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Co-ordinate expression of glycine betaine synthesis genes linked by the FMDV 2A region in a single open reading frame in *Pichia pastoris*

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Abstract The genes encoding the two enzymes choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) of glycine betaine synthesis in *Suaeda salsa* were cloned and fused with the *2A* region of foot-and-mouth disease virus in a single open reading frame. The fused genes were placed under the control of the alcohol oxidase (*AOX1*) promoter in pPIC3B and transformed into *P. pastoris* GS115. The expression of the fused genes in *P. pastoris* and the ability of recombinant yeasts to tolerate environmental stresses were studied. The results showed that induced with 0.5% methanol for 96 h, the maximal activities of CMO and BADH in the tested recombinant yeasts were 45- and 44-fold higher than those in the control yeast transformed empty vector only, respectively; the content of glycine betaine in the recombinant yeasts was 28- to 35-fold higher than that in the control. The fused genes linked by *2A* region of foot-and-mouth disease virus were expressed in *P. pastoris* successfully and the polyprotein was 'cleaved' to each functional protein. The yeasts transformed the fused genes, which were more resistant to salt, methanol, and high temperature stresses than the control as result of glycine betaine synthesis genes introduced.

Keywords Glycine betaine synthesis genes · 2A region of foot-and-mouth disease virus · Coexpression · Stress tolerance · *Pichia pastoris*

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Introduction

The methylotrophic yeast *Pichia pastoris* was extensively used for the high-yield expression of various foreign proteins (Cereghino and Cregg 2000). A benefit of the *P. pastoris* system is that strong promoters (*AOX1*) are available to driving the expression of a foreign gene of interest, thus enabling production of large amounts of the target protein with relative technical ease and at a cost lower than that of most other eukaryotic systems. However, it is apparent that yeast encounters various stresses such as high osmotic pressure, high or freezing temperature, methanol concentration, and low or high pH value when used in the production of heterologous proteins. These stresses have been shown to inhibit both yeast growth and fermentation. The protection of yeast under such adverse environmental conditions is a major requirement.

Glycine betaine (GB, *N, N, N*-trimethyl glycine) is a quaternary ammonium and an amphoteric compound, which is produced by a two-step oxidation of choline, catalyzed by choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), respectively, and this pathway was described in both prokaryotes and eukaryotes (Boch et al. 1994; Vijaranakul et al. 1997; Weretilnyk and

Hanson 1990; Landfald and Strøm 1986). GB is an important osmoprotectant present in bacteria, fungi, cyanobacteria, algae, animals, and several plant families (Rhodes and Hanson 1993). GB stabilizes the structures and activities of enzymes and protein complexes and maintains the integrity of membranes against the damaging effects of excessive salt, cold, heat, and freezing (Hayashi et al. 1998; Alia et al. 1998; Chen et al. 2000b). The stress tolerance of *P. pastoris* may be improved by introducing *CMO* and *BADH* genes together.

There are several approaches currently available for coordinate expression of multiple transgenes in eukaryotes. However, achieving co-ordinate, high-level and stable expression of multiple transgenes is difficult at present. Due to loss, rearrangement, or silencing of transgenes, instability of expression may occur, and it is exacerbated by an increasing number of transgenic loci and repeated use of homologous sequences (Francois et al. 2002; Halpin et al. 2001; Amendola et al. 2005). In the foot-and-mouth disease virus (FMDV) and some other picornaviruses the oligopeptide (~20 amino acids) 2A region of the polyprotein mediates cleavage at its own C terminus to release it from the 2B region. Dissociation occurs at the carboxy terminus of the 2A sequence without any requirement for cytosolic factors (de Felipe et al. 2003). Although the mechanism of its 'self-cleaving' remains largely unknown, some studies have demonstrated that the 'cleavage' is not a proteolytic process but a discrete translation of a 2A protein and its downstream product through a ribosomal 'skip' from one codon to the next with the formation of a peptide bond (Donnelly et al. (2001a, b)). 2A is also active when placed between heterogeneous proteins and effects efficient cotranslational dissociation of the polyprotein into discrete protein products (Ryan et al. 1991). This polyprotein system has been used for fusing genes in a single open reading frame (ORF) and coexpressing two or more genes in some eukaryotic systems, such as in the mammalian (Ryan and Drew 1994; Mattion et al. 1996) and plant systems (Santa Cruz et al. 1996; Halpin et al. 1999; Amrani et al. 2004). To the best of our knowledge, co-ordinate expression of multiple genes fused by *2A* sequence in *P. pastoris* has not been reported, although de Felipe et al. (2003) previously reported *2A* sequence was functional in *Saccharomyces cerevisiae* using report genes *GUS* and *GFP* fused by the sequence. It will be an exciting prospect for the future coexpressing multiple proteins in *P. Pastoris*.

To explore the potential application of coexpressing multiple proteins using 2A peptide in *P. pastoris* and the role of GB in yeasts to tolerate adverse environmental conditions, we cloned *CMO* and *BADH* from *Suaeda salsa* (a halophyte that can grow normally in the fields containing 200–300 mmol l^{-1} NaCl. The content of GB in *S. salsa* was increased greatly when induced with NaCl) and constructed yeast expression vector harboring *CMO* and *BADH* linked with *2A* region of FMDV in a single ORF. The cleavage of the artificial polyprotein 'CMO-2A-BADH' in *P. pastoris* was evaluated and the ability of recombinant yeasts to tolerate environmental stress was examined in this report.

Materials and methods

Plant materials

Seedlings of *S. salsa* were grown in the greenhouse at temperatures ranging from 23 to 26°C, relative humidity of 70–80% and 16 h/8 h (light/dark) photoperiod. Four-week-old plants were subjected to salt stress by watering with 400 mmol l^{-1} NaCl. Leaves and roots of *S. salsa* were collected 24 h or 1 week after salt treatment and frozen in liquid nitrogen rapidly for RNA isolation.

Strains, plasmids, and reagents

P. pastoris strain GS115 (a histidine auxotroph, which allows transformants to be selected by growth on complete media without histide) and the yeast expression vector pPIC3 were purchased from Invitrogen Corporation (San Diego, CA, USA). pBluescript KS(+), *E.Coli.* DH5α were stored in our laboratory. pMD18-T vector was purchased from Takara Co.

T4 DNA ligase, restriction enzymes, avian myeloblastosis virus (AMV) reverse transcriptase and DL15000 DNA marker were purchased from Takara Co. or NEB (New England Biolabs). TRIZOL regent was bought from Invitrogen. Go 3S Spin Plasmid Miniprep Kit was a product of Shenergy Biocolor BioScience and Technology. PCR Fragment Recovery Kit 2.0 was purchased from Takara Co. Primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. (Shanghai, People's Republic of China). Other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Primers design

According to the oligopeptide of 2A (RQLLNFDLLKLAG DVESNPGP) published by Ryan and Drew (1994), two oligonucleotide primers 2A-F and 2A-R, a reverse complementary sequence of 2A-F, were designed. According to the sequence of *CMO* cDNA (Genbank accession number: DQ656523) and *BADH* cDNA (Genbank accession number: DQ641924), two pairs of primers PC1E and PC2E, PB1E and PB2E were designed. PC1E was the sense primer containing the initiation codon ATG in *CMO*. PC2E was the antisense primer and the stop codon TGA was removed. PB1E was the sense primer to clone *BADH*, and

PB2E was the antisense primer containing stop codon TAA in *BADH*. *Sal*I, *Xba*I, *Apa*I, and *Kpn*I cleavage sites were introduced at each primers 5′ end, respectively. Primers PC and PB were designed to amplify the fused genes introducing *Bam*HI and *Sac*I at the 5′ends, respectively. Primers T1 and T2 were designed for site mutagenesis to remove *Sac*I cleavage site in the fused genes (Table 1).

Cloning *CMO* and *BADH* from *S. salsa*

Total RNA was extracted from leaves and roots of *S. salsa* using TRIZOL reagent according to the manufacturer's instructions. Single cDNA was synthesized using Oligo dT (16) by AMV reverse transcriptase. The *CMO* and *BADH* genes in *S. salsa* were amplified by polymerase chain reaction using such cDNA as the template and two pairs of primers PC1E/PC2E, PB1E/PB2E, respectively. PCR reactions were performed in a Thermal Cycler (Peltier Thermal Cycler, PTC-200) in a 50 μl volume for 35 cycles with 40 s at 94°C, 40 s at 52°C (for *CMO*), or 54°C (for *BADH*) and 1 min at 72°C. After the last cycle, the amplification was extended for 10 min at 72°C. Amplified products were recovered by using PCR Fragment Recovery Kit 2.0 and cloned into pMD18-T vector. The plasmids inserting *CMO* or *BADH* genes were confirmed by sequencing, and the correct plasmids were named pMDC or pMDB, respectively.

Vector construction, transformation to *P. pastoris* and selection of transformants

The *2A* fragment with sticky ends by cleavage with *Xba*I and *Apa*I was generated by annealing two long primers 2A-F, 2A-R in one tube. The fragment was then inserted *Xba*I/*Apa*I-digested pBKSM [modified from pBluescript KS(+), the restriction enzyme sites of MCS in which are *Sac*I/*Pst*I/*Sal*I/*BamH*I/*Xba*I/*Hin*dIII/*Apa*I/*Spe*I/*Kpn*I in order]. Five-microliter ligation mixtures were transformed into *E. coli* DH5α as described by Sambrook and Russell (2001). Positive clones containing pBKSM2A plasmid were confirmed by PCR and sequencing. Plasmids pMDC, pMDB were double-digested with *Sal*I/*Xba*I and *Apa*I/*Kpn*I, respectively. The *CMO* and *BADH* fragments were recovered from agarose gel and subcloned into pBKSM2A at *Sal*I/*Xba*I and *Apa*I/*Kpn*I sites successively to generate the transitional plasmid pBKSMC2AB, which harbored the fused genes '*CMO-2A-BADH*'.

The fused genes were amplified with primers PC/T1, PB/T2, respectively, and recovered by overlapped extension PCR using the amplified fragments as the templates and PC/PB as the primers to remove the *Sac*I cleavage site (Peng et al. 2006). The modified fused genes were digested by *Bam*HI/*Sac*I and subcloned into *Bam*HI/*Sac*I-digested pPIC3B (modified from pPIC3, *Eco*RI was replaced by *Sac*I in MCS, and *Sac*I in *AOX1* promoter was removed) under the control of *AOX1* promoter. The plasmid was designated as pYG5242.

Ten microgramme of pYG5242 linearized with *Bgl*II (NEB) were transformed into *P. pastoris* GS115 (*his4*) by electroporation (Bio-Rad Genepuler; Bio-Rad, Hercules, CA, USA). Transformants were grown on histidine deficient RD medium [1 mol l^{-1} sorbitol, 2% glucose, 1.34% yeast nitrogen base with ammonium sulfate without AAs (YNB), 4×10^{-5}% biotin, 0.005% L-glutamic acid, 0.005% L-methionine, 0.005% L-lysine, 0.005% L-leucine, 0.005% L-isoleucine, and 2.0% agar] and then they were selected on MM (1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, and 1.5% agar) and MD (1.34% YNB, 4×10^{-5}% biotin, 2% glucose, and 2.0% agar) media for identifying His^{+}/Mut^{S} phenotype.

Induction of recombinant yeasts

Expression of the recombinant yeasts induced by methanol was performed according to '*Pichia* expression kit instruc-

Table 1 Oligonucleotides used in this study

Primers name	Sequences(5′to 3′)
PC1E	ACGC*GTCGAC*TTG**ATG**GCTGCATCAGCAAGTGC *Sal*I
PC2E	TGC*TCTAGA*(**TCA**)CTTCAAAATTTGGTGCAACCAGCAG *Xba*I
PB1E	ACGA*GGGCCC***ATG**TCGATCCCTATACCTTCTCG *Apa*I
PB2E	CGG*GGTACC*CCT**TTA**AGGAGACTTGTACCACCCC *Kpn*I
PC	AAA*GGATCC***ATG**GCTGCATCAGCAAGTGCTACC *BamH*I
PB	AAA*GAGCTC*T**TAA**GGAGACTTGTACCACC *Sac*I
T1	ACGATGAGCACCAGAAGTAGCAGC
T2	GCTACTTCTGGTGCTCATCGTGCTAG
2A-F	CTAGACAGCTGTTGAATTTTGACCTTCTTAAGCTTGCGGGA GACGTCGAGTCCAACCCCGGGCC
2A-R	CGGGGTTGGACTCGACGTCTCCCGCAAGCTTAAGAAGGT CAAAATTCAACAGCTGT

Italicized nucleotides represent recognization sequences for the restriction enzymes. The bold-faces refer to initial codons or stop codons; the stop codon in parenthesis of *CMO* was removed

tion manual, Invitrogen'. Single colonies of His^+/Mut^S recombinants A764, A765, and A767 were inoculated into 100 ml buffered glycerol complex (BMGY) medium [1% yeast extract, 2% peptone, 2% dextrose, 100 mmol l^{-1} potassium phosphate (pH 7.0), 1.34% YNB, 4×10^{-5}% biotin, and 1% glycerol] in a 1 l baffle flask, respectively, with vigorous shaking (250 rpm) at 30°C and continually grown for 36–48 h until the optical density at 600 nm (OD 600) was between 10.0 and 14.0. The cells were harvested by centrifugation at 4,000×*g* for 10 min at room temperature. The supernatant was discarded, and the pellets were resuspended in 20 ml of BMMY (like BMGY but glycerol was replaced by 0.5% methanol) in a 100 ml baffle flask covered with two layers of sterile gauze. The flasks were incubated at 30°C with vigorous shaking (250 rpm) for another 5 days. The cells were induced with fresh methanol every 24 h to a final concentration of 0.5% (*v*/*v*) and 1-ml samples were taken every 24 h. The samples were centrifuged at 12,000×*g* for 10 min and the cell pellets kept at −80°C for SDS-PAGE analysis, extracting crude enzymes and glycine betaine. *P. pastoris* GS115 transformed with the empty pPIC3 vector, YPIC3 was assayed in parallel and used as a negative control. The fermentations were replicated three times.

SDS-PAGE electrophoresis

Protein expression was analyzed by Coomassie-stained SDS-PAGE (Laemmli 1970). The cell pellets were resuspended in 100 μl Breaking Buffer [50 mmol l^{-1} sodium phosphate (pH 7.4), 1 mmol l^{-1} PMSF (phenylmethylsulfonyl fluoride), 1 mmol l^{-1} EDTA and 5% glycerol] for each 1 ml sample and added an equal volume of acid-washed glass beads. The cells were broken up on vortex agitator for 30 s then incubated on ice for 30 s and eight cycles were repeated. The broken cells were centrifuged at 12,000×*g* for 10 min at 4°C. The clear supernatant was transferred to a fresh microcentrifuge tube. A 50-μl supernatant (cell lysate) mixed with 50-μl 2×SDS-PAGE sample buffer was boiled for 10 min and loaded 10 μl per well. The expressed proteins were analyzed on 12% SDS/polyacrylamide gels. Protein bands were stained with Coomassie brilliant blue solution.

Assay for enzyme activities and GB accumulation

The cell pellets were resuspended in 3 ml of extraction buffer [50 mmol l^{-1} Hepes-KOH (pH 8.0), 1 mmol l^{-1} EDTA, and 5 mmol l^{-1} dithiothreitol] and homogenized with sonication on ice. Crude extracts were centrifuged at 10,000×*g* for 10 min at 4°C and supernatant was desalted by dialysis.

The oxidase activity of CMO was measured with 10 mmol l^{-1} choline as the substrate in air-saturated 50-mmol l^{-1} potassium phosphate (pH 7.0), thermostated at 25°C by monitoring the absorbance of cytochrome.c at 549 nm in electron transportation system phenazine methosulfate-cytochrome.c (Wu and Lin 1984; Gadda and McAllister-Wilkins 2003). One unit of enzyme activity was defined as the amount of enzyme that increased 1.0 OD_{549} per minute under the assay conditions.

The activity of BADH was measured spectrophotometrically at 340 nm at 1 min intervals in 1 ml assay buffer [50 mmol l^{-1} HEPES-KOH (pH 8.0), 1 mmol l^{-1} EDTA, 5 mmol l^{-1} dithiothreitol, 1 mmol l^{-1} NAD^+ and 1 mmol l^{-1} betaine aldehyde] at 25°C, supplemented with 50-μl protein extract to start the reaction. The activity was calculated using an extinction coefficient of 6,220 M^{-1} cm $^{-1}$ for NADH. One unit of enzyme activity was defined as the amount converting 1 nmol of NAD^+ per min under the assay conditions.

Glycine betaine extraction and quantification were based on the method by Chen et al. (2000a). GB was extracted with boiling water and measured with a reverse phase ion-pair HPLC (RP-HPLC; HP 1050 modular 3D; Hewlett Packard, Boise, ID, USA). The aqueous phase was fractionated by Dowex 1×8 (100–200 mesh, Cl^- form, Sigma) and Dowex 50 w×2 (50–100 mesh, H^+ form, Sigma) ion-exchange chromatography. The glycine betaine fraction was eluted with 2 mol l^{-1} NH_4OH, dried 65°C in vacuum, and dissolved in 1 ml 50 mmol l^{-1} KH_2PO4 (pH 4.45). Separation was performed on a 250×4.6 mm C_{18} column eluted with 50 mmol l^{-1} KH_2PO4 (pH 4.45) containing ion-pair agent 0.1% PIC B-8 (1-octane sulfonic acid, Waters). Detection was performed by UV absorbance at 192 nm.

Assay for the ability of recombinant yeasts to tolerate stresses

Yeast strains A764, A765, A767 and YPIC3 were streaked on YPD medium supplemented with 0.5% methanol and different concentrations (0, 0.5, 0.8, 1.0, 1.2, and 1.5 mol l^{-1}) of NaCl. Their salt tolerance was examined after they incubated at 30°C for 3 days. To demonstrate the methanol tolerance of them, all the yeast strains were exposed to the high-methanol condition on YPD medium containing 5, 7, 8, and 10% of methanol and incubated at 30°C for 3 days. To investigate the ability to tolerate high temperature, they were grown on YPD medium containing 0.5% of methanol at 36°C for 3 days. All of the yeast strains on the above stresses without methanol induction were also assayed in parallel and used as negative control. The experiment was repeated three times.

Results

Cloning the glycine betaine synthases in *S. salsa*

The *CMO* and *BADH* genes were amplified using cDNA of *S. salsa* as the template and the primers PC1E/PC2E and PB1E/PB2E, respectively. The amplified fragments were recovered from 0.8% agarose gel and inserted into pMD18-T vector. Positive clones were sequenced on ABI377 (PE Applied Biosystems, CA, USA). Sequence analysis showed that the coding sequence of *CMO* cDNA was 1,329 bp in length encoding 442 amino acids; the coding sequence of *BADH* cDNA was 1,506 bp in length encoding 501 amino acids. The plasmids carrying *CMO* or *BADH* gene were named pMDC or pMDB respectively.

Vector construction and transformation into yeast

The *2A* sequence flanking *Xba*I- and *Apa*I-digested stick ends was generated when the two primers 2A-F, 2A-R annealed and was then introduced to pBKSM at *Xba*I/*Apa*I sites to generate pBKSM2A. *CMO* and *BADH* were subcloned into pBKSM2A successively to generate the transitional plasmid pBKSMC2AB. The plasmid was digested by restriction enzymes *Sal*I, *Kpn*I and *Pvu*I to detect the correctness of construction. The expected bands about 2.9 kb in size appeared on 0.8% agarose gel. Sequence analysis showed that the open reading frame of '*CMO-2A-BADH'* was 2,898 bp in length. *CMO* and *BADH* were placed in correct position of the fused genes '*CMO-2A-BADH'*: *CMO* containing initial codon ATG and eliminating stop codon TGA placed the upstream of *2A*, and the integrity ORF of *BADH* placed the downstream of *2A*. Site-directed mutagenesis was performed by overlapped extension PCR to remove *Sac*I cleavage site in the fused genes. The mutation product was cloned into pMD18-T vector and transformed to *E.coli* DH5α. One of the positive clones was selected and proved to be correct by sequence analysis. The fused genes '*CMO-2A-BADH*' were then inserted to pPIC3B and placed under control of *AOX1* promoter (Fig. 1). The pYG5242 plasmid containing fused genes was digested with the restriction enzyme *Bgl*II to generate linearized DNA for transformation. His^+ transformants appeared in 3 to 7 days after transformation. Twelve from the His^+ transformants were of the Mut^s phenotype. To further confirm the site of integration, three of these Mut^s strains (designated for strains A764, A765 and A767, respectively) were randomly selected and analyzed by PCR (data were not shown).

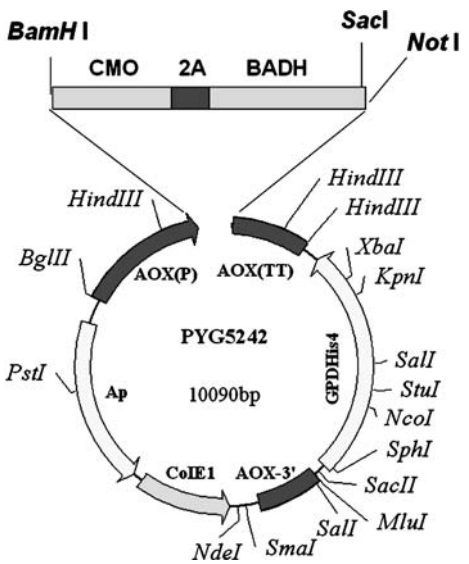

Fig. 1 The sketch of plasmid pYG5242 coexpressing *CMO* and *BADH* in *P. pastoris*. The fused genes '*CMO-2A-BADH*' were cloned into the downstream of the *AOX1* promoter of the vector pPIC3B

SDS-PAGE analysis of the recombinant yeasts

The protein expression of recombinant yeasts A764, A765, and A767 was analyzed by SDS-PAGE in comparison with that of the *P. pastoris* strain YPIC3 to test whether the fused genes translated and 'cleaved' the polyprotein at 2A to produce CMO and BADH proteins in the *P. pastoris* '*CMO-2A-BADH*' integrant clones. It was found that the expression of methanol-induced proteins was produced in a stable pattern. As shown in Fig. 2, among the profiles of SDS-PAGE bands with Coomassie brilliant blue R-250 staining, the recombinant yeasts A764, A765 expressed two extra protein bands compared with the control. The molecular mass of the two bands was about 49 and 54 kDa, which match with the proteins CMO and BADH in size as predicted. However, the two bands were undetectable in the recombinant yeast A767. The CMO and BADH proteins were visible in A764 and A765 cell extracts on tricine-SDS-polyacrylamide gels induced with 0.5% of methanol for 72 h and appeared to be at a maximum induced for 96 h (Fig. 2).

Assay for enzyme activities, GB accumulation in the recombinant *P. pastoris*

The BADH and CMO activities in A764, A765, and A767 were analyzed to determine whether the BADH and CMO polypeptides produced in the recombinant yeasts were enzymatically active. Soluble and active enzymes were extracted from the cells incubating at 30°C for 96 h in the presence of 0.5% of methanol. The activity of CMO was tested by monitoring the absorbance of cytochrome c at 549 nm in electron transportation system phenazine

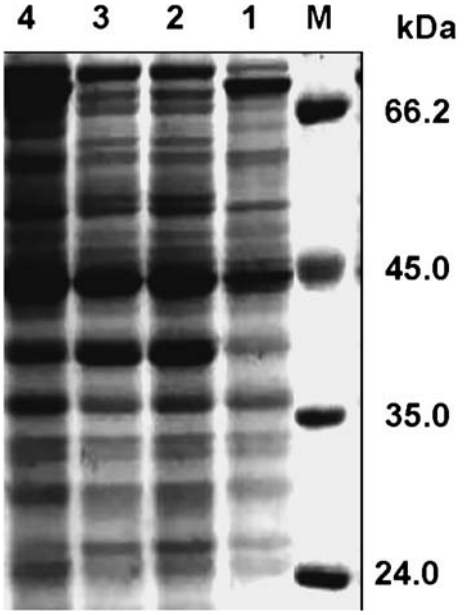

Fig. 2 SDS-PAGE analysis of total-cell lysate of the recombinant yeasts induced with 0.5% methanol for 96 h. *Lane 1*: the cell lysate band of *P. pastoris* transformed empty vector pPIC3 as a control; *lanes 2*, *3* and *4* the cell lysate band of the recombinant yeastsA764, A765 and A767 respectively; *lane M* the protein MW marker, from *top* to *bottom*: 66.2, 45.0, 35.0 and 24.0 kDa

methosulfate-cytochrome c. The NAD-dependent BADH activity was determined spectrophotometrically as described before (HolmstrÖm et al. 1994). In the presence of betaine aldehyde, BADH enzyme reduces NAD^+ to NADH and the rate of this reaction was measured by an increase in OD 340 nm due to the reduction of NAD^+. As shown in Table 2, all the three strains transformed the fused genes exhibited CMO and BADH activities. In A764 and A765, CMO activity was 19.57 and 21.87 U mg^{-1} protein, respectively, and in A767 it was only 4.30 U mg^{-1} protein, whereas CMO activity was almost undetectable (0.49 U mg^{-1} protein) in YPIC3. The three strains also exhibited higher BADH activity, which was 19.63 and 29.22 U mg^{-1} protein in A764 and A765, respectively, and 4.56 U mg^{-1} protein in A767, whereas BADH activity in YPIC3 was also very low, to give 0.67 U mg^{-1} protein.

Glycine betaine content was determined in the yeast cells transformed the fused genes or empty vector pPIC3 by RP-HPLC (Table 2 and Fig. 3). As shown in Table 2 and Fig. 3, the yeast YPIC3 cells contained very little GB (0.14 mg g^{-1} cell pellets), whereas the yeast A764, A765 and A767 cells contained 28- to 35-fold over the GB content in YPIC3, to give 3.93 to 4.82 mg g^{-1} cell pellets. The content of betaine in A765 was 4.82 mg g^{-1} cell pellets, the highest among the tested yeast strains, and in A764, A767 the content was 4.51 and 3.93 mg g^{-1} cell pellets, respectively. These results indicated that the levels of GB in the recombinant yeasts fluctuated and correlated with the activity of CMO and BADH. The recombinant yeasts kept higher activities of CMO and BADH also contained higher GB content. However, it was found that the activities of CMO and BADH in A764 and A765 were much higher, but the GB content did not increase accordingly compared with in A767, which may be the reason that the substrate of the two enzymes, choline was insufficient for GB metabolization in A764 and A765. The study of genetic engineering of GB production in plants also indicated such a phenomenon (Huang et al. 2000).

The ability of *P. pastoris* recombinants to tolerate stresses

Yeast strains A764, A765, A767, and YPIC3 were analyzed for tolerance to salt, methanol and high temperature stresses. All the yeast strains grew normally and there were no significant differences among A764, A765, A767, and YPIC3 on YPD medium or YPD medium containing 0.5% methanol at 30°C (Fig. 4a, 1). The growth of A764, A765, A767, and YPIC3 was severely suppressed on YPD medium containing 0.8 mol l^{-1} NaCl and completely inhibited on 1.0 mol l^{-1} NaCl without methanol induction. However, induced with 0.5% of methanol, A764, A765, and A767 grew well but YPIC3 was suppressed greatly on YPD medium containing 1.0 mol l^{-1} NaCl (Fig. 4a, 2). A764, A765, and A767 can resist 1.2 mol l^{-1} NaCl and could even grow a little on 1.5 mol l^{-1} NaCl with 0.5% of methanol induction. All the yeast strains were treated with 5–10% of methanol on YPD medium to test the ability

Table 2 Comparison of the CMO and BADH activities and GB content in A764, A765, A767, and YPIC3 (yeast strain GS115 transformed with the empty pPIC3 vector) after induction with 0.5% methanol for 96 h

Yeast strains	CMO activity (U·mg^{-1})	BADH activity (U·mg^{-1})	GB content (mg)
A764	19.57±0.93aA	19.63±1.84aA	4.51±0.24aA
A765	21.87±1.70bA	29.22±3.45bB	4.82±0.29aAB
A767	4.30±0.96cB	4.56±0.70cC	3.93±0.25bB
YPIC3	0.49±0.14dC	0.67±0.12dC	0.14±0.032cC

Each value represents the average value and SD of three parallel samples. Small and capital letters indicate significance at $P<0.05$ and $P<0.01$, respectively

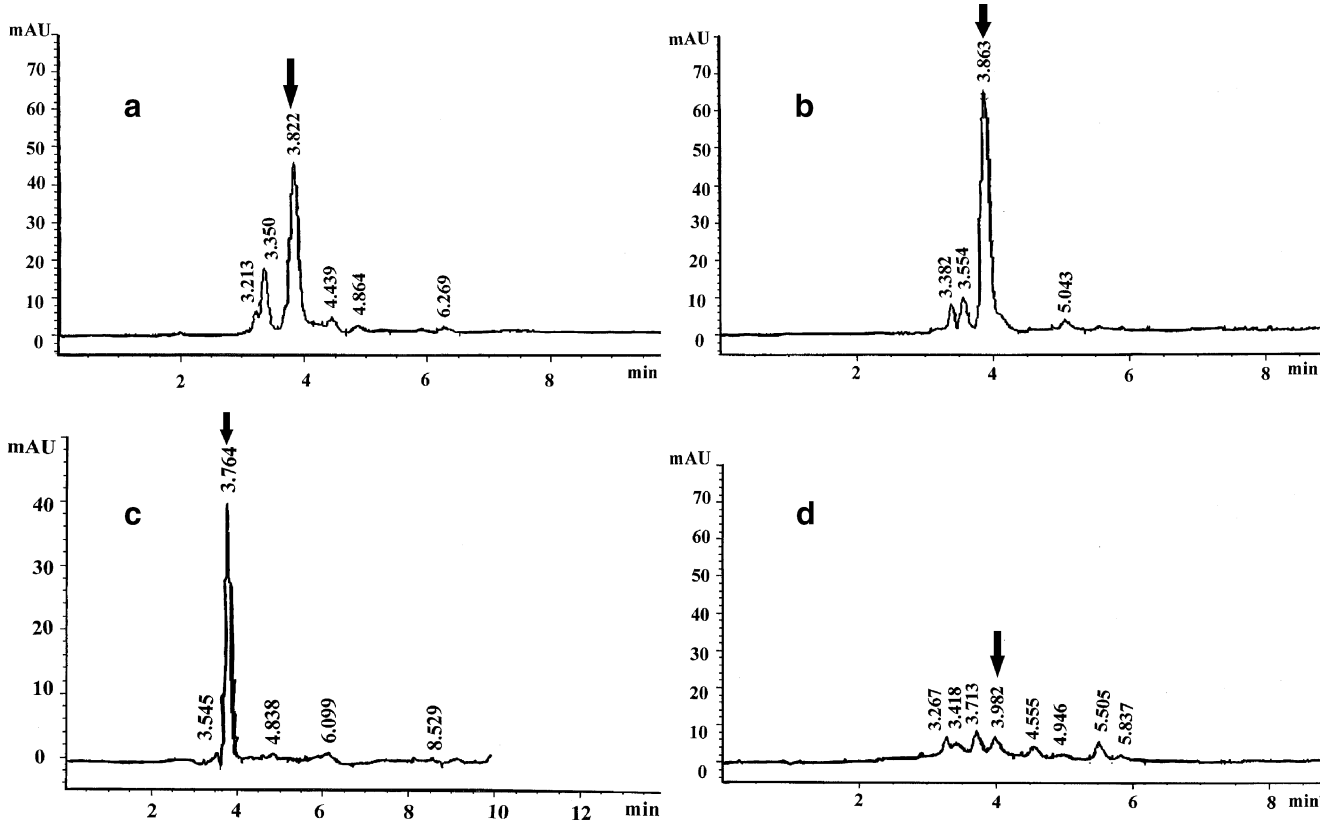

Fig. 3 RP-HPLC chromatograms of glycine betaine derived from the recombinant yeasts A764 (**a**), A765 (**b**), A767 (**c**) and YPIC3, *P. pastoris* transformed empty vector pPIC3 (**d**). Glycine betaine was extracted and processed as described in "Materials and methods." The peaks pointed by the arrows represent glycine betaine

tolerant to methanol. A764, A765, and A767 were found to be methanol-tolerant. On YPD medium supplemented 7% of methanol, A764, A765, and A767 grew well, whereas the growth of YPIC3 was greatly inhibited (Fig. 4a, 3). The ability of the yeast strains tolerant to high temperature stress was also evaluated. They were incubated at 36°C on YPD medium containing 0.5% of methanol for 3 days. As illustrated in Fig. 4a (4), the growth of A764, A765, and A767 was slightly inhibited at 36°C on YPD medium containing 0.5% of methanol, whereas YPIC3 grew badly at the temperature on the same medium. In the control experiment without methanol induction, all the yeast strains

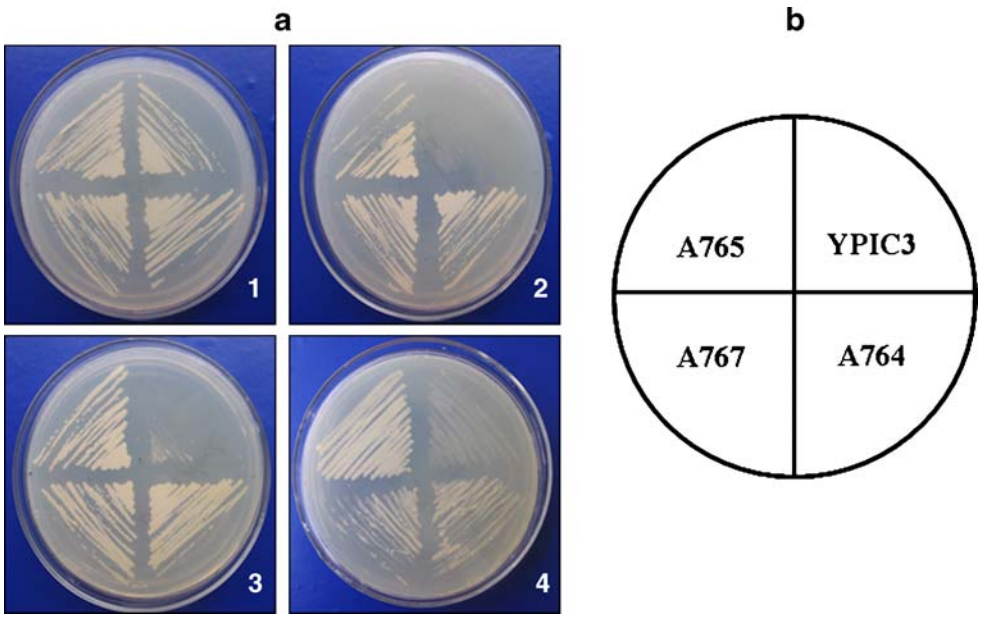

Fig. 4 Effects of salt, methanol and high temperature stresses on the growth of the recombinant yeasts. The behavior of A764, A765, A767 and YPIC3, *P. pastoris* transformed empty vector pPIC3 to different stresses on YPD medium (**a**). The yeast strains on YPD medium supplemented 0.5% methanol, incubated at 30°C for 3 days (*1*), or supplemented 0.5% methanol and 1.0 mol l^{-1} NaCl, incubated at 30°C for 3 days (*2*), or supplemented 7.0% methanol, incubated at 30°C for 3 days (*3*), and or supplemented 0.5% methanol, incubated at 36°C for 3 days (*4*). The sketch represents the position of A764, A765, A767 and YPIC3 on plates (**b**)

grew badly at 36°C. These results indicated that the tolerance of the recombinant yeasts to salt, methanol and high temperature stress was improved remarkably as a result of improving GB content in the yeasts expressing betaine synthesis genes from *S. salsa* in *P. pastoris*.

Discussion

Many organisms, including prokaryotic organisms and eukaryotic organisms can generate GB for osmoprotection by oxidation of choline (Csonka 1989; Rhodes and Hanson 1993). GB has been shown to protect enzymes and membranes from salt, heat, and cold stress (Mamedov et al. 1993; Papageorgiou and Murata 1995). GB accumulation is induced under stressed conditions, with its concentration being correlated with the level of tolerance (Saneoka et al. 1995). The introduction of a pathway for the biosynthesis of betaine into some organisms has been proven to be effective in increasing their tolerance to various abiotic stresses (McNeil et al. 1999). We transferred glycine betaine synthesis genes *CMO* and *BADH* from *S. salsa* to yeast. The recombinant yeasts transformed with the two genes exhibited higher tolerance to salt, methanol, and high temperature stress. The experiment also indicated that the glycine betaine synthesis genes from plants could function normally in yeast.

Previous studies demonstrated that proteins of reporter genes were efficiently released from an artificial polyprotein fusing with 2A, via the 'cleavage' mediated by the FMDV 2A region in both mammalian and plant cells (Halpin et al. 1999; Ryan and Drew 1994). Our study demonstrates that the 2A peptide derived from FMDV functions properly within different contexts and can facilitate efficient expression of functional proteins in *P. pastoris*, but the cleavage efficiency varies as different recombinants. In the recombinant yeasts A764 and A765, the artificial [CMO-2A-BADH] polyprotein undergoes efficient 'cleavage' to yield [CMO-2A] and BADH, but in the recombinant yeast A767, the 'cleavage' efficiency is low. In individual recombinants, there is a high degree correlation between CMO and BADH activities: the recombinant yeasts either expressed both enzymes at a high level, or expressed at a low level. Thus, with one transformation experiment yeasts coordinately expressing two genes were obtained. The recombinants expressing two genes coordinately at a high level can be screened from the numerous transformants. In addition, the FMDV 2A is a very small protein of only 16–20 amino acids, and 2A can be used a number of times in a polyprotein to effect efficient dissociation into multiple component polypeptides (Ma and Mitra 2002). Thus, the 2A protein may be an ideal system for coexpressing multiple genes in a single ORF in *P. pastoris*.

In industrial practices, yeast is often exposed to a variety of stresses. Environmental conditions such as temperature, osmotic pressure, cell viability and methanol concentration are crucial for expressing heterogenous proteins in *P. pastoris*. The role of trehalose in determinant of stress resistance of baker's yeast is widely believed (Dijck et al. 1995). In our experiment, we proved that the glycine betaine content in methylotrophic yeast is another major of determinant of stress resistance. The recombinant yeasts containing high-level of betaine were more tolerant to environmental stress. Although we only investigated high temperature, salt and methanol stress, we speculate that recombinant yeasts with high glycine betaine content could also be tolerant to cold, high pH, etc., adverse environment.

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