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ORIGINAL RESEARCH PAPER

Identification of functional *cis*-elements required for repression of the Taka-amylase A gene under secretion stress in *Aspergillus oryzae*

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Abstract The expression of secreted proteins in filamentous fungi is down-regulated by a transcriptional feedback mechanism under endoplasmic reticulum stress, termed repression under secretion stress (RESS). To investigate the RESS mechanism, we analyzed the expression of the Taka-amylase A gene (*amyB*) in *Aspergillus oryzae*, which was depressed under secreted protein stress. We conducted a truncation and deletion analysis of the *amyB* promoter to identify *cis*-elements required for RESS. A nucleotide sequence (positions −378 to −291) without any binding sites for the transcriptional activator AmyR, which is involved in amylolytic gene expression, was required for RESS. The octamer sequence TCACGG GC (positions −307 to −300) constituted the core sequence of the upstream activating element essential for *amyB* down-regulation under secretion stress. Both the inactivation of AmyR and RESS contributed to the down-regulation of *amyB* expression under ER stress.

Keywords *Aspergillus oryzae* · Quantitative real-time PCR · Repression under secretion stress · Taka-amylase A gene promoter · Truncation and deletion analysis

Introduction

Aspergillus oryzae, an organism generally recognized as safe (GRAS), can produce large quantities of proteins, making it a suitable host for protein production (Nevalainen et al. 2005; Ward et al. 2006). Compared to the large amounts of homologous proteins produced by *A. oryzae*, the production of heterologous proteins is often limited (Conesa et al. 2001; Gouka et al. 1997). Inefficient protein transit, sorting, and secretion of heterologous proteins result in the accumulation of unfolded proteins in endoplasmic reticulum (ER). The accumulated unfolded proteins in the ER activate the unfolded protein response which leads to the activation of a series of genes involved in protein folding, glycosylation, transport, and degradation of misfolded proteins (Mori 2000; Pakula et al. 2003; Travers et al. 2000). Under ER stress, the expression of secreted proteins is down-regulated in filamentous fungi, including *Trichoderma reesei* (Pakula et al. 2003), *Aspergillus niger* (Guillemette et al. 2007), *Aspergillus nidulans* (Sims et al. 2005), and *A. oryzae* (Wang et al. 2010), which is termed repression under secretion stress (RESS).

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RESS, as a transcriptional feedback mechanism, is activated in response to the impairment of protein folding or transport, and aims to reduce protein stress in the secretory pathway when ER stress is sensed (Pakula et al. 2003). The cellobiohydrolase I (*cbh1*) and glucoamylase (*glaA*) genes have been used as models to investigate the RESS mechanism in *T. reesei* and *A. niger*, respectively (Al-Sheikh et al. 2004; Pakula et al. 2003). However, the *cis*-element of the secretory protein promoters that is required for RESS is still unknown.

In *A. niger*, over-expression of glucoamylase and constitutive activation of the HacA bZIP transcription factor activate RESS, while the transcription factor AmyR and AmyR-dependent hydrolase genes are down-regulated during RESS. The down-regulation of AmyR and its target genes reflects a negative feedback mechanism (Carvalho et al. 2012; Kwon et al. 2012). However, the *cis*-elements and *trans*-acting factors that down-regulate the *amyR* gene are unknown.

We used the Taka-amylase A gene (*amyB*) promoter, which contained an AmyR binding site, to control the expression of the *Rhizomucor miehei* lipase (*rml*) gene, which was fused to an AmyB signal peptide, as a model to investigate the RESS mechanism, while the *amyB* gene itself was used as a control. A detailed truncation and deletion analysis of the *amyB* promoter was used to identify the *cis*-element required for RESS in *A. oryzae* under secretion stress, which was induced by treatment with dithiothreitol (DTT). A sequence from −378 to −291 in the *amyB* promoter, which lacked an AmyR binding site, was essential for RESS, and the octamer sequence TCACGGGC (positions −307 to −300) constituted the core sequence of the upstream activating element essential for *amyB* down-regulation under secretion stress.

Materials and methods

Strains and growth conditions

Aspergillus oryzae niaD300, a *niaD* mutant derived from the *A. oryzae* RIB40 wild-type strain, was obtained from the NITE Biological Resource Center in Japan and used in all transformation experiments. Dextrin/peptone/yeast extract (DPY) medium (Hisada et al. 2008) was modified to contain 2 % (w/v) glucose, 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.1 % (w/v) K_2HPO_4, and 0.05 % (w/v) $MgSO_4{\cdot}7H_2O$. Czapek-Dox (CD) medium (Wang et al. 2010) was modified to contain 2 % (w/v) glucose, 0.3 % (w/v) $NaNO_2$, 0.1 % (w/v) K_2HPO_4, 0.2 % KCl, 0.05 % (w/v) $MgSO_4{\cdot}7H_2O$, and 0.001 % (w/v) $FeSO_4{\cdot}7H_2O$, pH 5.5. The CD-P medium (Tsuchiya et al. 1992) was modified to contain 2 % (w/v) glucose, 0.3 % (w/v) polypeptone, 0.1 % (w/v) K_2HPO_4, 0.2 % (w/v) KCl, 0.05 % (w/v) $MgSO_4{\cdot}7H_2O$, and 0.001 % (w/v) $FeSO_4{\cdot}7H_2O$, pH 5.5. *Escherichia coli* DH5α and the pPTRI vector containing the pyrithiamine (PT)-resistance gene (*ptrA*) were used in DNA manipulations and purchased from TaKaRa (Shiga, Japan).

Transformation experiments

Aspergillus oryzae niaD300 mycelia were grown in DPY medium at 30 °C for 24 h and transformed as described (Gomi et al. 1987). Transformants were selected using CD medium containing 0.1 mg pyrithiamine/l (Kubodera et al. 2000).

Construction of *amyB* promoter deletion mutants

Using *A. oryzae* genomic DNA as a template, the 1.1 kb promoter of the *amyB* gene (AO090120000196) was amplified with primers pd0 (forward) and pd0d (reverse), and the alpha-glucosidase gene (*agdA*) terminator (E12508.1) was amplified with primers cdpu (forward) and cdp (reverse). The *rml* gene, fused to the *amyB* secretion signal, was amplified using primers RML-F (forward) and RML-R (reverse) with vector pNMA-RML (Wang et al. 2009) as the template. The above three amplification fragments were mixed and subjected to a second round of PCR amplification using primers pd0 and cdp to construct the *rml* expression cassette. Primers pd0 and cdp were tailed with 5′-ATACGGGGTACC-3′ sequences to generate a *Kpn*I site at the 5′-end. The expression cassette was digested with *Kpn*I and introduced into vector pPTRI using the same site, which resulted in the construction of plasmid pPTA1110. 5′-Truncations of the *amyB* promoter (Del1–Del8, PTA182) were amplified by PCR using vector pPTA1110 as the template. The forward primers (pd1–pd9) and the universal reverse primer (cdp) were tailed with a 5′-ATACGGGGTACC-3′ sequence to generate a *Kpn*I site at the 5′-end. Internal deletions were introduced

into the *amyB* promoter by the combined PCR method (Yu et al. 2004) using pPTA1100 as the template. Primer sets pd0/pd10u + pd10d/cdp, pd0/pd11u + pd11d/cdp, pd0/pd12u + pd12d/cdp, pd0/pd13u + pd13d/cdp, pd0/pd14u + pd14d/cdp, pd0/pd15u + pd15d/cdp, pd0/pd16u + pd16d/cdp were used to construct the following deletions in the *amyB* promoter region: −378 to −291, −378 to −358, −419 to −358, −358 to −291, −342 to −318, −329 to −291, and −291 to −234, respectively. The 5′-fragments were amplified by PCR using the universal forward primer pd0 and the reverse primers pd10u–pd16u, respectively. The 3′-fragments were amplified using the universal reverse primer cdp and forward primers pd10d–pd16d, respectively. To obtain site-specific, deletion promoter mutants, the 5′- and 3′-fragment PCR products were mixed and subjected to a second round of PCR amplification using primers pd0 and cdp. Promoter mutants with truncations or internal deletions were digested with *Kpn*I, and introduced individually into vector pPTRI using the same site. All primers are shown in Supplementary Table 1; primers pd10u and pd11u have the same sequence.

Quantitative real-time PCR (qRT-PCR) analysis of *A. oryzae* cultures treated with DTT

Spores (2×10^8) were inoculated into 200 ml modified CD-P medium and cultivated in conical flasks at 30 °C with shaking at 200 rpm. After cultivation for 24 h (mid-growth phase), cultures were diluted 1/10 (1:9, v/v) into fresh medium and grown for an additional 6 h in the presence of 20 mM DTT. The same amount of water was added to the control cultures. The mycelia were filtered through filter paper and frozen immediately in liquid nitrogen and stored at −80 °C. Total RNA was extracted using RNAisoPlus (TaKaRa) and then treated with RNase-free DNase I (TaKaRa) for 45 min according to the manufacturer's protocols. First-strand cDNA synthesis was performed with the PrimeScriptRT–PCR Kit (TaKaRa) according to the manufacturer's instruction. There was no PCR product in the negative control lacking reverse transcriptase.

qRT-PCR primers for glyceraldehyde-3-phosphate dehydrogenase (*gapdh*, AO090011000414) and *hacAi* (a spliced form of the *hacA* gene, AO090124000074) were used as previously described (Ye and Pan 2008). qRT-PCR primers for *amyB, rml* and *amyR* (AO090003001208) were designed with Primer3 Plus (Untergasser et al. 2007). All qRT-PCR primers were experimentally validated with two quality control (specificity and efficiency) assays; primers are listed in Supplementary Table 2. For each pair of primers, the amplification specificity was checked by both melting curve analysis and gel electrophoresis (Supplementary Figs. 1–5), and the amplification efficiency was calculated from the slope of a linear regression model according to the following equation for PCR efficiency: $(E) = 10^{-1/\text{slope}} - 1$ (Bustin et al. 2009). The efficiency of each primer pair (Supplementary Figs. 1–5) was assessed by plotting the cycle threshold value (Ct) against the logarithm of the dilution of the standard plasmids (Lee et al. 2006; Sun et al. 2012), pMDGHR and pMDGAA, which ranged from 10^4 to 10^8 copies/μl. A standard plasmid, pMDGHR, was constructed which contained the *gapdh*, *hacAi* and *rml* genes. The *gapdh* gene was amplified from *A. oryzae* cDNA by PCR using the primers Pg1/Pg2 (Supplementary Table 1); the full length of the *hacAi* cDNA was amplified by PCR using cDNA from DTT-treated mycelia as a template (Nakajima et al. 2006) and primers Ph1/Ph2 (Supplementary Table 1); the *rml* gene was amplified using the vector pPTA1110 as the template and primers RML-F/RML-R (Supplementary Table 1). The above three amplification fragments were mixed and subjected to a second round of PCR amplification using primers Pg1/RML-R and subcloned into the pMD20T vector using the TA cloning PCR kit (TaKaRa). A standard plasmid, pMDGAA, was constructed, which contained the *gapdh*, *amyB* and *amyR* genes. Using *A. oryzae* cDNA as a template, the *amyB* gene was amplified using primers Pb1/Pb2, and the *amyR* gene was amplified with primers Pa1/Pa2. The *gapdh*, *amyB* and *amyR* genes were mixed and subjected to a second round of PCR amplification using primers Pg1/Pa2, and then subcloned into the pMD20T vector using the TA cloning PCR kit (TaKaRa).

qRT-PCR was performed using the generated cDNA as the template, SYBR Premix Ex Taq II (Tli RNaseH Plus, TaKaRa) and a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Each 20 μl reaction mix contained 2 μl template, 10 μl 2× SYBR Premix and 0.4 μl (20 μM) of each primer. The reaction program was as follows: an initial denaturation 95 °C for 30 s, followed by 40 cycles of 5 s at

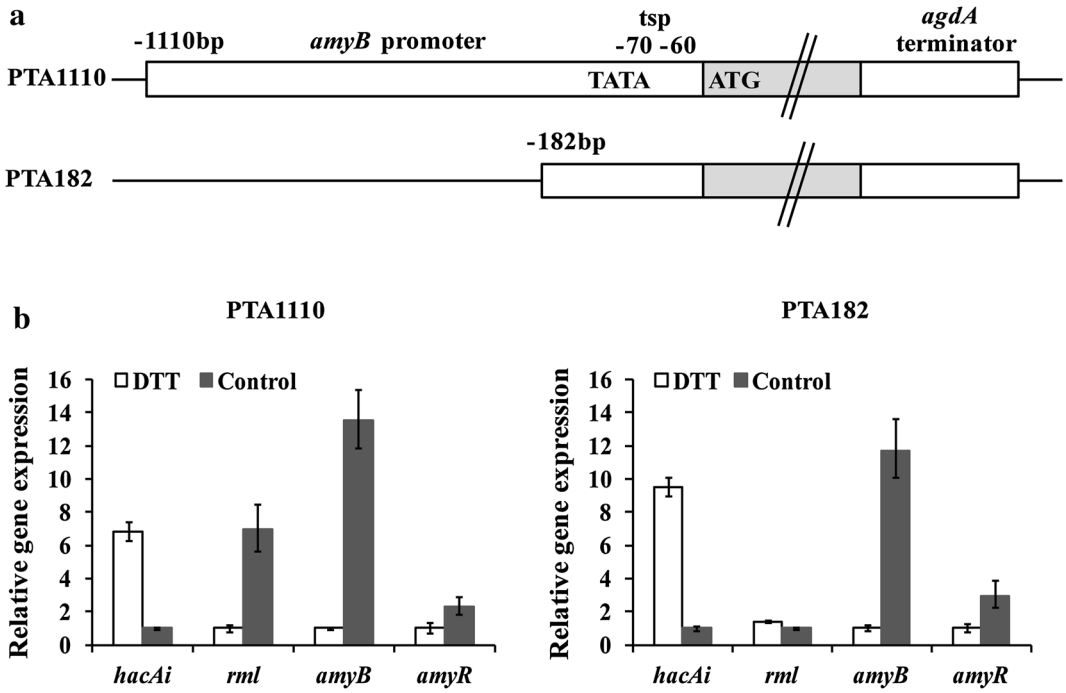

Fig. 1 Transcriptional down-regulation of *amyB* by a specific region of its promoter. **a** Schematic presentation of the *rml* expression cassette used in this study. *rml* was expressed under the control of the full-length *amyB* promoter in the strain transformed with the cassette PTA1110, and under a truncated promoter in the strain transformed with PTA182. The promoter regions of the *amyB* gene and the terminator region of the *agdA* gene are indicated by *open boxes*. The coding region of the *rml* gene is indicated by a *gray box*. *tsp* transcription start site. **b** qRT-PCR analysis of *hacAi, amyB*, *rml* and *amyR* genes expression in cultures 120 min after treatment with either water (control, *black bar*) or 20 mM DTT (*white bar*). The *gapdh* gene was an used as endogenous control to normalize the data (Ye and Pan 2008). All primers are listed in Supplementary Table 2. Each sample was analyzed in triplicate, and the values are the means of three replicates ± S.D

95 °C, and 34 s at 60 °C. The results were analyzed using the comparative CT ($2^{-\triangle\triangle CT}$) method (Bustin et al. 2009; Livak and Schmittgen 2001).

Results and discussion

Dependence of secretory protein mRNA regulation on the promoter region of *amyB* under RESS

To confirm that the feedback regulation of the mRNA encoding the endogenous secreted protein AmyB was mediated by its promoter in *A. oryzae* under secretion stress, we used *rml* fused to the AmyB signal peptide, which was expressed under the control of the full-length (1,110 bp) *amyB* promoter (PTA1110) Fig. 1 and a shortened promoter consisting of 182 bp (PTA182), respectively (Fig. 1a), as the secreted protein model (Fig. 1a). The 182 bp promoter contains a putative TATA box and the transcription start sites (Tsuchiya et al. 1992). qRT-PCR analysis indicated that the expression levels of *hacAi* with splicing the unconventional intron under DTT treatment were increased 6.8- and 9.5-fold relative to no DTT treatment in the PTA1110 and PTA182 transformants (Fig. 1b), respectively, which confirmed that the cells of *A. oryzae* were under ER stress. Under ER stress, the expression levels of *amyB* in the PTA1110 and PTA182 transformants were decreased 13.5- and 11.7-fold, respectively, as compared to controls (Fig. 1b), which indicated that the repression of *amyB* expression under RESS was consistent with that of the *cbh1* in *T. reesei* (Pakula et al. 2003) and the *glaA* in *A. niger* (Al-Sheikh et al. 2004).

The *rml* expression level of PTA1110 under the control of the full-length promoter following DTT treatment was decreased 6.9-fold as compared to control, which was similar to the behavior of *amyB*, while the *rml* expression level of PTA182 under the

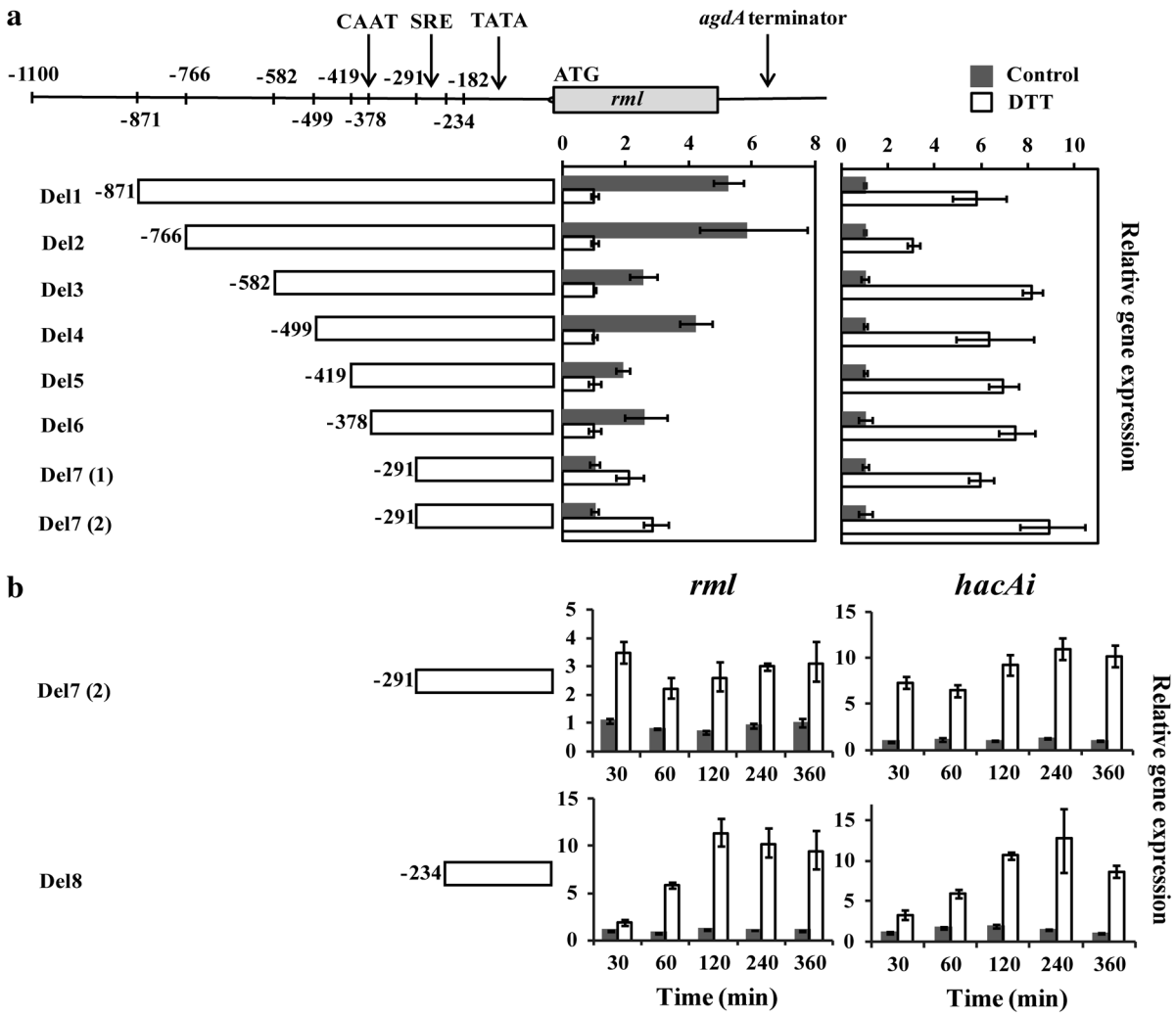

Fig. 2 Sequential 5′-truncation of the *amyB* promoter and the effect on *amyB* transcription. **a** qRT-PCR analysis of *rml* and *hacAi* gene expression of strains harboring deletion promoter plasmids in cultures 120 min after treatment with either water (control, *black bar*) or 20 mM DTT (*white bar*). The endogenous control gene was as described for Fig. 1. The promoter regions of the *amyB* gene are indicated by *open boxes*. The coding region of the *rml* gene is indicated by a *gray box*. The terminator region of the *agdA* gene is indicated by a *black line*. Numbers for the deletion mutants denote the distances in base pairs from the initiation ATG codon at +1 to the deletion endpoint. CAAT, a putative CAAT element at position −377 (Tsuchiya et al. 1992). TATA a putative TATA box at position −100 (Tsuchiya et al. 1992). SRE the binding site for the transcription factor AmyR at position −264 (Yoshino-Yasuda et al. 2013). **b** qRT-PCR analysis of *rml* and *hacAi* gene expression of the truncated *amyB* promoters in strains cultured for 30, 60, 120, 240 and 360 min after treatment with either water (control, *black bar*) or 20 mM DTT (*white bar*). Each sample was analyzed in triplicate, and the values are the means of three replicates ± S.D

control of the shortened promoter (182 bp) was increased 1.4-fold under ER stress (Fig. 1b). Therefore, we speculated that the sequence elements located between nucleotides −1110 and −182 in the *amyB* promoter were essential for the down-regulated expression of *rml* under ER stress. A similar result was found for sequence elements located between nucleotides −2200 and −161 in the *cbh1* promoter, which were required for RESS in *T. reesei* (Pakula et al. 2003).

The expression of *amyR*, which encodes a transcriptional activator required for amylolytic gene expression, were decreased 2.3-fold and threefold as compared to no DTT treatment in the PTA1110 and

PTA182 transformants, respectively (Fig. 1b). This was consistent with results from a study by Wang et al. (2010). It has been speculated that HacA activation could lead to inactivation of the transcriptional factor AmyR, thereby resulting in down-regulation of AmyR target genes (Carvalho et al. 2012), such as *amyB*, although the molecular mechanism of down-regulation of *amyR* under ER stress is unknown.

Identification of *cis*-acting sequence-elements in the *amyB* promoter

To identify the *cis*-elements required for RESS in the *amyB* promoter, we constructed the following truncations in the *amyB* promoter region: nucleotides −871 to −1110 (Del1), −766 to −1110 (Del2), −582 to −1110 (Del3), −499 to −1110 (Del4), −419 to −1110 (Del5), −378 to −1110 (Del6), −291 to −1110 (Del7), and −234 to −1110 (Del8) to control the expression of the model protein RML (Fig. 2). Variations in *hacAi* and *amyB* expression in all strains served as signals of UPR and RESS induction, respectively. A qRT-PCR assay indicated that the expression levels of *rml* were decreased 5.2-, 5.8-, 2.5-, 4.2-, 1.9-, and 2.7-fold as compared to controls following treatment with DTT in the Del1, Del2, Del3, Del4, Del5, and Del6 strains, respectively (Fig. 2a). However, the expression levels of *rml* under the control of nucleotides −291 to the end of the *amyB* promoter were increased 2.1- and 2.8-fold during DTT treatment in the replicated strains Del7(1) and Del7(2), respectively (Fig. 2a).

To confirm that the truncated *amyB* promoter released RESS under ER stress, we analyzed *rml* expression levels under −291 to −1110 and −234 to −1110 regions of the *amyB* promoter during the time-course of DTT treatment. The expression levels of *rml* under both truncated *amyB* promoters were increased during the 360 min time-course of DTT treatment (Fig. 2b). The results indicated that sequence elements of the *amyB* promoter located between nucleotides −377 to −291 were required for the down-regulation. Furthermore, the AmyR binding site is present at nucleotides −264 to −251 of the *amyB* promoter (Yoshino-Yasuda et al. 2013). However, the expression levels of *rml* under the control of the Del7(2) construct containing the AmyR binding site and the Del8 construct without the AmyR binding site were both up-regulated during the time-course of DTT treatment (Fig. 2b). To confirm whether the AmyR binding site was essential for the down-regulated transcription of *amyB* under ER stress, we deleted nucleotides −291 to −234 of the *amyB* promoter region (Del16, Fig. 3), which included the AmyR binding site (−264 to −251). The expression level of *rml* was decreased 3.2-fold as compared to control under DTT treatment (Del16, Fig. 3). This was not consistent with the studies by Carvalho et al. (2012) and Kwon et al. (2012). We inferred that both AmyR inactivation and RESS contribute to the depression of *amyB* expression under ER stress.

Characterization of *cis*-elements conferring repression under secretion stress

To characterize the *cis*-elements conferring RESS, we deleted and fused sequence elements of the *amyB* promoter located at nucleotides −378 to −291 (Del10), −378 to −358 (Del11), −419 to −358 (Del12), −358 to −291 (Del13), −342 to −318 (Del14), and −329 to −291 (Del15) to control the expression of the model protein RML (Fig. 3). The transcriptional level of *rml* under the control of the *amyB* promoter mutant lacking region −378 to −291 was increased 2.9-fold as compared to the control (Fig. 3), which further confirmed that sequence elements located between −377 and −291 in the *amyB* promoter were required for the down-regulation of the targeted gene under ER stress. The *amyB* promoter region located from −378 to −291 is also responsible for high-level expression (Kanemori et al. 1999; Tada et al. 1991; Tsuchiya et al. 1992), and contains three functional elements: a putative CAAT element (−377 to −372), an inverted repeat (−342 to −318), and an octamer sequence TCACGGGC, also termed region I (−307 to −300) (Tsuchiya et al. 1992).

To confirm whether the CAAT element was essential for the down-regulated transcription of *amyB* under DTT stress, the expression levels of *rml* under the control of mutant promoters Del11 and Del12, which did not contain the CAAT element, were decreased 1.8- and 1.4-fold as compared to controls, respectively, under DTT treatment (Fig. 3). The results indicated that the CAAT element in the *amyB* promoter was not essential for the down-regulated expression.

Aspergillus oryzae has three α-amylase genes (*amyA–C*). Although they have almost identical nucleotide sequences, the *amyA* gene was suggested to have a lower transcriptional level than *amyB* or

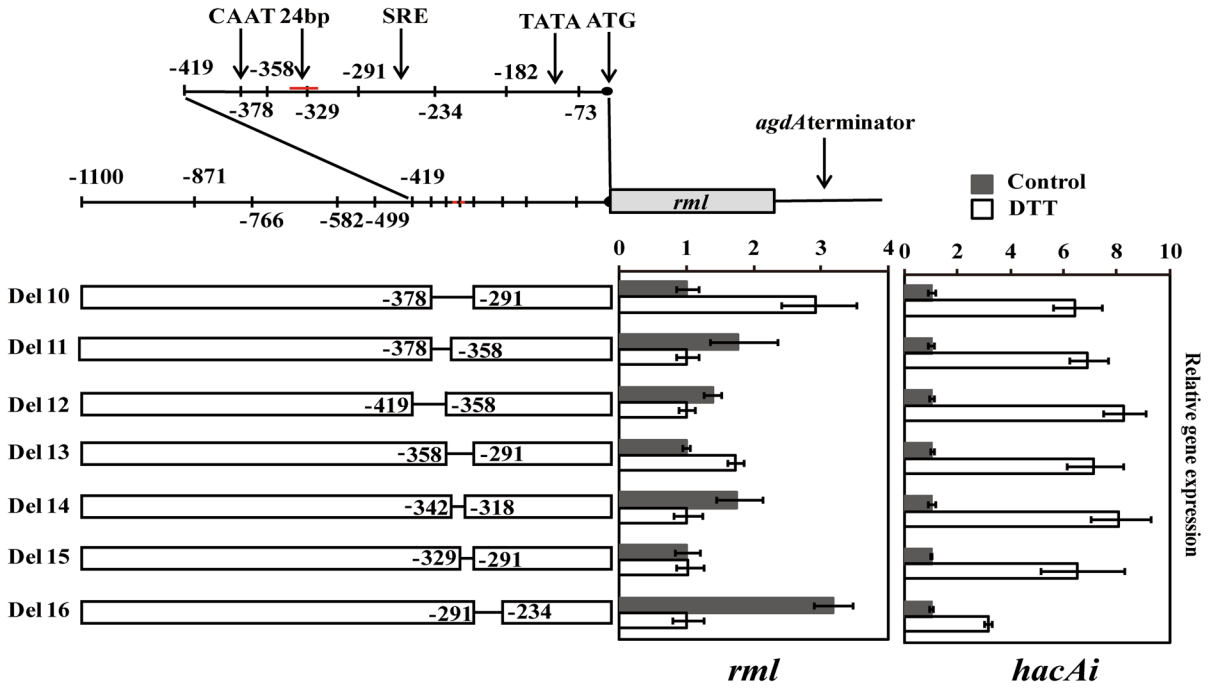

Fig. 3 Internal deletion analysis of the CAAT element, inverted repeat, octamer sequence, and AmyR binding site in the *amyB* promoter. qRT-PCR analysis of *rml* and *hacAi* gene expression in cultures 120 min after treatment with either water (control, *black bar*) or 20 mM DTT (*white bar*). The endogenous control gene was as described for Fig. 1. The promoter regions of the *amyB* gene are indicated by *open boxes*. The numbers noted in each mutant denote the distance from the start codon ATG. Deleted regions are indicated by *black lines*. The 24-bp inverted repeat region is indicated by a *red line*. Each sample was analyzed in triplicate, and the values are the means of three replicates ± S.D

amyC. A single base change of the CCAAT motif could lead to a great difference in transcription (CCAAT in *amyB* and *amyC*, and CCAAA in *amyA*) (Nemoto et al. 2012). Under secretion stress, *amyB* and *amyC* were down-regulated strongly, but the expression of *amyA* could not be detected (Wang et al. 2010). The *A. oryzae* nuclear protein, AoCP, binds specifically to the CCAAT sequence and this CCAAT-binding factor consists of three subunits: AohapB, -C and E (Tanaka et al. 2000). RNA interference of the *AohapC* gene in *A. oryzae* strain niaD300 revealed *amyB* was down-regulated strongly (data not shown). Taken together, these results indicated that the CCAAT element was essential for high-level gene expression, but not for the down-regulation of expression under secretion stress.

To confirm whether the inverted repeat (−342 to −318) and the octamer sequence (−300 to −307) were essential for the down-regulated transcription of *amyB* under DTT treatment, the expression levels of *rml* under the control of mutant promoters in which these elements were deleted were analyzed (Fig. 3). The expression level of *rml* of the mutant promoter containing the deletion from −358 to −291 (Del13, containing an inverted repeat and an octamer sequence) was increased 1.7-fold as compared to control under DTT treatment (Fig. 3). By deleting nucleotides −342 to −318 (Del114, containing an inverted repeat), the level of *rml* mRNA was decreased 1.7 fold under DTT treatment as compared to control (Fig. 3). Deletion of nucleotides −329 to −291 (Del15, containing an octamer sequence) did not alter the expression of *rml*.

These results indicate that the octamer sequence was essential for the down-regulation of *amyB* expression under stress conditions and the octamer sequence was the main element required for the down-regulation, which is designated as region I. Region I is suggested to have important roles in high-level expression (Tsuchiya et al. 1992) and maltose induction (Kanemori et al. 1999). Therefore, the octamer sequence TCACGGGC (−307 to −300) was the core

of the upstream element essential for the down-regulated transcription of *amyB* under secretion stress. We term this core sequence and its related sequences repression under secretion stress element (RESSE). This is the first detailed analysis of the RESS mechanism in *A. oryzae*. Due to the widespread existence of RESS in many industrially useful filamentous fungi, such as *A. niger* (Al-Sheikh et al. 2004), *T. reesei* (Pakula et al. 2003), and *A. oryzae* (Wang et al. 2010), the *cis*-elements identified in this study could contribute to relieving the repression of fungal protein expression under secretion stress, alleviate ER stress, and facilitate their industrial application as cell factories for the production of valuable recombinant enzymes and proteins.

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Supporting information Supplementary Table 1—Primers used for vector construction

Supplementary Table 2— Primers used for quantitative real-time PCR

Supplementary Fig. 1—Confirmation of PCR amplification specificity (**a**) and efficiency (**b**) of the *gapdh* gene

Supplementary Fig. 2—Confirmation of PCR amplification specificity (**a**) and efficiency (**b**) of the *hacAi* gene

Supplementary Fig. 3—Confirmation of PCR amplification specificity (**a**) and efficiency (**b**) of the *rml* gene

Supplementary Fig. 4—Confirmation of PCR amplification specificity (**a**) and efficiency (**b**) of the *amyB* gene

Supplementary Fig. 5—Confirmation of PCR amplification specificity (**a**) and efficiency (**b**) of the *amyR* gene

References

Al-Sheikh H, Watson AJ, Lacey GA, Punt PJ, MacKenzie DA, Jeenes DJ, Pakula T, Penttilä M, Alcocer MJ, Archer DB (2004) Endoplasmic reticulum stress leads to the selective transcriptional downregulation of the glucoamylase gene in *Aspergillus niger*. Mol Microbiol 53:1731–1742

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Carvalho ND, Jørgensen TR, Arentshorst M, Nitsche BM, van den Hondel CA, Archer DB, Ram AF (2012) Genome-wide expression analysis upon constitutive activation of the HacA bZIP transcription factor in *Aspergillus niger* reveals a coordinated cellular response to counteract ER stress. BMC Genomics 13:350

Conesa A, Punt PJ, van Luijk N, van den Hondel CA (2001) The secretion pathway in filamentous fungi: a biotechnological view. Fungal Genet Biol 33:155–171

Gomi K, Iimura Y, Hara S (1987) Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. Agric Biol Chem 51:2549–2555

Gouka RJ, Punt PJ, van den Hondel CA (1997) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. Appl Microbiol Biotechnol 47:1–11

Guillemette T, van Peij N, Goosen T, Lanthaler K, Robson GD, van den Hondel CA, Stam H, Archer DB (2007) Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*. BMC Genomics 8:158

Hisada H, Sano M, Hata Y, Abe Y, Machida M (2008) Deletion analysis of the catalase-encoding gene (*catB*) promoter from *Aspergillus oryzae*. Biosci Biotechnol Biochem 72:48–53

Kanemori Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1999) Insertion analysis of putative functional elements in the promoter region of the *Aspergillus oryzae* Taka-amylase A gene (*amyB*) using a heterologous *Aspergillus nidulans amdS-lacZ* fusion gene system. Biosci Biotechnol Biochem 63:180–183

Kubodera T, Yamashita N, Nishimura A (2000) Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. Biosci Biotechnol Biochem 64:1416–1421

Kwon MJ, Jørgensen TR, Nitsche BM, Arentshorst M, Park J, Ram AF, Meyer V (2012) The transcriptomic fingerprint of glucoamylase over-expression in *Aspergillus niger*. BMC Genomics 13:701

Lee C, Kim J, Shin SG, Hwang S (2006) Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J Biotechnol 123:273–280

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25:402–408

Mori K (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101:451–454

Nakajima K, Asakura T, Maruyama J, Morita Y, Oike H, Shimizu-Ibuka A, Misaka T, Sorimache H, Arai S, Kitamoto K, Abe K (2006) Extracellular production of neoculin, a sweet-tasting heterodimeric protein with taste-modifying activity, by *Aspergillus oryzae*. Appl Environ Microbiol 72:3716–3723

Nemoto T, Maruyama J, Kitamoto K (2012) Contribution ratios of *amyA*, *amyB*, *amyC* genes to high-level alpha-amylase expression in *Aspergillus oryzae*. Biosci Biotechnol Biochem 76:1477–1483

Nevalainen KM, Te'o VS, Bergquist PL (2005) Heterologous protein expression in filamentous fungi. Trends Biotechnol 23:468–474

Pakula TM, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M, Penttilä M (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei*.

Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. J Biol Chem 278:45011–45020

Sims AH, Gent ME, Lanthaler K, Dunn-Coleman NS, Oliver SG, Robson GD (2005) Transcriptome analysis of recombinant protein secretion by *Aspergillus nidulans* and the unfolded-protein response in vivo. Appl Environ Microbiol 71:2737–2747

Sun YF, Lin Y, Zhang JH, Zheng SP, Ye YR, Liang XX, Han SY (2012) Double *Candida antarctica* lipase B co-display on *Pichia pastoris* cell surface based on a self-processing foot-and-mouth disease virus 2A peptide. Appl Microbiol Biotechnol 96:1539–1550

Tada S, Gomi K, Kitamoto K, Kumagai C, Tamura G, Hara S (1991) Identification of the promoter region of the Taka-amylase A gene required for starch induction. Agric Biol Chem 55:1939–1941

Tanaka A, Kato M, Hashimoto H, Kamei K, Naruse F, Papagiannopoulos P, Davis MA, Hynes MJ, Kobayashi T, Tsukagoshi N (2000) An *Aspergillus oryzae* CCAAT-binding protein, AoCP, is involved in the high-level expression of the Taka-amylase A gene. Curr Genet 37:380–387

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 101:249–258

Tsuchiya K, Tada S, Gomi K, Kitamoto K, Kumagai C, Tamura G (1992) Deletion analysis of the Taka-amylase A gene promoter using a homologous transformation system in *Aspergillus oryzae*. Biosci Biotechnol Biochem 56:1849–1853

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acid Res 35:W71–W74

Wang B, Pan L, Guo Y (2009) Construction of heterologous gene expression system for filamentous *Aspergillus oryzae*. J South China Univ Technol (Nat Sci Ed) 37:84–90

Wang B, Guo G, Wang C, Lin Y, Wang X, Zhao M, Guo Y, He M, Zhang Y, Pan L (2010) Survey of the transcriptome of *Aspergillus oryzae* via massively parallel mRNA sequencing. Nucleic Acids Res 38:5075–5087

Ward OP, Qin WM, Dhanjoon J, Ye J, Singh A (2006) Physiology and biotechnology of *Aspergillus*. Adv Appl Microbiol 58:1–75

Ye L, Pan L (2008) A comparison of the unfolded protein response in solid-state with submerged cultures of *Aspergillus oryzae*. Biosci Biotechnol Biochem 72:2998–3001

Yoshino-Yasuda S, Fujino E, Matsui J, Ono N, Kato M, Kitamoto N (2013) Molecular analysis of *AsamyR* gene encoding transcriptional factor for amylolytic gene from Shoyu Koji Mold, *Aspergillus sojae* KBN1340. Food Sci Technol Res 19:505–511

Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Dominguez Y, Scazzocchio C (2004) Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol 41:973–981