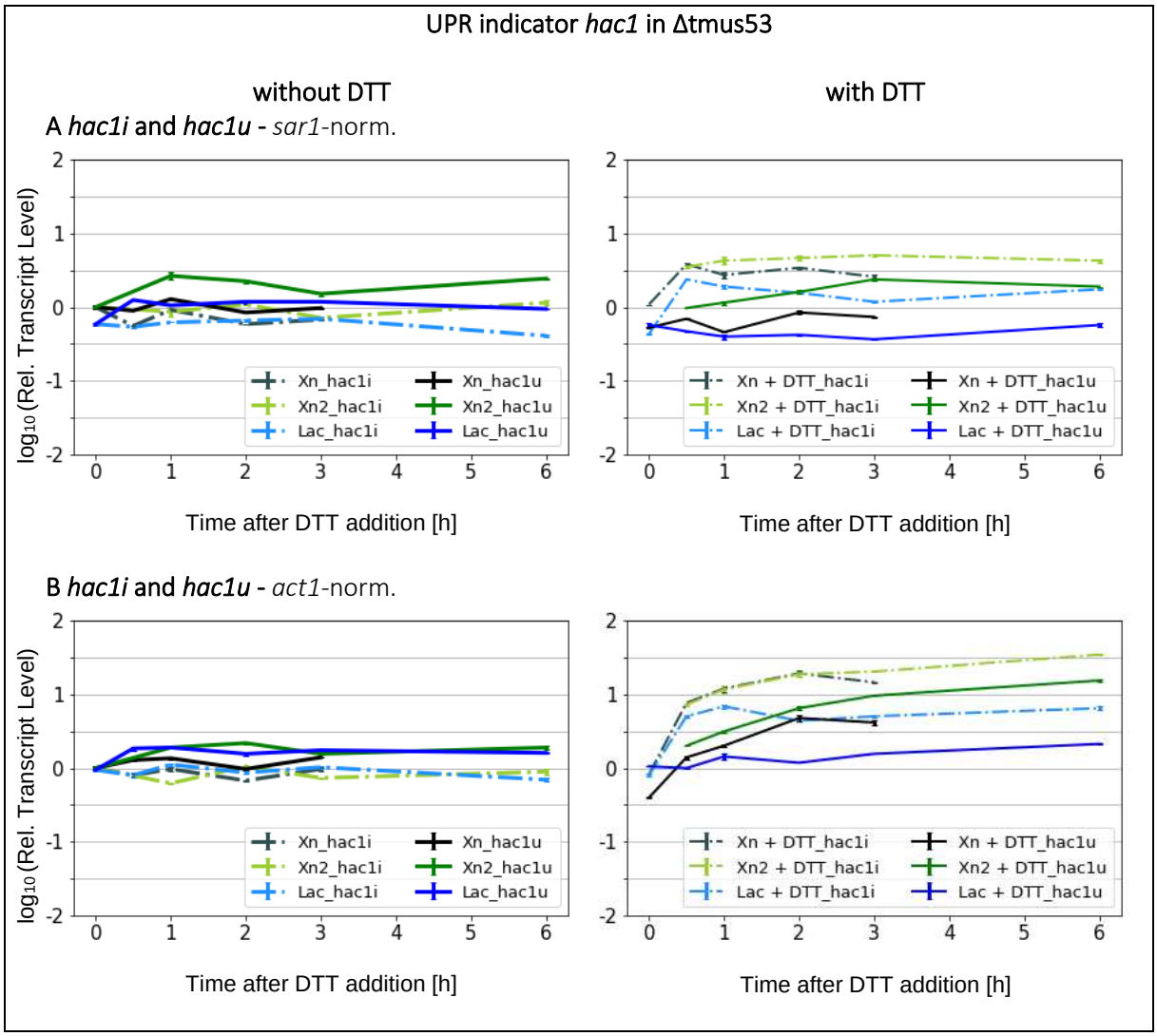

Figure 8: Logarithmic relative transcript levels of the UPR indicator hac1 without and with DTT in Δtmus53. A: Data normalized by sar1, B: Data normalized by act1. qPCR efficiencies were set to 1.76. Δtmus53 was grown on lactose (Lac) for 36.5h, and on xylan (Xn, Xn2) for 24 h in two independent experiments. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample. Dashed lines correspond to the spliced version of hac1, hac1i, and solid lines to the unspliced version, hac1u. Vertical bars indicate the difference between two technical replicates.

Wild-type like strain Δ*tmus53*. The UPR was increasingly induced by DTT until 1 h after DTT addition on both xylan and lactose in Δ*tmus53*. Then, the transcript levels of the UPR indicators remained almost constant.

Without DTT, the relative mRNA levels of *hac1i* were slightly lower than those of *hac1u* (**Figure 8B** (left graph)). Slightly means maximally 0.4 $\log_{10}$-units lower. This shows that there is a basal level of *hac1u* mRNA splicing into *hac1i* even when the UPR is not activated (Heimel 2015). In contrast, transcript levels of *hac1i* exceeded *hac1u* levels by 0.5 - 0.8 $\log_{10}$-units, when the UPR was activated by DTT (**Figure 8B** (right graph)). The findings are in accordance with previous reports of an increasing *hac1u* to *hac1i* splicing due to accumulated unfolded proteins in the ER in presence of DTT. An increasing translation of *hac1i* mRNA into to the transcription factor Hac1

leads eventually to the UPR induction (Saloheimo et al. 2003; Heimel 2015).

The induction of the UPR was clearly visible after 0.5 h and kept rising until 1h after DTT addition, comparing *hac1i* and *hac1u* levels in Δ*tmus53* (**Figure 8B**, right graph). The ratio of *hac1i* to *hac1u* remained then almost constant until 6 h after DTT addition. This temporal development was observed on both carbon sources, lactose and xylan. Hence, the inducibility of the UPR by DTT was similar for both carbon sources. However, the UPR induction seemed quantitatively stronger on xylan in Δ*tmus53*: *hac1i* levels on xylan surpassed those on lactose clearly later than 2 h after adding DTT (**Figure 8B**, right graph). In addition, *hac1u* levels rose barely on lactose but rose steadily on xylan in presence of DTT. A true rise of *hac1u* transcription, but also a DTT-caused drop of *act1* levels, could account for the observed increase. In the latter case, the difference between xylan and lactose data for *hac1u* could be explained by the carbon-source dependent regulation of the reference gene *act1* (Saloheimo et al. 1999; Margolles-clark et al. 1997). Nevertheless, *hac1i* and *hac1u* levels were also higher on xylan than on lactose when data were normalized by *sar1* (**Figure 8A** (right graph)). The fact that, *sar1*-normalized *hac1u* levels on xylan seemed to increase less than *act1*-normalized data, was probably due the UPR-caused up-regulation of *sar1*.

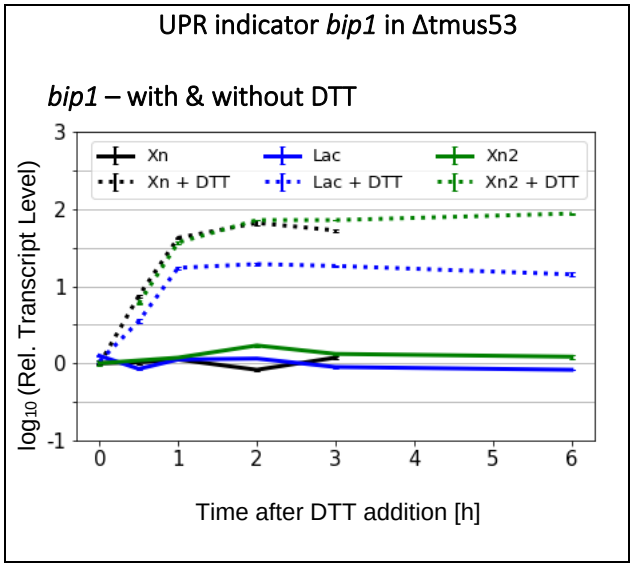

Figure 9: Logarithmic relative transcript levels of the UPR indicator bip1 without and with DTT in Δtmus53. Data normalized were normalized by act1 and qPCR efficiencies were set to 1.76. Δtmus53 was grown on lactose (Lac) for 36.5h, and on xylan (Xn, Xn2) for 24 h in two independent experiments. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. Vertical bars indicate the difference between two technical replicates.

In Δ*tmus53*, transcript levels of the ER chaperone *bip1* were increased by 0.9 – 2.0 $\log_{10}$-units (factor 7.8 - 100) within 0.5 – 6 h after DTT addition (**Figure 9**). Transcription of *bip1* was induced the strongest by DTT between 0 – 0.5 h. But 1 h after DTT addition, transcript levels of *bip1* did not change significantly and stayed on an elevated level like *hac1* mRNA. In absence of DTT,

relative transcript levels of *bip1* remained almost constant. Compared to *hac1i* and *hac1u*, DTT-caused changes were greater for *bip1* mRNA levels. Graphs of *bip1* transcript levels allowed therefore more reliable conclusions about the time point of UPR induction than *hac1i* or *hac1u* curves. However, *bip1* transcript levels alone are generally not sufficient as UPR indicator (Arvas et al. 2006) and *hac1i* and *hac1u* transcript levels should always be measured next to *bip1* levels. *Bip1* transcript levels indicated a qualitatively similar, but quantitatively different UPR induction by DTT on lactose and xylan. Like for *hac1*, DTT affected *bip1* levels on xylan to a greater extent than on lactose.

Transcript levels were additionally measured 24 h after DTT addition in one experiment with Δ*tmus53* on xylan ("Xn2"-data): *bip1* as well as *hac1i* and *hac1u* levels changed insignificantly between 6-24 h after DTT addition, indicating an active UPR even 24 h after DTT addition (data not shown here, see section 4.4, **Figure 19)**

Xyr1 overexpression strains TX(WT) and TXY(1). The strains TX(WT) and TXY(1) showed a similar inducibility of the UPR by DTT as the wild-type like strain Δ*tmus53.* In presence of DTT, relative transcript levels of the spliced mRNA *hac1i* exceeded levels of the unspliced mRNA *hac1u* (**Figure 10A**). In contrast to Δ*tmus53*, no induction of *hac1u* between 0 - 3 h after DTT addition on xylan was found in the Xyr1 overexpression strains (compare **Figures 8B & 10A**).
Interestingly, *hac1u* levels seemed to be lowered whereas *hac1i* levels seemed unchanged in presence of DTT in TXY(1) on lactose (**Figure 10A**). This contrasts data of TX(WT) and Δ*tmus53* on xylan and lactose where DTT increased both, *hac1i* and *hac1u* levels, or at least *hac1i* levels. Transcript levels of *bip1* increased by more than 1.2 $\log_{10}$-units within 1 h after DTT addition in TXY(1) and TX(WT) on xylan. After 1 h, *bip1* levels stayed on a relative constant elevated level (**Figure 10B**). In TX(WT), the UPR inducibility by DTT was tested only on xylan because lactose did not show to induce cellulases in TX(WT). In TXY(1), the induction of *bip1* on lactose was observed 2 h after DTT addition but less pronounced than in Δ*tmus53* (compare **Figures 9 & 10B**).

The unique time course of *hac1i* and *hac1u* and the little increase of *bip1* in TXY(1) on lactose with DTT coincided with a unique growth behaviour of TXY(1) on lactose in this experiment. In general, TXY(1) sporulated significantly less than TX(WT) and Δ*tmus53* on MEX plates. Therefore, cultures were inoculated with an OD_{600} of only 0.02 instead of 0.05, resulting in barely detectable growth within 24 h. The cultivation was then repeated with 6-month-old conidiospores due to the shortage of fresh spores. Only a few tiny balls of mycelium were observed instead of a dense network of filaments, like in cultivations for section 4.3. These few balls were sufficient for sampling only twice. In conclusion, the data of TXY(1) on lactose are hard to compare with the other data sets.

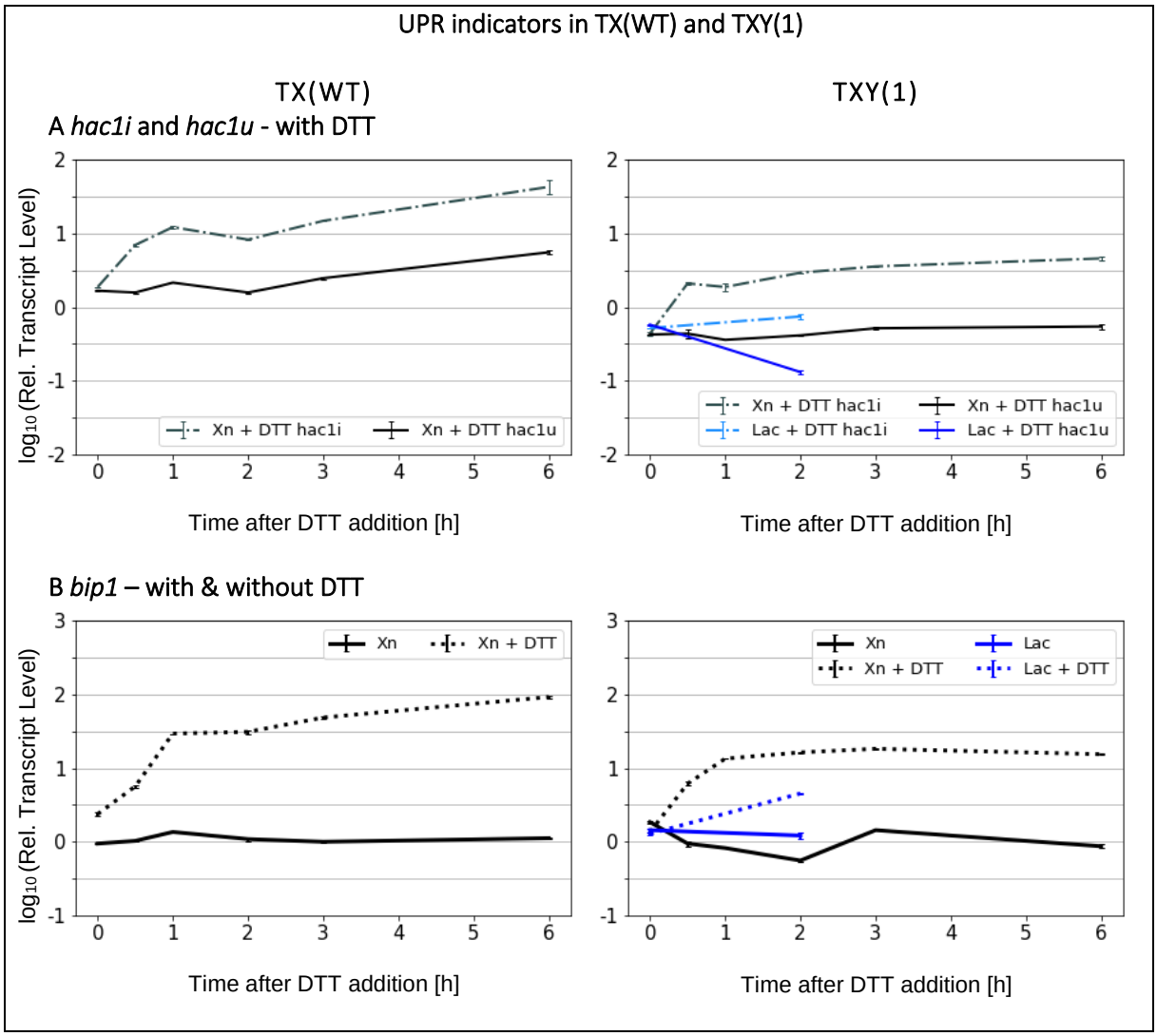

Figure 10: Logarithmic relative transcript levels of the UPR indicators hac1i and hac1u (A) and bip1 (B) in TX(WT) and TXY(1). Data were normalized by act1 and qPCR efficiencies were set to 1.76. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample (compare to Figure 8B & 9). TXY(1) was grown on lactose (Lac) for 69.5 h, and on xylan (Xn) for 47 h. TX(WT) was grown on xylan (Xn) for 46 h. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. Vertical bars indicate the difference between two technical replicates.

Transcript levels of the UPR indicators *hac1* and *bip1* in TXY(1) and TX(WT) in absence of DTT are discussed in the next section 4.3.

In conclusion, the UPR was found to be a universal mechanism, which is in accordance with previous literature (Heimel 2015). Nevertheless, its degree of induction varied between carbon sources. Looking at the *hac1i:hac1u* ratio, the UPR induction reached a high level 1 h after DTT addition and then only changed minimally during the following five hours (or 23 h in Δ*tmus53* on xylan).

Bip1 transcript levels showed the same qualitative temporal development, but a greater absolute change compared to *hac1i* and *hac1u* levels. Solely the combination of *bip1* and *hac1* transcript level allow reliable information about the UPR induction.

4.3 Influence of Xyr1 overexpression on (hemi-)cellulase transcription and ER stress

One of the research goals was to find out whether overexpression of Xyr1 induces the UPR by increasing (hemi-)cellulase production. To this end, the wild-type-like control strain, Δ*tmus53*, and the two Xyr1 overexpression strains, TX(WT) and TXY(1), were cultivated on different carbon sources: media contained either (hemi-)cellulase-repressing glucose, xylanase-inducing xylan, cellulase-inducing lactose or (hemi-)cellulase-inducing carboxymethyl cellulose (CMC). Samples were collected at different growth stages between 20 h to 168 h of cultivation and the RNA was isolated. Next, the mRNA was reverse transcribed into cDNA which was analysed by qPCR. Relative transcript levels of the genes encoding the UPR indicators Hac1 and BiP1, the transcription factor Xyr1, the (hemi-)cellulases CBHI, XYNII and the xylose reductase Xyl1 were assessed. The sample of Δ*tmus53* grown for 20 h on glucose served as reference sample. As reference genes served *sar1* and *act1*.

Xyr1. In accordance with previous results (Derntl et al. 2019), constitutive Xyr1 expression in strains TX(WT) and TXY(1) resulted in elevated *xyr1* mRNA levels nearly independent of the carbon source. In contrast, the carbon source influenced *xyr1* transcript levels in the control strain Δ*tmus53* as expected. (**Figure 11**).

The transcript levels of *xyr1* in TX(WT) and TXY(1) exceeded those in the control strain Δ*tmus53* by max. 1-2 $\log_{10}$-units, depending on the carbon source (**Figure 11**). *Xyr1* levels were only similarly high in all three strains on CMC and lactose at 38.5 h and 48 h.

In both overexpression strains, Xyr1 or fusion-Xyr1 is expressed under the control of the same constitutive promoter (*tef1*). However, *xyr1* transcript levels showed slightly different temporal developments in TX(WT) and TXY(1) (**Figure 11**). In addition, similar *xyr1* transcription trends were measured on all carbon sources in TXY(1), whereas they varied between carbon sources in TX(WT).

In the wild-type like strain Δ*tmus53*, the presence of glucose led to 1-1.2 $\log_{10}$-units lower *xyr1* transcription than on lactose and xylan in the beginning of cultivation (20 h, **Figure 11**). After 48 h incubation time, *xyr1* transcription on glucose reached an elevated, constant level similar to levels on lactose, xylan and CMC. This course of *xyr1* transcription on glucose in Δ*tmus53* has been observed before (Ilmén et al. 1997) and can be explained by the carbon catabolite repression (CCR) (Mach-Aigner et al. 2008; Antoniêto et al. 2014). At early time points, little glucose has been consumed so that high glucose concentrations repress *xyr1* transcription. Increasing glucose depletion in the medium abolishes the repression and induces *xyr1* together with (hemi-)cellulases to explore alternative carbon sources. On lactose and CMC, xyr1 transcription reached a maximum in the middle growth phase (38.5 h and 48 h, **Figure 11**). *Xyr1* mRNA levels on xylan did not change significantly during the observed cultivation time.

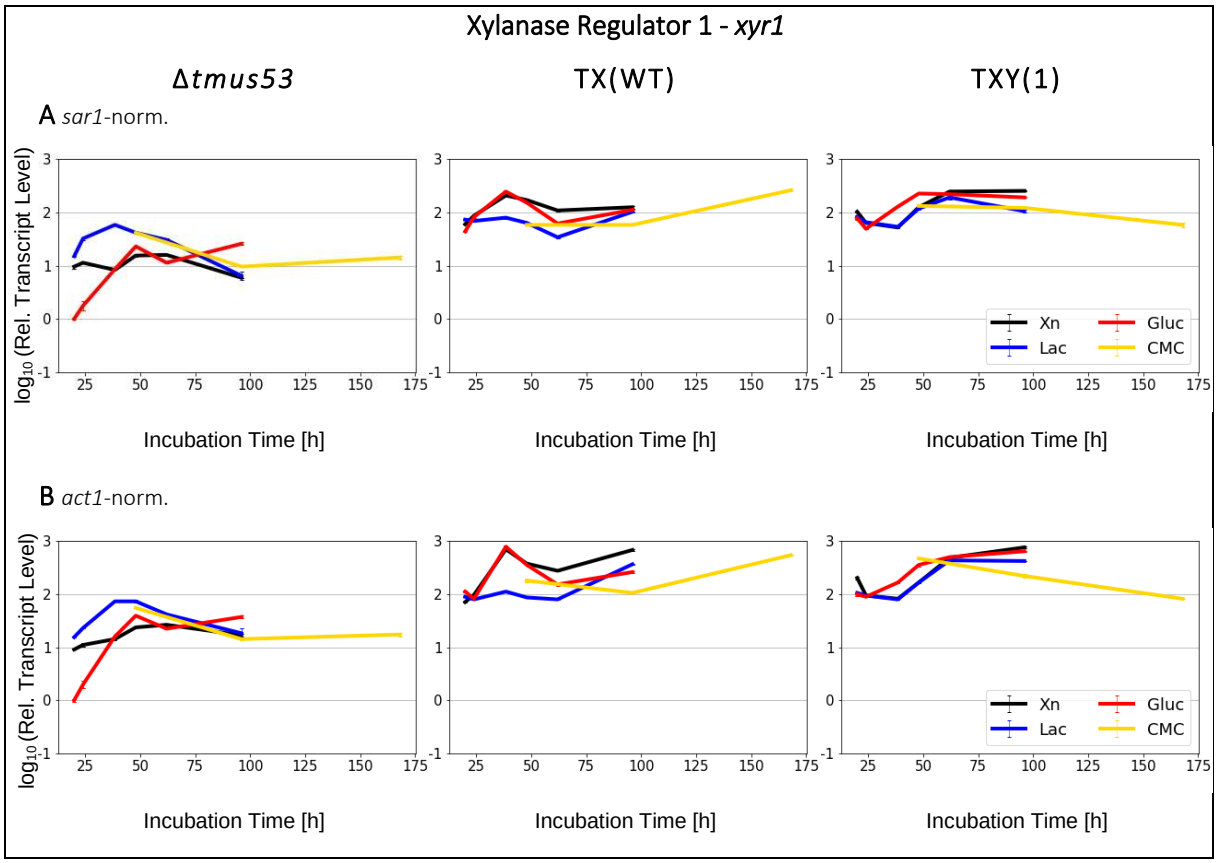

Figure 11: Logarithmic relative transcript levels of the gene encoding the xylanase regulator 1 (xyr1) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind Xyr1). A: sar1-normalized transcript levels, B: act1-normalized transcript levels. qPCR efficiencies were set to 1.76. Transcript levels were measured after 20 h, 24 h, 38.5 h, 48 h, 62 h and 96 h growth on glucose (Gluc, red), lactose (Lac, blue) and xylan (Xn, black), and after 48 h, 96 h and 168 h on CMC (yellow). The sample "Δtmus53 – Gluc – 20 h" served as reference sample. Vertical bars indicate the difference between two technical replicates.

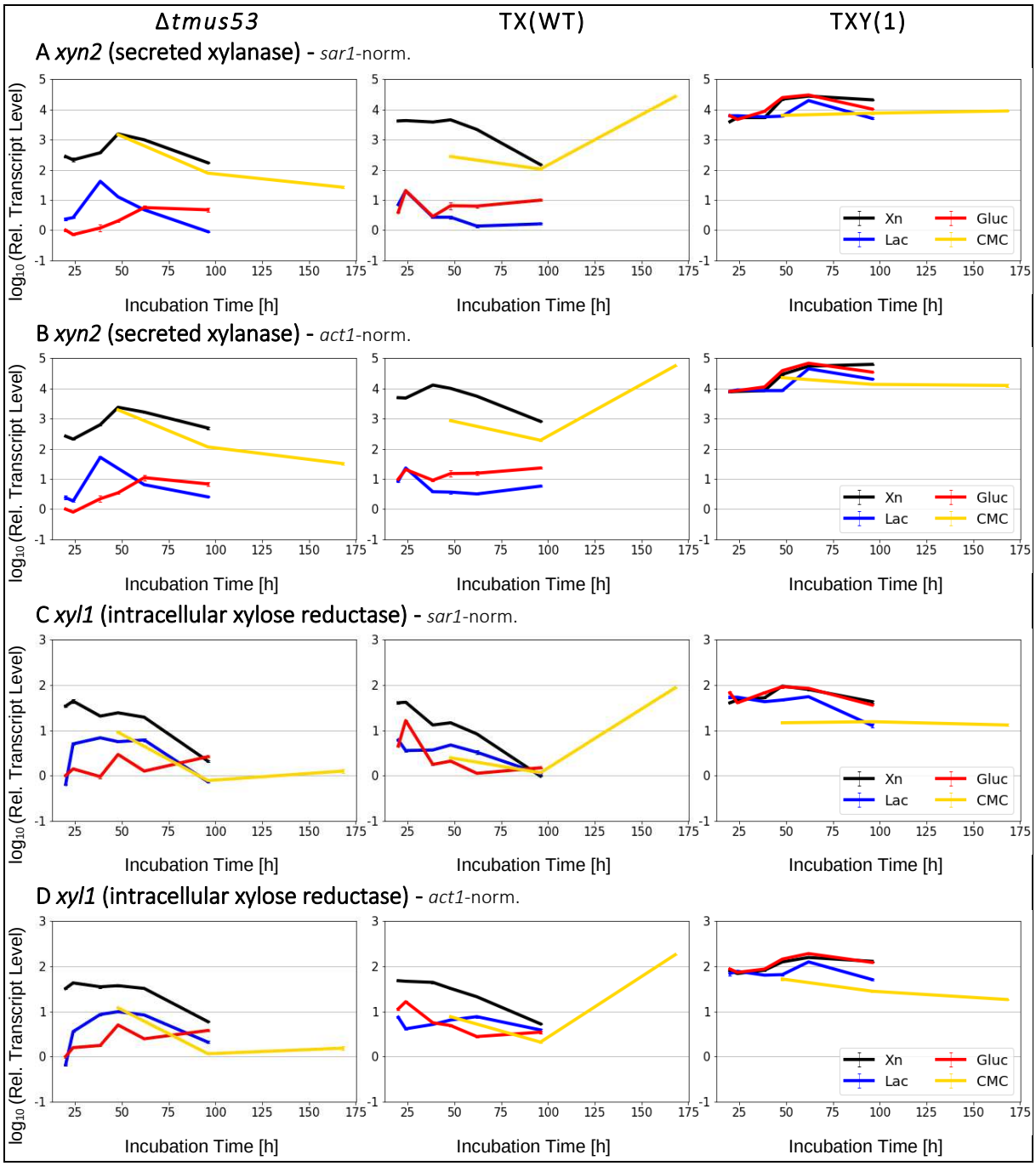

Figure 12: Logarithmic relative transcript levels of genes encoding the secreted xylanase 2 (xyn2, A, B) and of the intracellular xylose reductase 1 (xyl1)) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind Xyr1). A, C: sar1-normalized transcript levels. B, D: act1-normalized transcript levels. qPCR efficiencies were set to 1.76. Transcript levels were measured after 20 h, 24 h, 38.5 h, 48 h, 62 h and 96 h growth on glucose (Gluc, red), lactose (Lac, blue) and xylan (Xn, black), and after 48 h, 96 h and 168 h on CMC (yellow). The sample "Δtmus53 – Gluc – 20 h" served as reference sample. Vertical bars indicate the difference between two technical replicates.

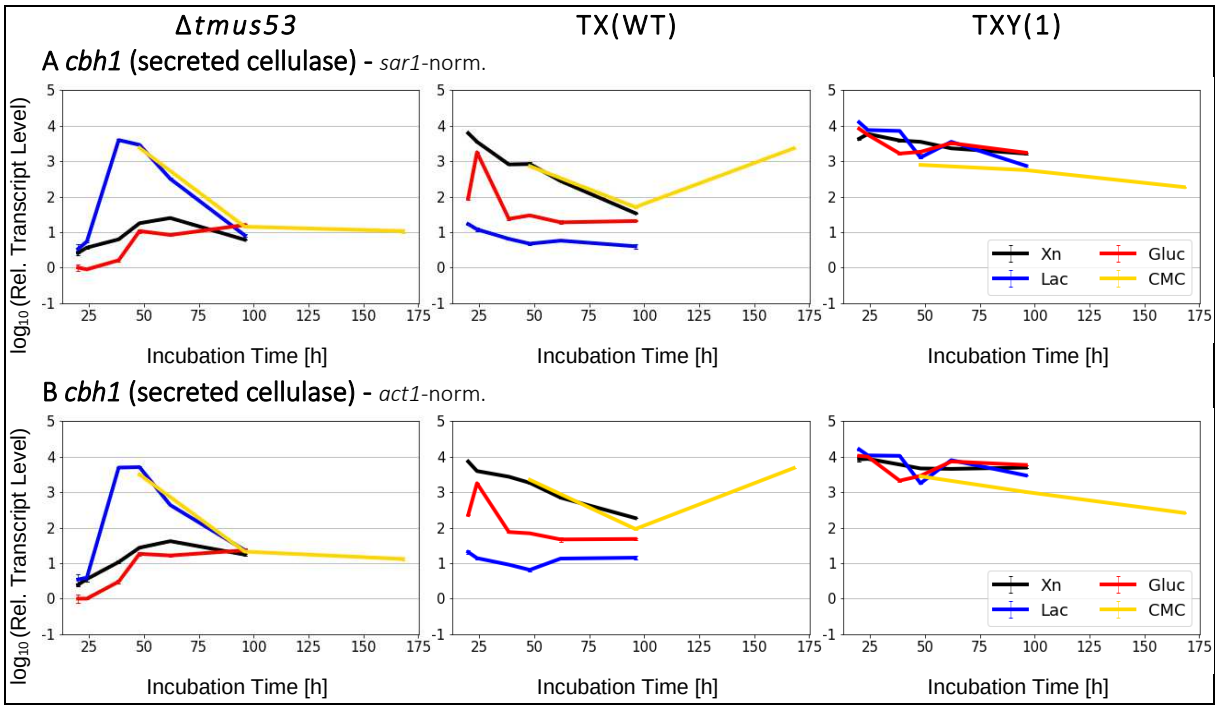

Figure 13: Logarithmic relative transcript levels of the gene encoding the secreted cellobiohydrolase 1 (cbh1) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind Xyr1). A: sar1-normalized transcript levels. B: act1-normalized transcript levels. qPCR efficiencies were set to 1.76. Transcript levels were measured after 20 h, 24 h, 38.5 h, 48 h, 62 h and 96 h growth on glucose (Gluc, red), lactose (Lac, blue) and xylan (Xn, black), and after 48 h, 96 h and 168 h on CMC (yellow). The sample "Δtmus53 – Gluc – 20 h" served as reference sample. Vertical bars indicate the difference between two technical replicates.

Secreted and intracellular enzymes. Overexpression of the fusion transcription factor Xyr1:Ypr1 in TXY(1) led to enhanced *xyn2, xyl1* and *cbh1* transcription independent of the carbon source (**Figures 12 & 13**).

In TXY(1), transcript levels of the secreted xylanase XYNII showed similar transcript levels on all carbon sources (**Figure 12, A and B**). In addition, a similar time course for lactose, xylan, and glucose with a maximum at 62 h was observed. The comparison of temporal trends between CMC and the other carbon sources was impeded by a lack of data points. Generally, the time course of *xyn2* was qualitatively comparable to the transcription of the *xyr1* in TXY(1) (compare **Figure 11 & 12**). Throughout the cultivation, overexpression of the carbon source-blind Xyr1 version did not only increase *xyn2* transcript levels by factors of 1000 – 10 000 under repressing conditions (glucose). It also enhanced *xyn2* transcription under inducing conditions by approx. 1-2 $\log_{10}$-units compared to the control strain Δ*tmus53*: x*yn2* levels in TXY(1) exceed those in Δ*tmus53* especially at later time points (see "CMC"- and "xylan"-data after 62 h in **Figure 12, A and B**). X*yn2* transcription in TXY(1) and TX(WT) was comparable only on xylan at early time points (20 – 48 h) and on CMC at 168 h (**Figure 12, A and B**).

The fusion transcription factor Xyr1:Ypr1 affected the transcription of the genes encoding the intracellular xylose reductase Xyl1 and the secreted XYNII similarly: *xyn2* and *xyl1* levels showed

comparable temporals trends and an independence of the carbon source – except on CMC (**Figure 12, C and D**). However, the significantly lower sar1-normalized transcript levels of *xyl1* on CMC seem to be an artefact when comparing them to *act1*-normalized data.

In comparison to *xyn2*, *xyl1* transcript levels were boosted less than *xyn2* transcription by the modified Xyr1 version in TXY(1). Maximal *xyl1* transcript levels in TXY(1) differed therefore only by a factor of max. 10 from those in Δ*tmus53* on xylan, CMC and lactose (**Figure 12**).

Transcript levels of the secreted cellobiohydrolase CBHI in TXY(1) were similar on all carbon sources. *Cbh1* levels and started at a similarly high level compared to *xyn2* but decreased over time (**Figure 13**). Temporal development differed from the development of *xyr1* as well, which indicates the dependence of *cbh1* transcription on further transcription factors (see section 2.2).

When wild-type Xyr1 was expressed (Δ*tmus53*, TX(WT)), transcription of the intracellular enzyme Xyl1 and of the secreted enzymes CBHI and XYNII depended not solely on Xyr1 expression. Also, carbon source-dependent mechanisms regulated *xyl1*, *xyn2* and *cbh1* transcription in Δ*tmus53* and TX(WT).

In Δ*tmus53* and TX(WT), *xyn2* was strongly transcribed on inducing carbon sources, on xylan and CMC, and to a 3-1000 times lower extent on the lactose or on repressing glucose (**Figure 12, A and B**). Overexpression of Xyr1 in TX(WT) enabled higher *xyn2* levels on xylan and glucose at early time points and – at late time points –on CMC as well.

Highest *xyl1* levels were measured on xylan in TX(WT) and Δ*tmus53* at early time points (**Figure 12, C and D**). At late time points, maximal *xyl1* levels were measured on CMC in TX(WT). X*yl1* levels in TX(WT) on glucose were 2 $\log_{10}$-units higher at 20 h compared to Δ*tmus53*. Hence, overexpression of Xyr1 seemly counteracts the carbon catabolite repression, i.e. *xyl1* repression at high glucose concentrations. On lactose, *xyl1* transcript levels showed a maximum at the middle time points in Δ*tmus53*, but *xyl1* levels did not change significantly in TX(WT). To sum up, overexpression of Xyr1 in TX(WT) increased *xyl1* transcription on glucose and lactose in the beginning and on CMC at late time points compared to Δ*tmus53*. Comparing *xyn2* and *xyl1*, which are both required for exploiting xylan as energy source (see section 2.1), differences between carbon sources for the intracellular *xyl1* were less prominent than for the secreted *xyn2* in Δ*tmus53* and TX(WT).

Regarding *chb1*, carbon sources influenced transcription differently in the two "wild-type Xyr1"-strains (**Figure 13**). In Δ*tmus53*, *cbh1* transcript levels reached a maximum between 38,5 – 48 h and then dropped by more than 2 $\log_{10}$-units under inducing conditions, on lactose and CMC. Low *cbh1* levels were measured in Δ*tmus53* under non-inducing (xylan) or repressing (glucose) conditions. Temporal development of *cbh1* transcription followed *xyr1* transcription – except that differences between carbon sources were much more prominent for *cbh1*. The influence of additional regulators on *cbh1* and *xyn2* transcription could be responsible for these differences.

In TX(WT), *cbh1* mRNA levels on CMC were similar to levels in Δ*tmus53* in the beginning but exceeded levels in Δ*tmus53* at later time points by 1 – 2 $\log_{10}$-units (**Figure 13**). Also, high *cbh1* transcript levels were measured on xylan which was not observed in Δ*tmus53*. In contrast, *cbh1* was hardly transcribed on lactose in TX(WT) compared to Δ*tmus53. Cbh1* levels on glucose were similar in Δ*tmus53* and TX(WT) - except at 20 h and 24 h. At 20 h, the *cbh1* levels on glucose were higher in TX(WT) and were not repressed compared to *Δtmus53* (like *xyl1*). At 24 h, the *cbh1* transcription on glucose was substantially higher in TX(WT) than on other time points. This glucose data point seems to be an artefact: The ratio of *sar1* to *act1* differed from other glucose samples of TX(WT) pointing towards an erroneous sample preparation. Therefore, relative transcript levels of all genes of the "TX(WT)-glucose-24 h"-sample might be biased.

Present results for *xyn2*, *xyl1* and *cbh1* match well transcripts levels of Derntl et al. (2019). In their paper, means of *sar1*- and *act1*-normalized transcript levels were measured in TXY(1), TX(WT) and *Δtmus53* on glucose, xylan and lactose after 24 h cultivation, and on CMC after 48 h. Only differences in present and previous transcript levels greater than 0.5 were estimated to be significant. Under this point of view, only transcript levels in *Δtmus53* on lactose were clearly lower for *xyn2* (-1 $\log_{10}$-unit) and *cbh1* (-2 $\log_{10}$-units) in the present study. In addition, it was confirmed that the remarkable higher transcript levels in TX(WT) on glucose after 24 h must be an artefact.

UPR indicators. Overexpression of the wild-type and fusion-Xyr1 enhanced the secretion of native enzymes without inducing the UPR. RNA level of the activated UPR inducer, *hac1i*, stayed lower or equal to the levels of the inactive form, *hac1u* (**Figures 14, A & B**). More specifically, *hac1i:hac1u* ratios ≤ 1 were found at all time on all carbon sources, in both Xyr1 overexpression strains as well as in the control strain *Δtmus53*. In addition, similar absolute *hac1u* levels were measured on all carbon sources in all strains.

Comparable transcript levels of the gene encoding another UPR indicator, the ER-located chaperone BiP1, were measured on all carbon sources in TXY(1), TX(WT) and *Δtmus53* (**Figure 14, C and D**). Even in TX(WT) and *Δtmus53*, where *cbh1* and *xyn2* transcription varied strongly from one carbon source to another, no significant difference between *bip1* levels was found. If the UPR had been activated by the elevated enzyme production, then *bip1* levels on glucose would have been significantly higher in TXY1 than in *Δtmus53.* In addition, significantly lower *bip1* levels in TX(WT) would have been measured on glucose than on the other carbon sources. Thus, *bip1* data confirm the absence of ER stress, i.e. no accumulation of unfolded protein in the ER, due to Xyr1 overexpression.

Comparing transcript levels of *xyn2*, *xyl1*, *chb1* and *xyr1* with *bip1* levels, no correlation with *bip1*

was found in TX(WT), TXY(1) or *Δtmus53*. This, and equal *bip1* levels in all three strains, disprove the suggestion of Pakula et al. (2016) that *bip1* (and other ER-related genes) are regulated by Xyr1.

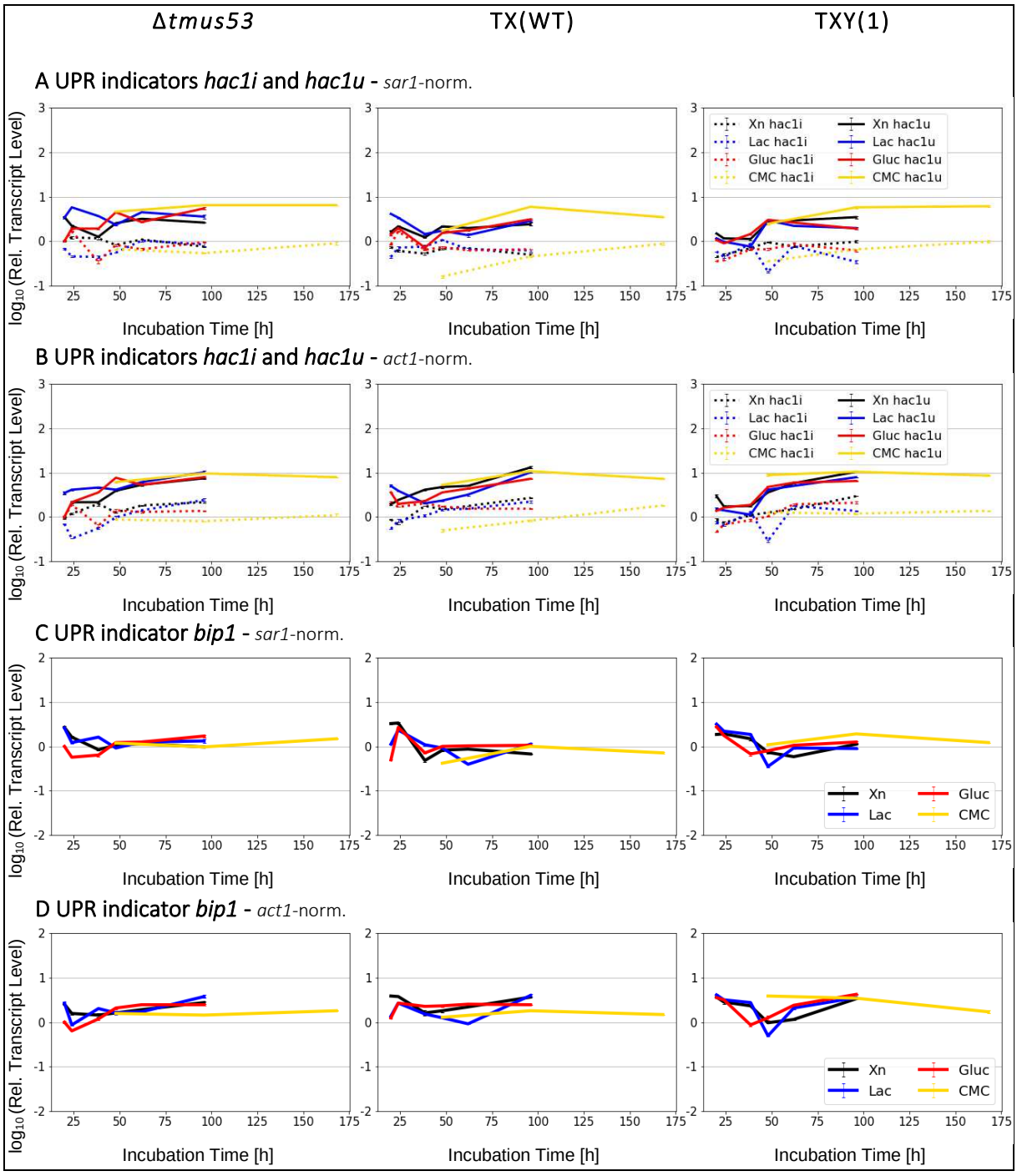

Figure 14: Logarithmic relative transcript levels of the UPR indicator hac1u (solid lines) and hac1i (dashed lines) (A, B), and of the UPR indicator bip1 (C,D) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind Xyr1). A, C: sar1-normalized transcript levels. B, D: act1-normalized transcript levels. qPCR efficiencies were set to 1.76. Transcript levels were measured after 20 h, 24 h, 38.5 h, 48 h, 62 h and 96 h growth on glucose (Gluc, red), lactose (Lac, blue) and xylan (Xn, black), and after 48 h, 96 h and 168 h on CMC (yellow). The sample "Δtmus53 – Gluc – 20 h" served as reference sample. Vertical bars indicate the difference between two technical replicates. The data points of "TXY(1)-Lac-48 h" are probably an artefact.

Conclusions. Overexpression of the wild-type and fusion-Xyr1 enhance the secretion of native enzymes without inducing the UPR. Hence, the protein folding capacity of the ER is not surpassed yet in TX(WT) and TXY(1) strains.

The result, that the UPR is not activated in Xyr1 overexpression strains under the chosen conditions, validated these conditions as "UPR-inactive" control condition for UPR inductions experiments by DTT (see next section).

Outlook. It would be interesting to compare the expression levels of the (hemi-)cellulases in the Xyr1 overexpression strains to those in Rut-C30 under conditions that have been shown to induce the UPR in Rut-C30 (Pakula et al. 2005). This will reveal whether the UPR was inactive in TXY(1) because of significantly lower enzyme secretion levels in TXY(1) than in Rut-C30 or because of other factors, like the cultivation condition or genomic differences.

4.4 The role of the transcription factors Xyr1, Ace3 and Rxe1 in RESS upon UPR

Regulatory mechanisms of RESS and the role of the (hemi-)cellulase activators Xyr1, Rxe1 and Ace3 in the induction of RESS are analysed in this section.
To this end, the two Xyr1 overexpression strains, TX(WT) and TXY(1), and the wild-type-like strain, Δ*tmus53,* were cultivated on xylan and lactose. After a certain pre-growth time, 20 mM DTT was added to induce the UPR. Mycelium of min. two biological replicates was sampled and pooled before and up to 6 h after DTT addition. Transcript level in TXY(1) on lactose were only measured before and 2 h after DTT addition because of the extraordinarily little growth of TXY(1) on lactose (discussed in section 4.2). The RNA was isolated, and reverse transcribed into cDNA for qPCR analysis. Relative transcript levels were assessed of the following genes encoding: the UPR indicators Hac1 and BiP1, the transcription factors Xyr1, Ace3, Rxe1, the (hemi-)cellulases CBHI, EGLI, BGLII, XYNII, and the xylose reductase Xyl1. The transcript levels of the target genes were normalized by *act1*. The sample "Δtmus53 – without DTT – 0 h – xylan" was chosen as reference sample for all genes except for the cellulase genes. For the cellulases genes, the reference sample was "Δtmus53 – without DTT – 0 h – lactose".

UPR induction by DTT. *Hac1i, hac1u* and *bip1* transcript levels indicated a highly active UPR 1 h after DTT addition (**Figure 15**). During the following five (or 23) hours, *hac1i, hac1u* and *bip1* levels hardly changed. The UPR induction was more pronounced on xylan than on lactose. More details have been discussed in section 4.2 using the same data.

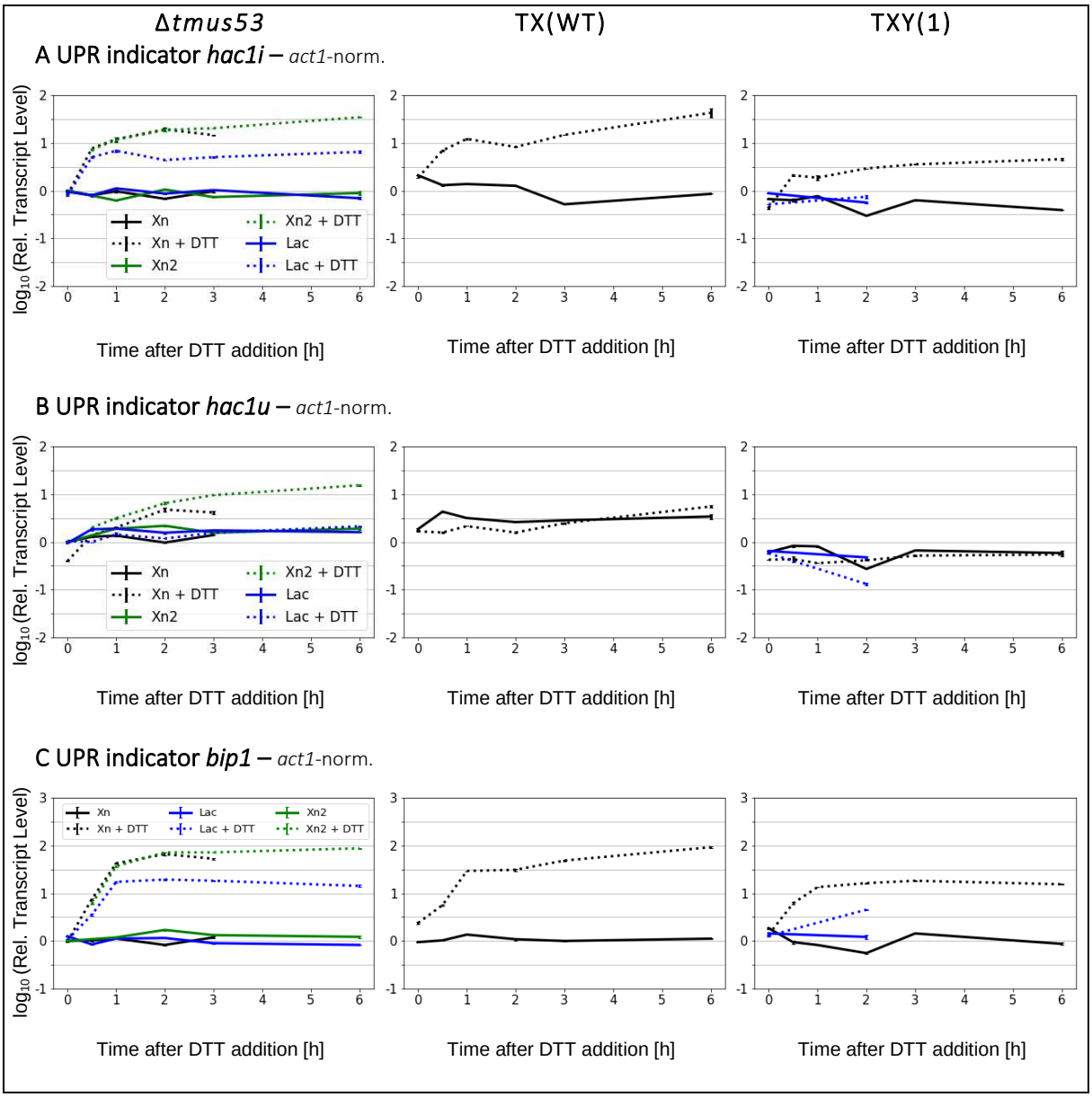

Figure 15: Logarithmic relative transcript levels of the UPR indicators hac1u and hac1i (A, B), and of the UPR indicator bip1 (C) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind Xyr1). Transcript levels were act1-normalized and qPCR efficiencies were set to 1.76. Δtmus53 was grown on lactose (Lac) for 36.5h, and on xylan (Xn, Xn2) for 24 h in two independent experiments. TXY(1) was grown on lactose (Lac) for 69.5 h, and on xylan (Xn) for 47 h. TX(WT) was grown on xylan (Xn) for 46 h. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. Vertical bars indicate the difference between two technical replicates.

Transcript levels on lactose. The transcript levels of the genes encoding the transcription factors Xyr1 and Rxe1 and the cellulases CBHI and EGLI clearly decreased during the UPR in Δ*tmus53* on lactose (**Figures 16 & 17**). Clearly means by more than 0.5 log10-units 2 h after DTT-addition. The temporal development of *xyr1, cbh1* and *egl1* was similar: The transcript levels dropped until 2 h after DTT addition and then stayed almost constant. Transcript levels of *rxe1* kept decreasing later than 2 h after DTT addition. In contrast, transcript levels of the genes encoding the (hemi-)cellulase activator Ace3 and the intracellular ß-glucosidase BGLII were so slightly lowered by DTT (0.5 $\log_{10}$-units, factor of 3) that it was not considered significant (**Figures 16 & 17**). The observations for BGLII are in accordance with a previous study (Pakula et al. 2003) that found BGLII to be unaffected by RESS.

On lactose in TXY(1), minimal changes (< 0.5 $\log_{10}$-units) were measured due to the presence of DTT for the transcription of the constitutively expressed gene encoding the modified Xyr1 (**Figure 16**). Also, *ace3* and *bgl2* were insignificantly affected by the RESS in TXY(1) on lactose, like in Δ*tmus53* (**Figures 16 & 17**). DTT decreased clearly (> 0.5 $\log_{10}$-units) the transcription of genes encoding the transcription factor Rxe1, and the cellulases CBHI and EGLI (see 2 h after DTT addition, **Figures 16 & 17**). However, the decrease of *cbh1* and *egl1* levels was less pronounced than in the wild-type like strain Δ*tmus53*. The cultivation should be repeated with fresh conidiospores to check whether it was related to the unique growth behaviour of TXY(1) on lactose.

Transcription in TX(WT) was not investigated on lactose, as lactose does not induce cellulases in TX(WT) (see section 4.3).

In summary, overexpression of the modified Xyr1 version on lactose did not interfere with RESS: *rxe1* and the known RESS targets (Pakula et al. 2003), *cbh1*, and *egl1* were down-regulated irrespective of *xyr1* levels. These results suggest that Xyr1 is not the mediator of RESS.

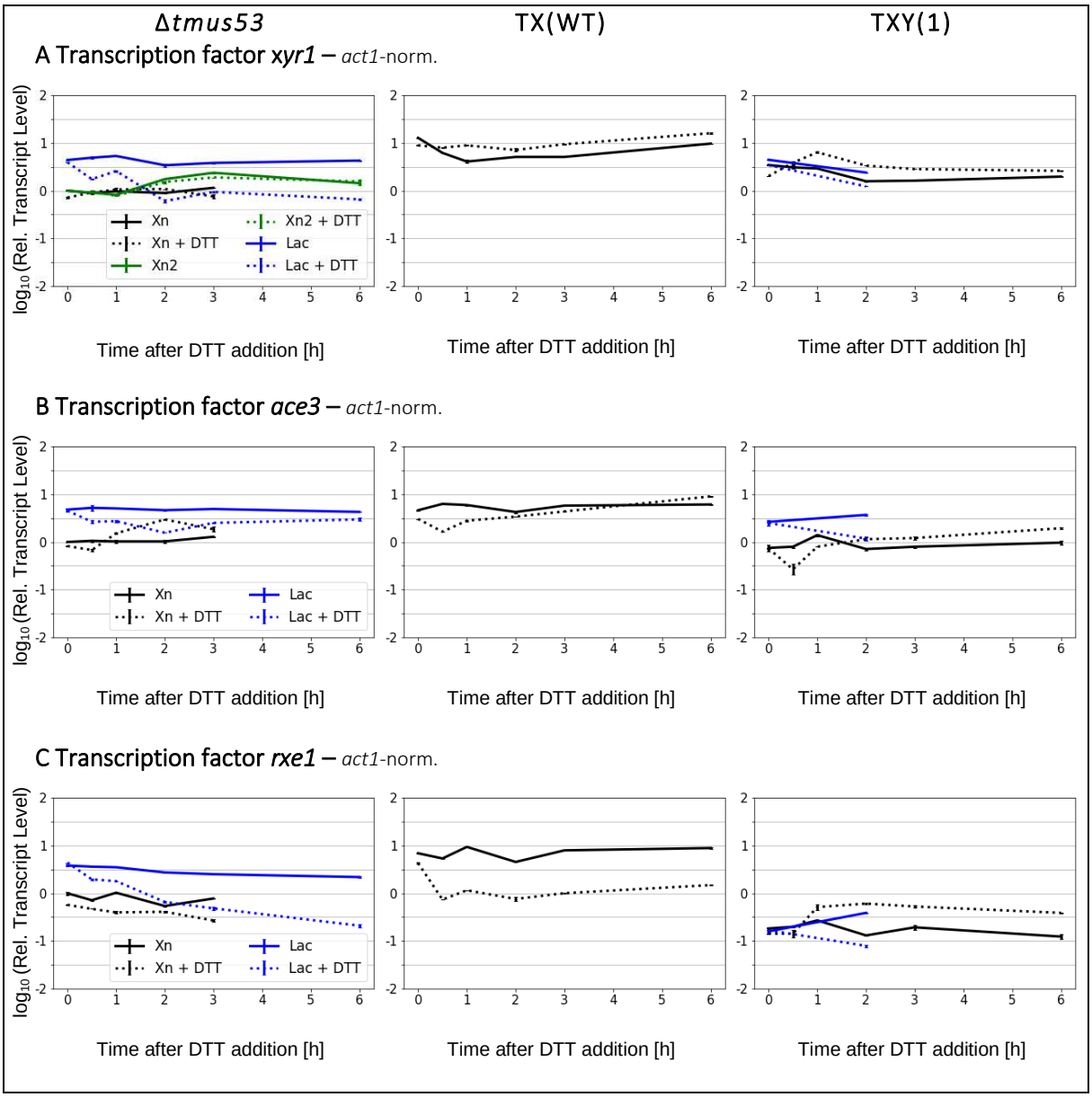

Figure 16: Logarithmic relative transcript levels of genes encoding the (hemi-)cellulase activators Xyr1 (A), Ace3 (B) or Rxe1 (C) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind Xyr1). Transcript levels were act1-normalized and qPCR efficiencies were set to 1.76. Δtmus53 was grown on lactose (Lac) for 36.5h, and on xylan (Xn, Xn2) for 24 h in two independent experiments. TXY(1) was grown on lactose (Lac) for 69.5 h, and on xylan (Xn) for 47 h. TX(WT) was grown on xylan (Xn) for 46 h. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. Vertical bars indicate the difference between two technical replicates.

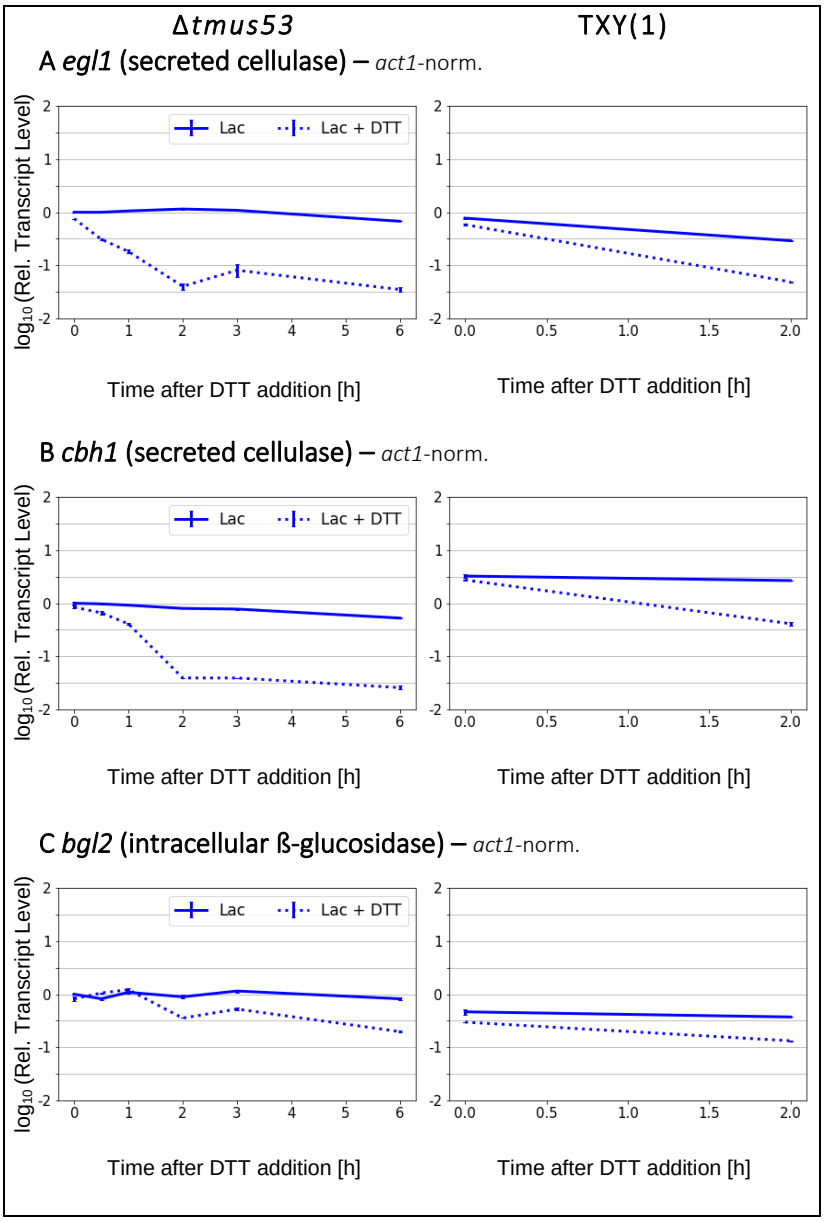

Figure 17: Logarithmic relative transcript levels of the genes encoding the secreted endoglucanase 1 (egl1, A), the secreted cellobiohydrolase (cbh1, B) and the intracellular ß-glucosidase 2 (bgl2, C) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind xyr1). Transcript levels were act1-normalized and qPCR efficiencies were set to 1.76. Δtmus53 and TXY(1) were grown on lactose (Lac) for 36.5h and 69.5 h, respectively. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h), at 2 h after DTT addition in both strains, and at 0.5 h, 1 h, 3 h and 6 h after DTT addition in Δtmus53. The sample "Δtmus53 – Lac – without DTT – 0 h" served as reference sample. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. Vertical bars indicate the difference between two technical replicates.

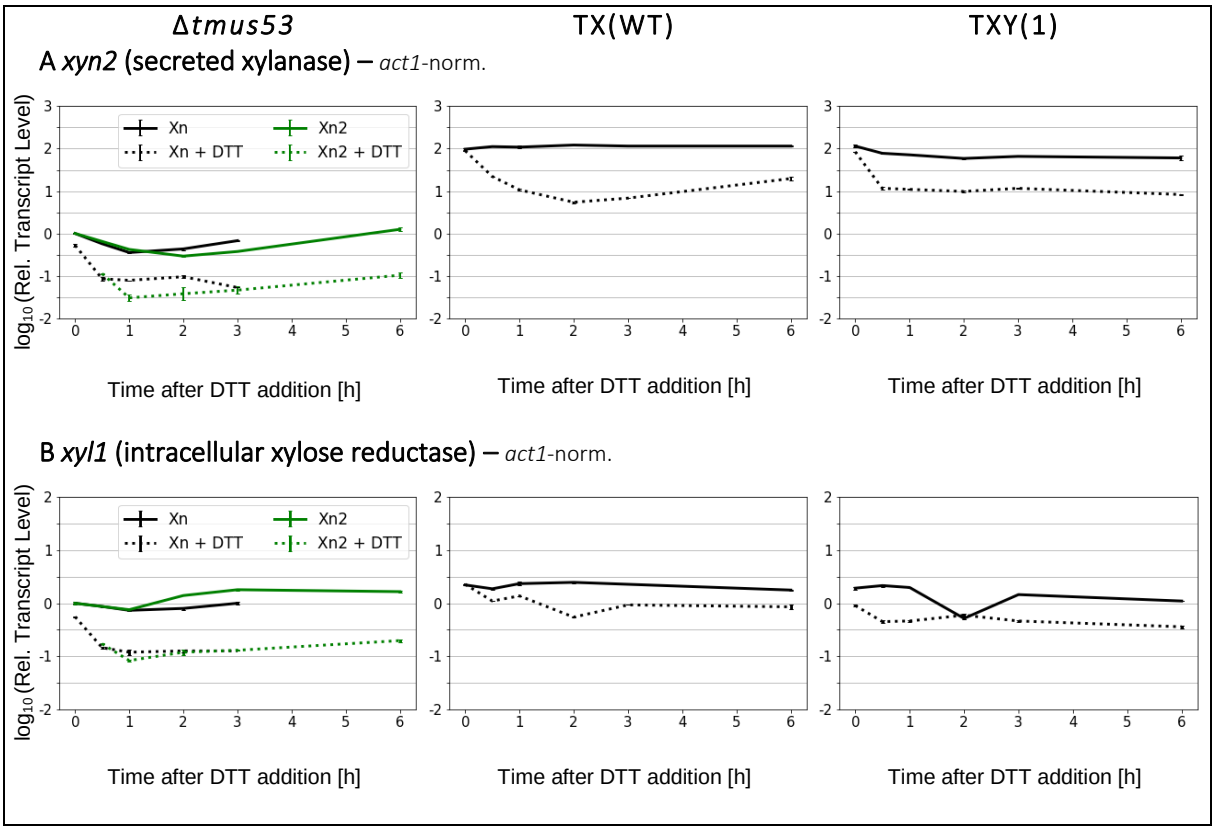

Figure 18: Logarithmic relative transcript levels of the genes encoding the secreted xylanase 2 (xyn2, A) and the intracellular xylose reductase 1 (xyl1, B) in the control strain Δtmus53, in TX(WT) (overexpressing wild-type Xyr1) and in TXY(1) (overexpressing a carbon source-blind Xyr1). Transcript levels were act1-normalized and qPCR efficiencies were set to 1.76. Δtmus53 was grown on lactose (Lac) for 36.5h, and on xylan (Xn, Xn2) for 24 h in two independent experiments. TXY(1) was grown on lactose (Lac) for 69.5 h, and on xylan (Xn) for 47 h. TX(WT) was grown on xylan (Xn) for 46 h. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured before (0 h) and at 0.5 h, 1 h, 2 h, 3 h and 6 h after DTT addition. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. Vertical bars indicate the difference between two technical replicates.

Transcript levels on xylan. In Δ*tmus53*, the transcript levels of genes encoding the xylanase XYNII and the xylose reductase Xyl1 dropped clearly under secretion stress induced by DTT. The most prominent decrease of transcript levels was observed 0.5 h after DTT addition (**Figure 18**). At later time points, *xyn2* and *xyl1* levels changed insignificantly. Although results for *xyl1* were consistent in both "Xylan + DTT" data sets, a deviation of 0.5 $\log_{10}$-units was found for *xyn2* (**Figure 18**). The lower *xyn2* levels of the green "Xn2"-data set could be explained by C_t-values close to the blank.

The transcription of genes encoding the (hemi-)cellulase activators – Xyr1, Rxe1 and Ace3 – reacted differently to DTT in Δ*tmus53* on xylan (**Figure 16)**. The transcript levels of *rxe1* decreased insignificantly (< 0.5 $\log_{10}$-units) after DTT addition. The other transcription factor, Ace3, seemed to be up-regulated 1 h after DTT addition as transcript level increased by approx. 0.5 $\log_{10}$-units. This increase of *ace3* levels on xylan contrasts the decreasing *ace3* levels on lactose. The relative transcript levels of *xyr1* seemed completely unaffected by the DTT addition

on xylan. The surprising result was observed for both independent experiments with Δ*tmus53* on xylan (**Figure 16**, "Xn"- and "Xn2"-data). However, a parallel down-regulation of *act1* and *xyr1* in presence of DTT could be the reason for the apparent constant *xyr1* levels on xylan as well. The results must be therefore validated, e.g. by using an alternative reference gene.

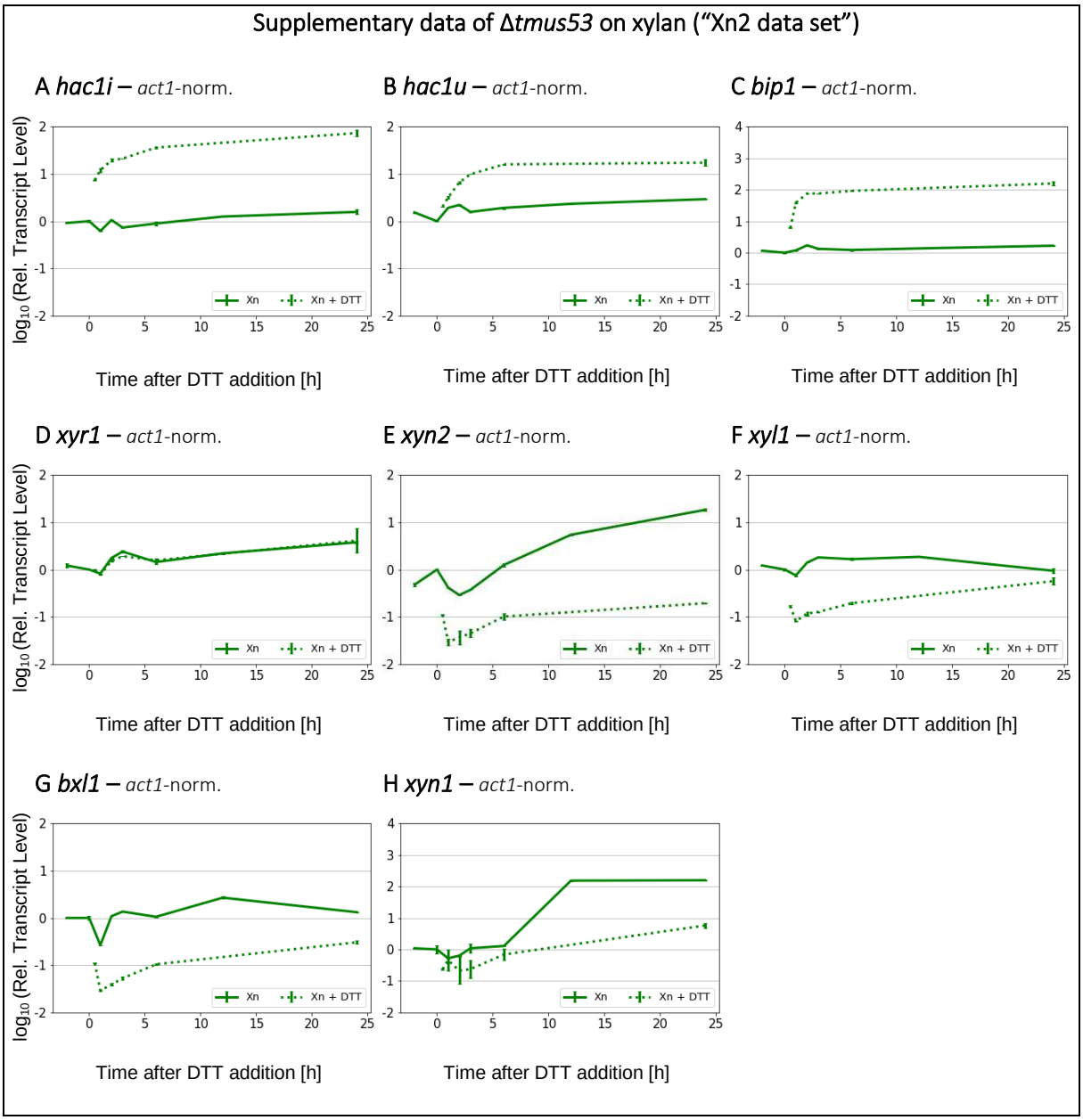

Figure 19: Supplementary logarithmic relative transcript levels of wild-type like strain Δtmus53 on xylan ("Xn2"-data set): here, data for 24 h after DTT addition, as well as transcript levels of the genes encoding the secreted ß-xylosidase BXLI and the secreted xylanase XYNI are shown. Transcript levels were act1-normalized and qPCR efficiencies were set to 1.76. Δtmus53 was grown for 24 h on xylan. Then, 20 mM DTT was added to liquid cultures. Transcript levels were measured at 0.5 h, 1 h, 2 h, 3 h, 6 h, and 24 h after DTT addition in the DTT-containing samples. In the DTT-free control samples, levels were measured at -2 h, 0 h, 0.5 h, 1 h, 2 h, 3 h, 6 h, 12 h and 24 h relative to the DTT addition. The sample "Δtmus53 – Xn – without DTT – 0 h" served as reference sample. Dashed lines correspond to DTT-treated samples and solid lines to DTT-free control samples. Vertical bars indicate the difference between two technical replicates.

In one cultivation experiment, transcript levels of *xyr1*, *xyn2* and *xyl1* were also measured 24 h after DTT addition in Δ*tmus53* on xylan ("Xn2"- data set, **Figure 19**). At 24 h after DTT addition, *xyr1* levels were still identical in the sample with and without DTT. In the DTT-free control samples, *xyn2* transcription was strongly induced by more than a factor of 10 between 6-24 h relative to DTT addition (**Figure 19**). But *xyn2* mRNA levels increased insignificantly during this period when DTT was present. This matches *hac1i, hac1u* and *bip1* data, indicating an activated UPR even 24 h after adding DTT. An insignificant increase was also observed for *xyl1* levels in presence of DTT. *Xyl1* levels of the DTT-free samples decreased insignificantly during this period. Hence, *xyl1* levels of "Xn2" and "Xn2 + DTT" seemly assimilated, contrasting *xyn2* levels.

In the same experiment, transcript levels of the secreted ß-xylosidase BXLI and of the secreted xylanase 1 XYNI were additionally assessed in Δ*tmus53* on xylan ("Xn2"- data set, **Figure 19**). Both genes, *bxl1* and *xyn1*, seem to be RESS targets.

On xylan, DTT affected transcript levels similarly in the Xyr1 overexpression strain TX(WT) as in Δ*tmus53* – except *xyl1* and *ace3* (**Figures 16 & 18**). Like in Δ*tmus53, xyr1* transcript levels did not change significantly (< +0.5 $\log_{10}$-units) and those of *xyn2* decreased after DTT addition. Transcription of *rxe1* was also lowered by DTT like in Δ*tmus53,* but this decrease is considered significant only in TX(WT) (-0.7-(-1) $\log_{10}$-units). Remarkably, *rxe1* levels were 10-times higher in TX(WT) than in *Δtmus53* in the samples without DTT. However, when DTT was added similar *rxe1* levels were measured in both strains. X*yl1* transcript levels did not change significantly after the UPR induction in TX(WT) (ignoring the "2 h after DTT"-data point), what contrasts *xyl1* levels in Δ*tmus53.* Transcript levels of *ace3* were comparable in presence and absence of DTT, what differs from rising *ace3* levels after DTT addition in Δ*tmus53*.

The effects of secretion stress were similar in TXY(1) on xylan and in TX(WT) on xylan – except for *rxe1* (**Figures 16 & 18**). Transcript levels of the modified *xyr1* gene increased as slightly as in TX(WT) after DTT addition (<+0.5 $\log_{10}$-units) (**Figure 16**). This slight increase of *xyr1* in presence of DTT could be an artefact due to data variance or could be caused by a down-regulation of *act1* in presence of DTT. Transcript levels of the modified *xyr1* could have also risen due to an up-regulation of *tef1* by the UPR, as the modified *xyr1* gene is under the control of the *tef1* promoter in TXY(1). Transcription of the gene encoding the (hemi-)cellulase activator Ace3 slightly decreased 30 min after DTT addition (**Figure 16**). Then, ace3 levels slightly rose and exceeded levels of the untreated sample 2 h after DTT addition. In all three strains, the temporal trend of *ace3* levels were similar: a minimum 30 min after UPR induction followed by rising *ace3* mRNA levels. However, ER stress significantly enhanced (by > +0.5 $\log_{10}$-units) *ace3* levels only in the wild-type strain Δ*tmus53*. Interestingly, the presence of DTT increased *rxe1* transcript levels by a factor of 3 (0.5 $\log_{10}$-units) in TXY(1) on xylan (**Figure 16**). This contrasts findings for TX(WT) and Δ*tmus53,* where *rxe1* level were decreased by DTT. Therefore, the seemly increased *rxe1* levels could also be an artefact. It should be noted that absolute *rxe1* levels were 1.5 $\log_{10}$-units

lower in TXY(1) than in TX(WT). Regarding the enzymes XYNII and Xyl1, RESS seemed to affect them both as transcript levels were decreased by DTT in TXY(1) (**Figure 18**). The relatively low value for "TXY(1) - *xyl1* – 2 h" is supposedly an artefact. Noticeably, 100-times higher transcript levels of *xyn2* were measured in the Xyr1 overexpression strains, TX(WT) and TXY(1), in comparison to the wild-type like strain Δ*tmus53*. When transcription of the xylanase-encoding gene *xyn2* was lowered by a factor of >3-10 due to ER stress in all strains, *xyn2* levels were still 100-times higher in the Xyr1 overexpression strains compared to Δ*tmus53.*

Conclusions. The results on xylan and lactose show that Xyr1 is not an indirect mediator of RESS. This means RESS is not indirectly mediated by a down-regulation the main (hemi-)cellulase activating transcription factor Xyr1. This finding was indicated by the levels of the mRNA encoding the secreted enzymes, xylanase XYNII and cellobiohydrolase CBHI: *xyn2* and *cbh1* were down-regulated regardless of Xyr1 overexpression. ER stress caused by DTT influenced *xyr1* transcription levels significantly on lactose but not on xylan. Hence, it seems to depend on the cultivation conditions if Xyr1 is a RESS-target. The (hemi-)cellulase activator Ace3 is not a RESS-target as its transcript levels were almost unaffected by DTT. It is safe to assume Ace3 is also not a mediator of RESS. If the transcription factor Rxe1 is mediating RESS by its own down-regulation, was not evident. Data of Δ*tmus53* and TXY(1) on lactose, and of TX(WT) on xylan indicate that *rxe1* is a target of RESS. In contrast, *rxe1* transcription even seemed to be up-regulated by the ER stress response on xylan in TXY(1).

The amount of wild-type Xyr1 seems to influence *rxe1* transcription as overexpression of the wild-type Xyr1 increased rxe1 levels approx. 10-times. In contrast, *rxe1* levels similar as in Δ*tmus53* were measured when overexpressing the DNA-binding domain of Xyr1 fused to the C-terminus of Ypr1 in TXY(1). Therefore, Xyr1 might be a trans-regulatory element for *rxe1* transcription interacting via its C-terminus. Notably, Rxe1 has been shown to be an activator of Xyr1 (Wang et al. 2019). This means, my theory would only hold true, if there was a mutual regulatory relationship between Rxe1 and Xyr1. Research is needed to support this theory. Transcript levels of *xyr1* and *rxe1* decreased temporarily in parallel on lactose with DTT. This indicates rather a co-regulation of both genes during RESS than an indirect regulation of *xyr1* by a down-regulation of *rxe1*, or vice versa.

Further studies are required to elucidate which transcription factor - other than the repressor Cre1 (Pakula et al. 2003), or the activators Hac1, Xyr1, Rxe1 and Ace3 - mediates the repression under secretion stress. A study by Pakula et al. (2003) has implied that the cellulase-encoding gene *cbh1* is repressed by a transcription factor under secretion stress. This was indicated by the dependence of the down-regulation on the length of the *cbh1* promoter. Pakula et al. also found that the *cbh1* promoter does not contain consensus UPR elements which means that RESS of *cbh1* is unlikely to be mediated by the UPR transcription factor Hac1. The strongest reduction of *xyr1*,

cbh1 and *egl1* mRNA on lactose occurred 1 – 2 h after adding DTT in the present study The apparent lag time of the down-regulation of *cbh1*, *egl1* and *xyr1* in Δ*tmus53* on lactose might therefore stem from the time required to express the repressor. This would match the study by Hollien and Weissman (2006), reporting a time lag between UPR activation and RESS when genes were down-regulated by a transcription factor (by the Hac1-analogue XBP-1 in Drosophila S2 cells). Hence, a transcription factor-mediated RESS mechanism might exist for the *xyr1* gene and both cellulases-encoding genes, *cbh1* and *egl1*, in presence of lactose.
As *xyr1* and *rxe1* were also down-regulated on lactose, a cumulative effect of direct *cbh1* and *egl1* down-regulation by binding of a repressor, and an indirect down-regulation by lowered amounts of transcription activators is assumed.

Interestingly, the down-regulation mechanism under secretion stress seems to depend on the carbon-source and on the secreted enzyme itself.
The ER stress caused by DTT decreased *xyr1* and *rxe1* transcription levels significantly on lactose but not on xylan. Even if the constant relative transcript levels of *xyr1* and *rxe1* in Δ*tmus53* on xylan with DTT were an artefact (as discussed above), a decrease of *xyr1* levels by DTT must have been still less pronounced on xylan than on lactose. This was indicated by the sar1-normalized transcript levels of *xyr1* for example (data not shown).
Another difference between xylan and lactose cultures was that the gene encoding the secreted xylanase XYNII was down-regulated on xylan immediately after DTT addition, but the *cbh1* gene on lactose particularly 1 – 2 h after DTT addition. In addition, *xyn2* mRNA level decreased in total less than those of *cbh1* (**Figures 17 & 18**). A post-transcriptional mechanism, that rapidly reduces transcript levels to alleviate the ER stress caused by accumulated unfolded proteins, is called regulated Ire1-dependent degradation (RIDD) (Hollien et al. 2009; Hollien and Weissman 2006). RIDD describes the sequence specific cleavage of mRNA by Ire1, which also cleaves *hac1u* mRNA to signal unfolded proteins in the ER (Maurel et al. 2014). Even though the existence of RIDD in filamentous fungi has not been proved yet, the fast decline of *xyn2* levels here might be a first clue for its existence in *T. reesei*. To confirm this and other RIDD targets, further research is needed. As a first step, for example, the consensus sequence of the Ire1-cleaving sites located in a stem-loop (Saloheimo et al. 2003; Maurel et al. 2014) could be searched for in the *xyn2* mRNA sequence. Next, it must be ensured that the fast reduction of *xyn2* mRNA is not a result of a quickly binding transcription factor in combination with a low mRNA stability of *xyn2*.
A further difference between xylan and lactose data were the temporal developments of the intracellular carbohydrate-active enzymes BGLII and Xyl1. BGLII and Xyl1 are involved in catabolizing lactose and xylan, respectively. *Bgl2* transcript levels on lactose seemed in another study (Pakula et al. 2003) and here to be independent of the UPR. In contrast, *xyl1* transcript levels on xylan were reduced quickly by a factor of approx. 10 in the wild-type like strain. It remains

unclear whether *xyl1* is a target of RESS or RIDD, as a clear mRNA reduction was observed in Δ*tmus53* and TXY(1), but not in TX(WT). However, due to the immediate decrease of *xyl1* transcript levels in Δ*tmus53* and TXY(1), mRNA reduction by RIDD seems more likely than a transcription factor mediated-down-regulation like RESS. As for *xyn2*, the consensus cleaving sequence of Ire1 should be searched for in the mRNA sequence of *xyl1* next. In addition, the mRNA stability should be assessed to exclude it as the reason for the fast *xyl1* mRNA decline in presence of DTT.

Outlook. In order to validate my results on a statistical basis, the experiment must be repeated. For future research, I suggest using an additional housekeeping gene for normalization of qPCR data, like for example *gpd1 (Pakula et al. 2003; Valkonen et al. 2004),* and RNA-Seq to validate qPCR data.

Further experiments will unravel the RESS-mediating transcription factor, the existence of RIDD in *T. reesei*, as well as the role of Rxe1 in the regulation of Xyr1 - and vice versa.

In the context of exploiting the knowledge about the UPR for industrial enzyme production, not only RESS and RIDD as mechanisms reducing transcript levels should be investigated. In other to reduce the workload of the ER, *Aspergillus niger* seems to have developed another strategy than RESS to control transcript levels upon ER stress post-transcriptionally. A study in *Aspergillus niger* showed that some genes of secreted proteins and of proteins involved in ribosomal biogenesis and assembly were affected by differential translation upon DTT-mediated ER stress. (Guillemette et al. 2007). However, a similar study in *Aspergillus fumigatus* yielded contradictory results to those of *Aspergillus niger* (Krishnan et al. 2014). It would be interesting to find out whether such post-transcriptional regulation exists in *T. reesei*.

5 Conclusion

Overexpression of the wild-type and fusion-Xyr1 enhances the secretion of native enzymes without inducing the UPR. Hence, the protein folding capacity of the ER is not surpassed yet in these Xyr1 overexpression strains.

Xyr1 appears to be a UPR- or RESS-target on lactose and might be significantly less (or not all) repressed under secretion stress on xylan. *Ace3* transcription seems to be also no UPR- or RESS-target. Regarding the mediation of RESS during the UPR, we speculated an indirect mediation of RESS by the down-regulation of the (hemi-)cellulase activators Xyr1, Ace3 or Rxe1. Neither of the two the (hemi-)cellulase activators - Xyr1 and Ace3 – seems to mediate RESS. The role of Rxe1 in RESS and as UPR- or RESS-target remains unclear due to inconsistent findings in the three strains investigated – but worth future research.

Different mechanisms for the down-regulation of secreted enzymes in dependence of the cultivation conditions might exist in *T. reesei.* On the one hand, transcript levels of hemicellulose- and cellulose-degrading enzymes showed a different temporal decrease during ER stress. On the other hand, the absolute change of transcript levels of the genes encoding UPR indicators and transcriptions factors was different for xylan- and lactose-grown samples.

In addition, elevated rxe1 transcript levels by the overexpression of wild-type Xyr1 suggest a mutual regulatory influence between Rxe1 and Xyr1.

6 Experimental

6.1 Fungal strains

All *T. reesei* strains (Table 1) used in this study were pre-grown on malt extract agar (per 1 L: 1 g peptone, 30 g malt extract, 15 g agar, tap water) at 30 °C in darkness until sporulation and stored at 4 °C for maximal two weeks.

Table 1: Trichoderma reesei strains used in this study.

Designation	Description	Source
Δ*tmus53*	Wild-type-like strain with deficiency of the non-homologous end joining repair pathway	(Steiger et al. 2011)
TX(WT)	Xyr1-overexpressing strain; the wild-type *xyr1* under the control of the strong constitutive *tef1* promoter inserted at the *pyr4* locus; uridine prototrophy re-established	(Derntl et al. 2019)
TXY(1)	Overexpression of the fusion transcription factor XY1: N-terminal DNA-binding domain of Xyr1 fused to the C-terminal transactivation domain of Ypr1; the fusion gene under the control of the strong constitutive *tef1* promoter is inserted at the *pyr4* locus; uridine prototrophy re-established	(Derntl et al. 2019)

6.2 Transcription Analysis

Cultivation Conditions

For cultivation in liquid medium, *T. reesei Δtmus53*, TX(WT), and TXY(1) conidiospores younger than 2 weeks were harvested from agar plates and suspended in 0.8% (w/v) NaCl/ 0.05% (v/v) Tween 80 by glaswool filtration. *T. reesei* strains were grown in 50 ml Mandels-Andreotti (MA) medium (Mandels 1985) containing 10 g L^{-1} glucose monohydrate, xylan from beechwood (Carl Roth GmbH + Co KG, Karlsruhe, Germany), or lactose in 250 ml shake flasks at a starting OD_{600} = 0.05. Flasks were shaken at 180 rpm at 30 °C. In case of 10 g L^{-1} carboxymethyl cellulase (CMC) containing MA medium, *T. reesei* was grown in 60 ml medium in 1 L flasks at 30 °C without shaking. To induce the UPR, 20 mM Dithiothreitol (DTT) was added to liquid cultures, when enough mycelium for sampling had grown (Table 2). For the cultivation of TXY(1) on lactose, 6 month-old conidiospores were exceptionally used because the cultivation had to be repeated and then no fresh conidiospores were available anymore. The reason for the repetition was that fresh conidiospores hardly germinated and outgrew within 24 h. Due to a shortage in conidiospores, the starting OD_{600} was only 0.02 for TXY(1) on lactose.

Table 2: Incubation time before DTT addition in dependence of the strain and the medium.

Medium	Incubation Time Before DTT Addition
MA + Xylan	24 h (*Δtmus53*), 46 h (TX(WT)), 47 h (TXY(1))
MA + Lactose	36.5 h (*Δtmus53*), 69.5 h (TXY(1))

Table 3: Number of biological replicates per cultivation condition.

Number of biological replicates for:	Δtmus53	TX(WT)	TXY(1)
data shown in section 4.3	3	3	3
data shown in section 4.1, 4.2, 4.4	4 cultures with DTT 4 cultures w.o. DTT	2 cultures with DTT 2 cultures w.o. DTT	on xylan: 4 cultures with DTT 4 cultures w.o. DTT on lactose: 2 cultures with DTT 2 cultures w.o. DTT

Sampling

Mycelia were harvested by filtration through Miracloth (EMD Millipore, part of Merck KGaA, Darmstadt, Germany), washed with deionized water and dried with filter paper (Cat.-No. 1001090, Whatman, Maidstone, England). Mycelia of 2-4 biological replicates were pooled (Table 3). Samples were stored at -196 °C (liq. N_2) on the day of sampling and were then transferred to a -80 °C freezer.

RNA Extraction

Approx. 10-30 mg of harvested mycelia were homogenized by glass beads (0.37g 0.1 mm-diameter beads, 0.25 g 1 mm-diameter beads, one 5 mm-diameter bead) in 1 ml of acidic guanidinium thiocyanate-phenol-chloroform reagent (peqGOLD TriFast DNA/RNA/protein purification system reagent, VWR, part of Avantor Performance Materials, LLC, Radnor, PA, USA; or TRIzol reagent, Thermo Fisher Scientific, Waltham, USA (used for experiments of section 4.2 and 4.4 for strain TXY(1))). A FastPrep FP120 BIO101 ThermoSavant cell disrupter (Qbiogene, Carlsbad, US) was used. RNA was isolated in accordance to the manufacturer's protocol (VWR peqlab 2020), and RNA concentration was measured using the NanoDrop ONE spectrometer (Thermo Scientific, Waltham, USA).

Transcript analysis by RT-qPCR

To remove residual DNA, 0.6875 µg of isolated RNA was treated by DNaseI (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions (Thermo Scientific 2020). Then, RNA was reverse transcribed into cDNA using LunaScript RT SuperMix (NEB, Ipswich, USA) according

to the manufacturer's instructions (New England BioLabs Inc. 2020b). For qPCR analysis, the cDNA was diluted 1:50 and 2 µl were used as template in a 15-µl reaction using Luna Universal qPCR Master Mix (NEB, Ipswich, USA) according to the manufacturer's instructions (New England BioLabs Inc. 2020a). Primers used are listed in Table 4. All reactions were performed in duplicates on a Rotor-Gene Q system (Qiagen, Hilden, Germany). Relative transcript levels were calculated based on the efficiency calibrated Double Delta C_t method (Pfaffl 2001): The expression level of a target gene is normalized with the transcript level of a stably expressed reference gene of the same sample. This accounts for differences in cDNA input between samples. To compare transcript levels between samples, the normalized expression level of the target gene in a sample is compared to a reference sample. The calculation of the relative transcript level is based on the qPCR efficiencies E given by the slopes of the fluorescence curves ($E = 10^{[-1/slope]}$), and the threshold cycle C_t where the fluorescence rises appreciably above the background fluorescence (**Formula 1**).

(1)

$$relative\ transcript\ level = \frac{\frac{RNA\ copies\ of\ target\ gene\ in\ sample}{RNA\ copies\ of\ ref.gene\ in\ sample}}{\frac{RNA\ copies\ of\ target\ gene\ in\ ref.sample}{RNA\ copies\ of\ ref.gene\ in\ ref.sample}} = \frac{\frac{E_{target_{ref.sample}}^{Ct_{target_{ref.sample}}}}{E_{ref.gene_{ref.samples}}^{Ct_{ref.gene_{ref.sample}}}}}{\frac{E_{target_{sample}}^{Ct_{target_{sample}}}}{E_{ref.gene_{sample}}^{Ct_{ref.gene_{sample}}}}} =$$

$$= \frac{E_{ref.gene_{sample}}^{Ct_{ref.gene_{sample}}} * E_{target_{ref.sample}}^{Ct_{target_{ref.sample}}}}{E_{target_{sample}}^{Ct_{target_{sample}}} * E_{ref.gene_{ref.sample}}^{Ct_{ref.gene_{ref.sample}}}}$$

$E_{target_{sample/ref.sample}}$ ..... RT-qPCR efficiency of target gene transcript in sample/ in reference sample
$Ct_{target_{sample/ref.sample}}$ .....Threshold cycle of target gene transcript in sample/ in reference sample
$E_{ref.gene_{sample/ref.sample}}$ ..... RT-qPCR efficiency of reference gene transcript in sample/ in reference sample
$Ct_{ref.gene_{sample/ref.sample}}$ .....Threshold cycle of reference gene transcript in sample/ in reference sample

Reference genes in this study were *sar1* (gene involved in secretion pathway: small GTPase involved in vesicle budding from the ER) and *act1* (actin 1 gene, important for exocytosis, endocytosis, organelle movement and cytokinesis in fungi (Berepiki et al. 2011)). As reference sample data points in section 4.3 served the mean C_t-value of the sample "*Δtmus53* – glucose – 20 h". In UPR induction experiments based on DTT addition (sections 4.1, 4.2, 4.4), the reference

samples were "*Δtmus53* – xylan - 0 h" for transcripts of *hac1i, hac1u, bip1, xyr1, ace3, rxe1, xyn2* and *xyl1*, and the sample "*Δtmus53* – lactose - 0 h" for transcripts of *egl1, bgl2, cbh1*.

qPCR efficiencies were set to 1.76, which corresponds to the mean efficiency of all data (as discussed in section 4.1).

Table 4: Primers used for RT-qPCR.

Name		Sequence (5'-3')
A772	**sar1fw**	TGG ATC GTC AAC TGG TTC TAC GA
A773	**sar1rev**	GCA TGT GTA GCA ACG TGG TCT TT
A774	**act1fw**	TGA GAG CGG TGG TAT CCA CG
A775	**act1rrev**	GGT ACC ACC AGA CAT GAC AAT GTT G
A860	**hac1i fw**	CAC AAC GTC CTG CAG TGT CA
A861	**hac1u fw**	ACA ACG TCC TGC AGA GAT GT
A862	**hac1rev**	AGA AGT TGG CTT TCG AGG AG
A863	**bip1 fw**	CAT TGT CTA CGA TCT CGG TGG
A864	**bip1 rev**	GAT TTC GAT ACG AGT GCT CATC
A347	**xyr1_q2f**	TCC GTC GCT ATT CTG CCT AC
A348	**xyr1_q2r_wt_1**	CAG CAG TAC CCG TTG AAT TC
A776	**cbh1fw**	GAT GAT GAC TAC GCC AAC ATG CTG
A777	**cbh1rev**	ACG GCA CCG GGT GTG G
A792	**bgl2fw**	ACG GCT GCC TAC CAG ATC G
A793	**bgl2rev**	AGC CGT CGG CGA TCT TGC
A408	**xyn2_q1f**	CCG TCA ACT GGT CCA ACT CG
A409	**xyn2_q1r**	GTG CGG TAA ATG TCG TAG ACG
A784	**XORf (xyl1-fwd)**	CTG TGA CTA TGG CAA CGA AAA GGA G
A785	**XORr (xyl1-rev)**	CAC AGC TTG GAC ACG ATG AAG AG
340	**bxl1_q1fwd**	GAA TGA CAT GAA CCT CCG ACC
341	**bxl1_q1rev**	CGA AGG TGA AGA CGG GAA TC
A782	**taqxyn1f**	CAG CTA TTC GCC TTC CAA CAC
A783	**taqxyn1r**	CAA AGT TGA TGG GAG CAG AAG

7 Abbreviations

CMC	Carboxymethyl-Cellulose
C_t	Threshold cycle of a qPCR
DTT	Dithiothreitol
ER	Endoplasmic reticulum
OD_{600}	Optical density at 600 nm

Proteins:

Ace1-3	Activator of cellulase expression 1-3
BGLII	ß-Glucosidase 2
BiP1	Binding protein 1
BXLI	ß-Xylosidase 1
CBHI	Cellobiohydrolase 1
Cre1	Carbon catabolite repressor protein 1
EGLI	Endoglucanase 1
Ire1	Inositol-requiring enzyme 1
Rxe1	Regulator 1 of Xyr1 expression
Xyl1	D-Xylose reductase 1
XYNI, II	Xylanase 1, 2
Xyr1	Xylanase regulator 1
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RESS	Repression under secretion stress
RIDD	Regulated Ire1-dependent decay
TX(WT)	*Trichoderma reesei* strain overexpressing the wild-type transcription factor Xyr1
TXY(1)	*Trichoderma reesei* strain overexpressing a fusion transcription factor of Xyr1 and Ypr1
UPR	Unfolded protein response

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