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Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in *Saccharomyces cerevisiae*



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ABSTRACT

Due to limitations associated with whole blood for transfusions (antigen compatibility, transmission of infections, supply and storage), the use of cell-free hemoglobin as an oxygen carrier substitute has been in the center of research interest for decades. Human hemoglobin has previously been synthesized in yeast, however the challenge is to balance the expression of the two different globin subunits, as well as the supply of the prosthetic heme required for obtaining the active hemoglobin ($\alpha_2\beta_2$). In this work we evaluated the expression of different combinations of α and β peptides and combined this with metabolic engineering of the heme biosynthetic pathway. Through evaluation of several different strategies we showed that engineering the biosynthesis pathway can substantially increase the heme level in yeast cells, and this resulted in a significant enhancement of human hemoglobin production. Besides demonstration of improved hemoglobin production our work demonstrates a novel strategy for improving the production of complex proteins, especially multimers with a prosthetic group.

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1. Introduction

Hemoglobin is the major oxygen transport protein in blood and is found in erythrocytes. The protein is a tetrameric metalloprotein consisting of four globin subunits ($\alpha_2\beta_2$) with each subunit containing a prosthetic heme group. One heme molecule is comprised of a protoporphyrin IX and a ferrous iron which has high affinity for binding oxygen (Belcher et al., 2010; Benesch and Benesch, 1963). Due to the concerns with whole blood for transfusion, e.g. limited resources, cross-typing requirements and transfusion transmitted disease, substantial efforts have been put into the development of safe and effective human blood substitutes (Looker et al., 1992; Wagenbach et al., 1991). Aside from synthetic perfluorocarbon (PFC) polymers (Biro, 1993) and erythrocyte products (which are purified hemoglobin from a variety of mammalian sources), cell-free hemoglobin-based oxygen carrier (HBOC) has been extensively studied for its high efficiency in oxygen transfer and its contribution to the maintenance of osmotic pressure (Lu et al., 2011). The main limitation with this product is protein instability due to tetramer dissociation into dimers, resulting in short retention time and renal toxicity, and high

oxygen affinity in the body caused by absence of a co-factor 2, 3-bis (phospho) glycerate (2,3-BPG) (Kroeger and Kundrot, 1997; Looker et al., 1992; Wagenbach et al., 1991). A series of advances have been achieved including chemical modifications of natural hemoglobin to extend its half-life in circulation (Harris and Palmer, 2008) which have greatly stimulated further studies on HBOC (Lu et al., 2011).

With the development of recombinant DNA technology, it became possible to engineer hemoglobin with genetic engineering, which enabled designed modification to improve its biological properties as well as to enhance its production. A variety of strategies have been applied in different organisms since the late 80s ranging from bacteria (Hoffman et al., 1990) to higher organisms, including plants (Dierckx et al., 1997) and animals (Behringer et al., 1989; Martinez et al., 2012). The main advances include (i) expression of α and β globin chains simultaneously and refold together, with addition of exogenous heme *in vitro* (Hoffman et al., 1990) (ii) Co-expression of the methionine amino-peptidase (Met-AP) gene with the globin genes to reduce the N-terminal methionine in modified globins produced in *Escherichia coli* (Shen et al., 1993) (iii) Site-directed mutations in β globin chain in order to reduce the extreme oxygen affinity due to the lack of 2,3-BPG allosteric regulation (Hernan et al., 1992) (iv) Tandem fusion of two α globins to prevent tetramer disassociation into $\alpha\beta$ dimers (Looker et al., 1992) (v) Co-expression of bacterial heme membrane transporter systems to increase heme uptake and further

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enhancement of hemoglobin production (Graves et al., 2008). In addition to this, several recent studies showed that α -hemoglobin stabilizing protein (AHSP) could prevent α globin precipitation and hereby have a positive effect on hemoglobin production (Feng et al., 2004; Feng et al., 2005; Vasseur-Godbillon et al., 2006).

The yeast *S. cerevisiae* is a well-known cell factory for production of fuels (Lee et al., 2008), chemicals (Chemler et al., 2006; Nevoigt, 2008) and heterologous proteins, especially industrial enzymes and pharmaceuticals (Hou et al., 2012; Martinez et al., 2012). There are several reasons why yeast can potentially be a highly competitive expression host for production of human hemoglobin: it is general regarded as safe (GRAS organism) which avoids the risk of pathogen contaminations from human or animal resources, it is able to perform post-translational modifications, including correct processing the globin termini, folding and subunit assembling (Porro et al., 2005), being the prerequisite for proper reconstitution of a soluble heme-containing tetramer in vivo. Furthermore, being a long-term eukaryal model organism, numerous studies have been carried out on *S. cerevisiae* from which extensive genome and physiological information have been generated (Petranovic and Nielsen, 2008). For these reasons several recombinant proteins such as human insulin (Novo Nordisk), human bovine serum albumin (Novozymes) and hepatitis vaccines (GlaxoSmithKline, Merck) among others, have been industrially produced and approved for medical applications in *S. cerevisiae* (Martinez et al., 2012; Walsh, 2010).

Hemoglobin synthesis is highly conserved through evolution and the non-globin part, the prosthetic group protoporphyrin IX, is common among divergent species such as human and yeast, so one can utilize the endogenous yeast heme pathway for the production of human recombinant hemoglobin (Kumar, 1995). Studies suggest that recombinant hemoglobin produced in yeast remains fully functional and shows identical rate of dissociation as the native human hemoglobin (Coghlan et al., 1992). However, with improved globin production it is questionable whether endogenous heme production is sufficient. Thus, it has been found that supplementation with hemin can accelerate hemoglobin synthesis in immature cultured erythroid cells, which suggests that heme availability is a rate-controlling step for hemoglobin production (Fibach et al., 1995). Additionally, heme association to globins was suggested to occur co-translationally and could therefore facilitate the formation of the proper tertiary structure on the ribosome (Komar et al., 1997). A coordinated optimization of endogenous heme level is therefore expected to improve heterologous hemoglobin production in yeast cells.

The heme biosynthesis pathway in yeast (Fig. 1) contains eight steps starting from 5-aminolevulinic acid (ALA) synthesis from succinyl-CoA and glycine (catalyzed by Hem1p) in mitochondria. After being transported to the cytosol to form porphobilinogen (PBG) (catalyzed by Hem2p), the intermediate is further converted to hydroxymethylbilane (catalyzed by Hem3p) and then uroporphyrinogen III (by Hem4p) and finally into coproporphyrinogen III (catalyzed by Hem12p). The coproporphyrinogen III is transported back to the mitochondria and converted to protoporphyrinogen IX by Hem13p following synthesis of protoporphyrin IX (by Hem14p). The last step is the incorporation of an iron atom into protoporphyrin IX to form the heme molecule catalyzed by Hem15p.

In this study, we evaluated the effect of metabolic engineering the heme biosynthetic pathway on heterologous hemoglobin production (Fig. 1): we overexpressed, individually or in different combinations (with promoters of different strengths), three genes coding for enzymes that represent confirmed (*HEM2* and *HEM3*) or suspicious (*HEM12*) rate-limiting steps in the heme biosynthetic pathway (Hoffman et al., 2003), and additionally co-expressed α and β globin genes in different ratios. Using this approach we could evaluate a large number of combinations with engineering the heme

biosynthesis and globin subunit co-expression and hereby identify the optimal combination for high level human hemoglobin production.

2. Materials and methods

2.1. Plasmids and strains

S. cerevisiae CEN.PK 113-11C (*MATa his3 Δ 1 ura3-52 MAL2-8c SUC2*) (Entian and Kötter, 1998) was used as the host strain for all the experiments performed in this study. Plasmid constructions were performed following standard molecular biology techniques using *E. coli* DH5 α (Bethesda Research Laboratories). All yeast transformations were performed following standard lithium acetate method (Daniel Gietz and Woods, 2002) and selected on synthetic dextrose media SD-His (heme overexpression strains), SD-Ura (globin overexpression strains), and SD-His-Ura (heme and globin double overexpression strains) respectively. For cultivation, yeast strains were grown in liquid SD-His, SD-Ura and SD-His-Ura accordingly.

All the heme overexpression plasmids were constructed using a bidirectional expression plasmid pLYC04 (Chen et al., 2013). The heme genes were amplified from the chromosomal DNA of CEN.PK 113-11C (Table S1). Plasmids H2, H3 and H12 were constructed by inserting the corresponding genes into the expression cassette of pLYC04 under the *TEF1* promoter. Plasmids H2H3 and H2H12 were constructed by inserting *HEM3* and *HEM12* respectively into H2 under the *PGK1* promoter. H3H2H12 was built by inserting a *HEM3* expression cassette amplified from H3 into H2H12. The globin overexpression plasmids were constructed from a different bidirectional expression plasmid pSP-GM1 (Chen et al., 2012). The DNA sequences (with restriction sites at both ends), namely *HBA* (coding α), *HBB* (coding β) and *HBAA* (coding di- α) were synthesized by GeneScript, Inc., and codon optimized to facilitate their expression in *S. cerevisiae*. The *HBAA* sequence coding di- α was made by linking two *HBA* gene sequences (first one without stop codon) with one glycine linker. The *HBB* fragment was digested with *SalI* and *KpnI* and ligated under the *PGK1* promoter of pSP-GM1 first. Then the *HBA* and *HBAA* fragments were digested by *NotI* and *SacI* and ligated under the *TEF1* promoter of pSP-GM1 to make B/A and B/AA respectively. Plasmid B/A/A was made by inserting the *HBA* expression cassette amplified from B/A into plasmid B/A between *PstI* and *MfeI*. Plasmid B/AA/B was constructed by inserting the *HBB* expression cassette amplified from B/A into the *MfeI* site of plasmid B/AA. All the primers and the synthesized globin gene sequences are listed in the supplementary file (Table S1 and S2).

CEN.PK 113-11C was transformed with the heme and globin overexpression plasmids, resulting in different recombinant host strains (Fig. 1B, Table 1).

2.2. Coproporphyrin assay

Coproporphyrin levels were measured by HPLC using the HPLC Porphyrin Kit from ALPCO Diagnostics Inc. Strains transformed with the heme overexpression plasmids were cultured in SD-His (containing 6.7 g/L of yeast nitrogen base w/o amino acids (Difco Laboratories, Sparks, MD), 0.77 g/L of complete supplement mixture (CSM, w/o histone) (MP Biomedicals, Solon, OH) and 2% glucose) medium at 30 °C with constant shaking at 180 rpm during 96 h before being harvested. Cell pellets collected from 35 ml of culture were resuspended into 1 mL of 0.6% HCl after being washed twice with ddH₂O. The suspended cell pellets were kept in dark at 4 °C before being loaded onto HPLC (PU-2089 Plus pump, AS-2057 Plus auto sampler, and FP-920 fluorescence

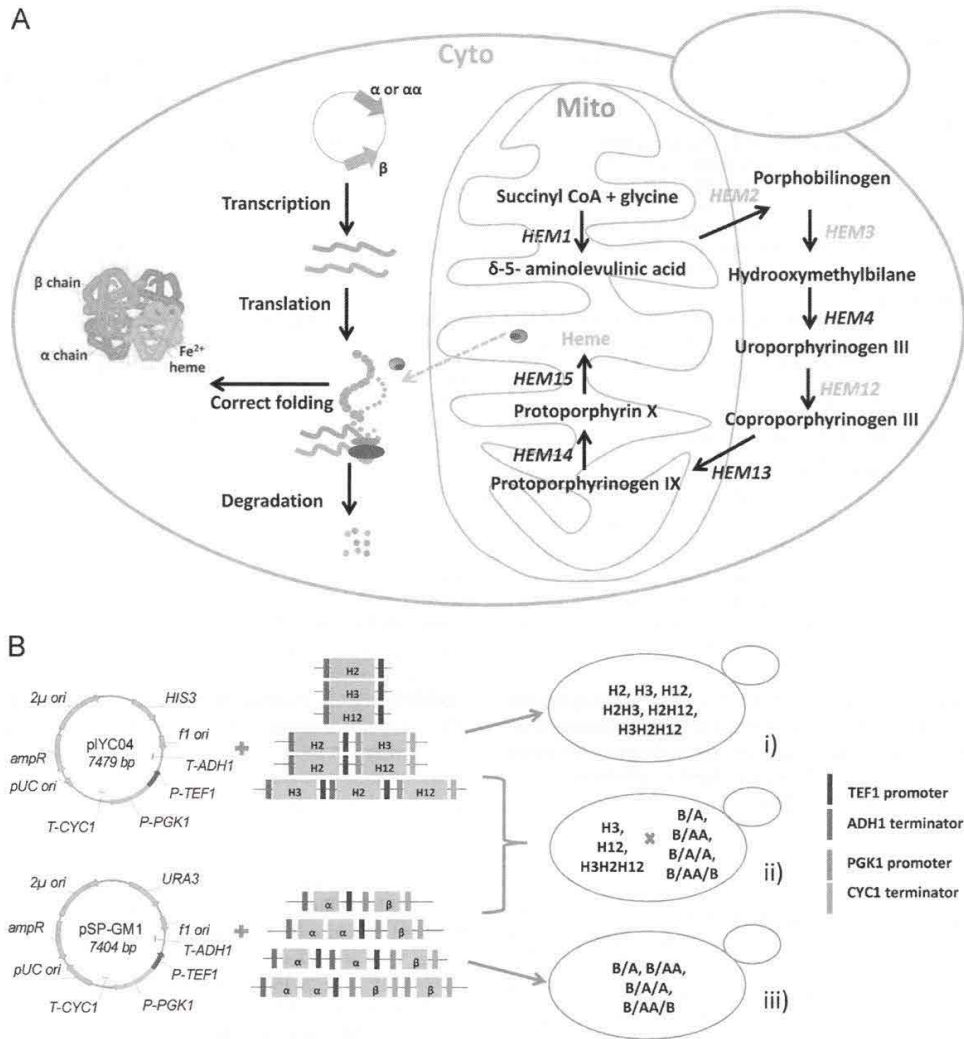


Fig. 1. Engineering strategy for optimizing the production of human hemoglobin. (A) Strategy overview: Yeast genes *HEM2*, *HEM3* and *HEM12* (highlighted in red) in heme biosynthesis pathway were overexpressed for an improved level of heme in cell cytosol. Human α and β globins were overproduced on plasmid in cytosol. Correct folding and assembling resulted in completed hemoglobin, failure in assembling resulted in degradation. (B) Strain construction: CEN.PK 113-11C as the expression host being transformed with i) heme overexpression plasmids: gene *HEM2*, *HEM3* and *HEM12* were inserted into the expression cassette *TEF1-ADH1*, either individually or in different combinations in the cassettes *TEF1-ADH1* and *PGK1-CYC1* on pLYC04. ii) globin overexpression plasmids: *HBA*, *HBB*, and *HBAA* were inserted into pSP-GM1 in the cassettes of *TEF1-ADH1* and *PGK1-CYC1* in different combinations. iii) heme and globin overexpression plasmids: The top three best heme/coproprophyrin producing plasmids *H3*, *H12* and *H3H2H12* were transformed in different combinations together with the globin overexpression plasmids *B/A*, *B/AA*, *B/A/A* and *B/AA/B*. Hemoglobin structure was modified from (Lu et al., 2011). Heme biosynthesis pathway was modified from (Hoffman et al., 2003). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detector, JASCO). The eluents, standard, and reversed phased C_{18} -column were included in the kit and the running conditions were referred to the kit.

2.3. Heme/porphyrin determination assay

The intracellular heme concentration was measured using a protocol derived from (Sassa, 1976) and slightly modified from (Michener et al., 2012). Cells were grown to a desired density and harvested at $OD_{600} \times mL = 8$ (e.g. if $OD_{600} = 2$, 4 mL of culture is harvested). The cell pellets were resuspended in 500 μL of 20 mM oxalic acid and then kept in dark at 4 $^{\circ}C$ overnight. 500 μL of preheated 2 M oxalic acid was added and half of the mixture was taken into an amber tube and heated up to 95–98 $^{\circ}C$ for 30 min during which the other half mixture was kept at room temperature. 200 μL of clear supernatant was transferred into a black 96

plate and fluorescence with excitation at $\lambda = 400$ nm and emission at $\lambda = 600$ nm was monitored. The total porphyrin level (bound heme and free porphyrins) corresponds to the heated sample, and the free porphyrins level corresponds to the non-heated sample.

2.4. SDS-PAGE assay

For protein extraction, yeast cell samples were taken at the indicated times and cells were harvested by centrifugation. Pellets were resuspended in 2 ml of NaOH containing 7% 2-mercaptoethanol plus 2 ml of trichloroacetic acid (TCA) and incubated 2 min at room temperature. After incubation, cells were washed with 1 M Tris-HCl (pH 7.5) and centrifuged. The cell pellet was resuspended in loading buffer (SDS 3%, Glycerol 10%, 2-mercaptoethanol 5%, Tris pH 6.8 0.0625 M, 0.01 mg bromophenol blue) and heated up to 95 $^{\circ}C$ during 5 min. Cells were discarded by centrifugation and the remaining protein

Table 1

Plasmids and strains used in this study.

Plasmid/Strain	Description	Marker	Reference
Heme over-expression plasmids			
pYCO4	Empty plasmid with bidirectional gene expression cassettes (<i>TEF1-ADH1</i> and <i>PGK1-CYC1</i> , 2 μ)	HIS3	(Chen et al., 2013)
H2	pYCO4 inserted with <i>HEM2</i> under <i>TEF1</i> promoter	HIS3	This study
H3	pYCO4 inserted with <i>HEM3</i> under <i>TEF1</i> promoter	HIS3	This study
H12	pYCO4 inserted with <i>HEM12</i> under <i>TEF1</i> promoter	HIS3	This study
H2H3	H2 inserted with <i>HEM3</i> under <i>PGK1</i> promoter	HIS3	This study
H2H12	H2 inserted with <i>HEM12</i> under <i>PGK1</i> promoter	HIS3	This study
H3H2H12	H2H12 inserted with an <i>HEM3</i> expression cassette under <i>TEF1</i> promoter	HIS3	This study
Globin over-expression plasmids			
pSP-GM1	Empty plasmid with bidirectional gene expression cassettes (<i>TEF1-ADH1</i> and <i>PGK1-CYC1</i> , 2 μ)	URA3	(Chen et al., 2012)
B/A	pSP-GM1 inserted with <i>HBA</i> under <i>TEF1</i> and <i>HBB</i> under <i>PGK1</i>	URA3	This study
B/AA	pSP-GM1 inserted with <i>HBA-Gly-HBA</i> under <i>TEF1</i> and <i>HBB</i> under <i>PGK1</i>	URA3	This study
B/A/A	B/A inserted with a <i>HBA</i> over-expression cassette under <i>TEF1</i>	URA3	This study
B/AA/B	B/AA inserted with a <i>HBB</i> over-expression cassette under <i>PGK1</i>	URA3	This study
Engineered strains for heterologous hemoglobin production			
H3 + B/A	CEN.PK 113-11C transformed with plasmids H3 and B/A		This study
H3 + B/AA	CEN.PK 113-11C transformed with plasmids H3 and B/AA		This study
H3 + B/AA/B	CEN.PK 113-11C transformed with plasmids H3 and B/AA/B		This study
H3 + B/A/A	CEN.PK 113-11C transformed with plasmids H3 and B/A/A		This study
H3 + pSP-GM1	CEN.PK 113-11C transformed with plasmids H3 and pSP-GM1		This study
H12 + B/A/A	CEN.PK 113-11C transformed with plasmids H12 and B/A/A		This study
pYCO4 + B/A/A	CEN.PK 113-11C transformed with plasmids pYCO4 and B/A/A		This study
pYCO4 + pSP-GM1	CEN.PK 113-11C transformed with plasmids pYCO4 and pSP-GM1		This study

Note: slash separates the globin overexpression cassettes.

suspension was used for loading into gradient (4–12%) polyacrylamide bis–tris gels (Novex, Invitrogen) at the selected protein concentrations. The marker ladder used was Novex Sharp Pre-stained Protein Standard from Invitrogen. Software GelPro Analyzer (Media Cybernetics) was used for quantification of band intensities.

2.5. Assay for active hemoglobin

Washed cell pellets were resuspended into 500 μ l of 100 mM of PBS buffer supplemented with 1 $\times$ protease inhibitor (Halt Protease Inhibitor Cocktail, Thermo), pH 7.0. The cell suspension was loaded into a FastPrepTM Lysing tube with an equal volume of glass beads (425–600 μ m diameter, Sigma). Cells were broken by applying 8 pulses of 20 s with the speed of 6 m/s using a FastPrep 24 instrument (MP, Biomedicals, LLC). The sample tubes were placed on ice for 3 min between pulses. Clear supernatants were harvested by centrifugation at full speed for active hemoglobin assay and protein quantification. Lipid removal agent Cleanascite (X2555-100, BIOTECH SUPPORT GROUP) was added according to product description in case of unclear suspensions in the supernatant. Protein concentration was measured by BCA protein assay kit (Cat 23,225, Pierce). The active hemoglobin was measured using the QuantiChromTM Hemoglobin Assay Kit (DIHEMOGLOBIN-250, Clonagen), based on an improved Triton/NaOH method, in which the hemoglobin is converted into a uniform colored end product that can be measured by spectrophotometer.

2.6. Relative gene transcription levels by qPCR

Yeast cells were cultured at 30 °C with shaking at 180 rpm in shake flask containing 50 ml of SD-His. Total RNA was isolated from 10 ml of cells with OD₆₀₀ around 1. The cells were disrupted by Fast-Prep 24 (MP-Biomedicals) using glass beads (Lysing matrix C, MP-Biomedicals). Extracted RNA was purified using the RNeasy kit (Qiagen). 200 ng of total RNA was used to synthesize cDNA from which 2 μ l of the synthesized cDNA was used as the template for the qPCR reaction to a final reaction volume of 20 μ l, using the Brilliant III Ultra Fast SYBRGreen QPCR Master mix (Stratagene) in

Mx3005P QPCR System (Agilent Technologies). Cycle thresholds (Ct) were normalized to the Ct value of *S. cerevisiae* ACT1.

2.7. Batch fermentations

Fresh colonies were picked to inoculate into 50 mL of SD- His-Ura media (containing 6.7 g/L of yeast nitrogen base w/o amino acids (Difco Laboratories, Sparks, MD), 0.75 g/L of complete supplement mixture (CSM, w/o histone and uracil) (MP Biomedicals, Solon, OH) and 2% glucose) to make seed cultures. After growing for 24 h at 30 °C at 180 rpm, the seed cultures were inoculated into the bioreactors with an initial OD₆₀₀ of 0.1. Fermentations were performed in DasGip 1.0-I stirrer-pro vessels (DASGIP GmbH, Germany) with a working volume of 600 mL in SD-His-Ura media. The temperature was controlled at 30 °C with agitation at 600 rpm and aeration at 1 vvm. Dissolved oxygen was controlled above 30% throughout the experiment. The impeller speed was automatically adjusted in response to the sensor. The pH was maintained at 6.0 using 2 M KOH in response to the pH sensor (Mettler Toledo, Switzerland). All fermentations were performed in biological duplicates. Dry cell weight was measured by filtering 5 mL of cell culture through a 0.45 μ m filter membrane and calculating the increased weight of the dried filter. Supernatants filtered through 0.45 μ m membrane were loaded to HPX-87G column (BIORAD, USA) on a Dionex Ultimate 3000 HPLC (Dionex Softron GmbH, Germany) to measure the concentrations of glucose, ethanol, and glycerol in the cultures. The samples were running with a flow rate of 0.6 mL/min at 65 °C using 5 mM H₂SO₄ as mobile phase.

3. Results and discussion

To investigate the impact of increased endogenous heme synthesis on heterologous hemoglobin production in *S. cerevisiae*, a series of recombinant strains were constructed by transforming different expression plasmids in the yeast strain CEN.PK 113-11C (Fig. 1, Table 1).

3.1. Increased endogenous heme level by overexpression of heme genes

By transforming the heme overexpression plasmids H2, H3, H12, H2H3, H2H12, and H3H2H12 into CEN.PK 113-11C, strains with the engineered heme biosynthesis pathway were constructed (Table 1). The strains were designated with the same names as the corresponding plasmids H2, H3, H12, H2H3, H2H12, and H3H2H12 (Table 1). The cytosolic intermediate of the heme biosynthetic pathway, coproporphyrin was measured in all the engineered strains and used as an indicator for heme and porphyrin levels in the cells (Fig. 2A). All strains were compared with the strain transformed with pLYC04 (empty plasmid) and the fold values were calculated relative to it. Strain H3 showed a four times increase on coproporphyrin level compared to the control strain, pLYC04, followed by H12 and H3H2H12 whose coproporphyrin levels were 3.2 and 2.2 times to pLYC04 respectively (Fig. 2A). In terms of bound heme level, H3 still stood out with almost 5 fold of pLYC04, followed by H3H2H12, 2.8 fold, and H12, 2.4 fold (Fig. 2B).

HEM13 transcription is known to be negatively regulated by oxygen and heme (Zagorec and Labbe-Bois, 1986) through *HAP1* and *ROX1* (Keng, 1992). Overexpression of the genes in the heme biosynthesis pathway (strains H2, H3, H12, H2H3, H2H12 and H3H2H12) resulted in certain improvements on heme levels (Fig. 2B) which might induce feedback inhibition on the synthesis of Hem13p which converts coproporphyrinogen III into protoporphyrinogen IX, causing subsequent accumulation of coproporphyrins (Michener et al., 2012). The coproporphyrin levels (Fig. 2A) may therefore represent reliable indicators of the heme levels in the strains, and Hem13p might be another potential bottleneck in addition to the reported rate-limiting steps catalyzed by Hem2p

and Hem3p. Both driven by *TEF1* promoter, *HEM3* overexpression (H3) showed a better effect than *HEM12* overexpression (H12) on coproporphyrin accumulation suggesting a greater rate-limiting effect of Hem3p than Hem12p during heme biosynthesis. Overexpression of *HEM3* released the bottleneck in this step providing more intermediate for further (catalyzed by Hem4p and Hem12p) conversion to coproporphyrin. On the other hand, also driven by the *TEF1* promoter, *HEM2* overexpression (H2) exhibited a very limited improvement on the coproporphyrin level in the cells although the Hem2p has been confirmed as a rate-controlling enzyme under aerobic conditions (Hoffman et al., 2003; Zhang and Hach, 1999). This is very likely due to the enzymes blocking the subsequent steps, especially Hem3p and Hem12p, were not overexpressed in H2. The point could be supported by the higher coproporphyrin levels presented in H2H3 and H2H12 than in H2. Since *HEM3* in H2H3 and *HEM12* in H2H12 are both under the *PGK1* promoter which has a reported weaker strength than *TEF1* promoter (Nacken et al., 1996) driving *HEM2* in these strains, the steps catalyzed by Hem3p and Hem12p may still being blocked to certain extends which could partly explain the not so obvious improvements on the coproporphyrin levels in these strains. Additional reason could be that with more gene cassettes compulsory for expression, the resource for expressing one gene has to be distributed to more genes and therefore each gene gets lesser/weaker expression. The expressions of *HEM2*, *HEM3* and *HEM12* in H2H3 and H2H12 might have been reduced to certain levels resulting in the minor improvement of coproporphyrin accumulation than H2. Similarly, the expression burden was even heavier for the cells in the H3H2H12 strain and consequently the proteins especially Hem3p which has been indicated to be the most effective enzyme compared to Hem2p and Hem12p to block heme synthesis, were less expressed, which could be the reason that

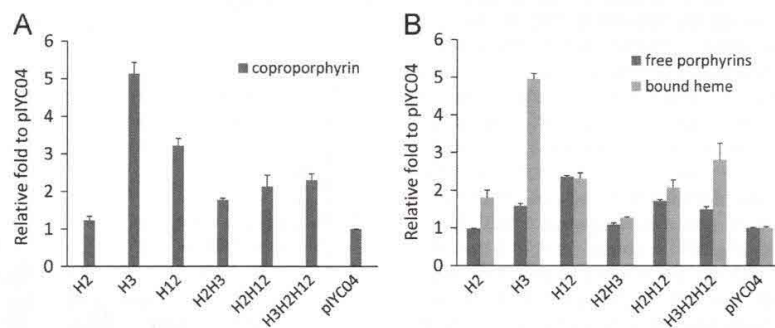


Fig. 2. Porphyrin/heme levels in heme over-expression strains. (A) Level of coproporphyrin. (B) Level of free porphyrins and bound heme. The relative fold change is calculated based on the fluorescence reads (excitation at $\lambda=400$ nm and emission at $\lambda=600$ nm) of the sample strains against the control strain, pLYC04.

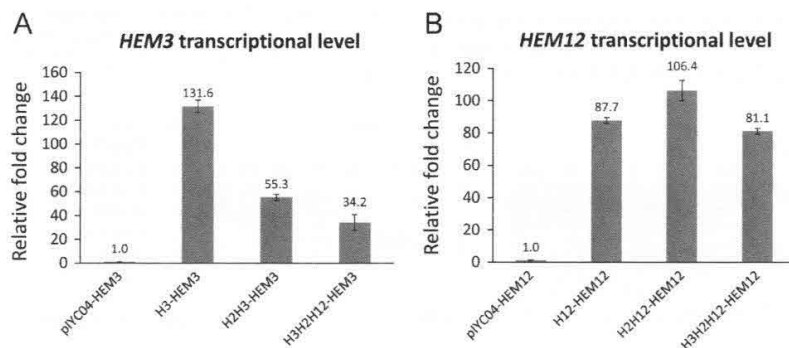


Fig. 3. Transcription level of (A) *HEM3* and (B) *HEM12* in the engineered strains transformed with Heme plasmids. In both cases, the relative fold change is calculated based on the transcription levels of the genes (relative to *ACT1*) in the sample strains against the control strain, pLYC04.

H3H2H12 did not show higher or even comparable level of coproporphyrin than H3 and H12.

The transcriptional levels of *HEM3* and *HEM12* were examined and as shown in Fig. 3, the mRNA levels of the heme genes *HEM3* and *HEM12* were much higher in the strains transformed with the corresponding vectors than with the empty vector. The transcriptional level of *HEM3* in strain H3 is extremely high (131.6 folds) correlating to the highest coproporphyrin accumulation in the H3 strain.

The acidic-heat method can discriminate between heme bound to proteins and the free porphyrins in cells. As shown in Fig. 2B, the heme level in H3 was the most abundant followed by H3H2H12 and H12, showing the same trend as that for coproporphyrin. The higher heme level in H3 than in H12 might indicate a bigger flux of coproporphyrins converted to protoporphyrin IX than in H12, even though the coproporphyrins accumulated in H3 were also more than in H12. The free porphyrins include more than coproporphyrins which might explain the higher free porphyrin levels in H12 compared to H3. Both assays indicate that *HEM3* overexpression alone generates the highest heme levels in cells compared to the other strains.

3.2. Co-overexpression of heme and globin constructs increased heterologous hemoglobin production

The top three heme overexpression plasmids H3, H3H2H12 and H12 were selected to be co-transformed together with a range of globin overexpression plasmids B/A, B/AA, B/AA/B, B/A/A and the empty vector pSP-GM1 (Fig. 1B). The combination of α and β globin genes on the globin vectors were designed based on (i) α globin is more unstable than β globin (Vasseur-Godbillon et al., 2006) and (ii) $\alpha\beta$ dissociation can be prevented by linking two α globin genes with a glycine linker (Looker et al., 1992). The expression levels were examined by SDS-PAGE (Fig. 4). By co-overexpression with H3, the globin construct B/A/A showed the highest protein expression, however, there was nearly no band of the right size (around 16 kDa) when H3 was expressed in combination with other globin constructs (Fig. 4A). Very weak signal of α and β globins were detected by ELISA (data not shown) and based on this result, co-overexpressions of B/A/A with the other two heme overexpression plasmids H12 and H3H2H12 were also checked by SDS-PAGE: with heme overexpressed, the globin synthesis was also improved (Fig. 4B). Expression of B/A/A with the empty plasmid pYC04 did not show clear expression, thus further supporting the hypothesis that endogenous heme levels are insufficient to achieve high heterologous globin production (Smith et al., 2011).

3.3. Effect of B/A/A co-overexpression on heme/porphyrin level

In order to understand if an improved hemoglobin expression is correlated with an increased heme level, the heme/porphyrin level in the strains co-overexpressed with B/A/A was examined.

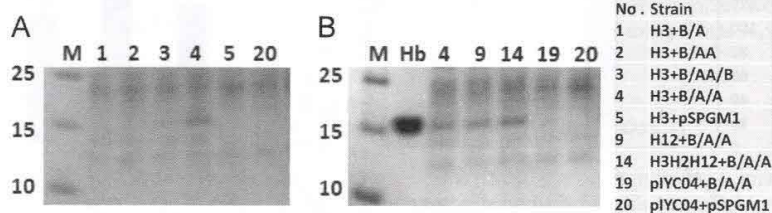


Fig. 4. SDS-PAGE analysis of the engineered strains with heme and globin co-overexpressed. (A) co-overexpression of H3 with the globin constructs. (B) co-expression of B/A/A with the heme constructs. M stands for the protein ladder, Hb stands for the standard hemoglobin (16 kDa) and the numbers indicate the strains listed in the table on the side. 50 μ g of total protein was loaded per lane.

The relative fold change was calculated relative to the heme level in pIYC04+pSP-GM1. As shown in Fig. 5, both the bound heme level and the free porphyrins level in H3+B/A/A are higher than in H3+pSP-GM1, and in the H3H2H12+B/A/A strain compared with H3H2H12+pSP-GM1. With B/A/A co-overexpressed, we found an increase of 87% with H3 co-overexpression. In erythroid cells, heme export from the mitochondria requires a corresponding synthesis of heme-binding proteins (Lynch et al., 2009). One hypothesis could be that with higher levels of globin expressed, an initial decrease in the pool of free cytosolic heme occurs which induces an increase in the production rate of porphyrins resulting in a global increase of both free porphyrins and consequently the hemoproteins. The bound heme levels that we measured would be thus higher.

3.4. Physiological characterization and active hemoglobin production in batch fermentations

The physiological properties of the recombinant hemoglobin strains were characterized in batch fermentations, as shown in Table 2. The specific growth rates did not vary significantly which were 0.25, 0.28, 0.28 and 0.27 respectively for H3+B/A/A, H3H2H12+B/A/A, pIYC04+B/A/A and pIYC04+pSP-GM1. At the end of cultivation, the percentages of the active hemoglobin in the whole extracted soluble proteins were assayed and calculated. The result showed that the strain H3+B/A/A had the most active hemoglobin accounting for 4.09% of the whole cell soluble protein followed by H3H2H12+B/A/A with 3.82%. There was activity accounted for 2.8% of the whole cell protein in the pIYC04+pSP-GM1 strain, most probably contributed by the endogenous flavo-hemoglobin (Zhao et al., 1996).

The specific growth rate of strain H3+B/A/A was slightly lower when compared with other strains. It also had the most soluble hemoglobin production and less biomass yield. Declines on

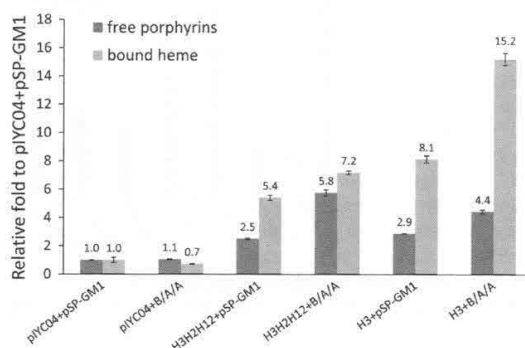


Fig. 5. Effects of B/A/A plasmid co-overexpression on heme/porphyrin level. The relative fold change is calculated based on the fluorescence reads (excitation at $\lambda = 400$ nm and emission at $\lambda = 600$ nm) of the sample strains to that of the control strain, pIYC04.

Table 2

The physiological parameters and soluble hemoglobin concentration.

Strain	$\mu_{\max}^a$	Y_{SX}^b	Y_{SE}^b	Y_{SG}^b	[Hb]% ^c
H3+B/A/A	0.25 ± 0.001	0.10 ± 0.005	0.29 ± 0.01	0.04 ± 0.006	4.09 ± 0.04
H3H2H12+B/A/A	0.28 ± 0.01	0.11 ± 0.006	0.30 ± 0.004	0.04 ± 0.001	3.82 ± 0.05
pLYC04+B/A/A	0.28 ± 0.006	0.12 ± 0.003	0.34 ± 0.004	0.04 ± 0.001	3.14 ± 0.06
pLYC04+pSP-GM1	0.27 ± 0.002	0.11 ± 0.004	0.32 ± 0.003	0.04 ± 0.001	2.8 ± 0.05

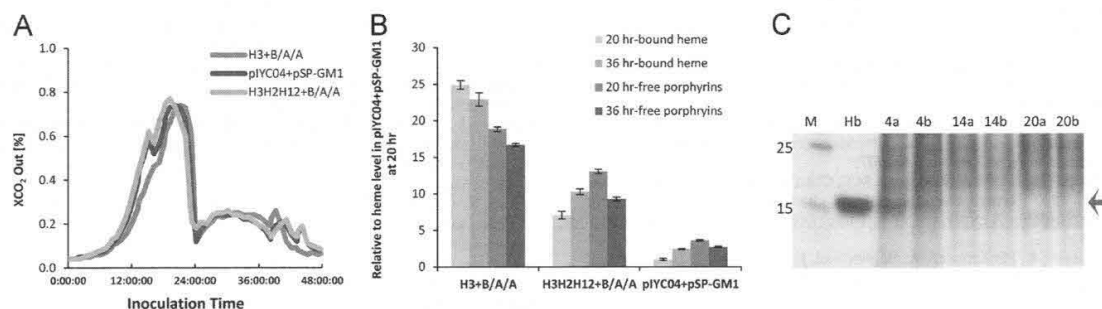
^a The maximum specific growth rate (h^{-1}) on glucose.^b Yield of biomass(X), ethanol(E), and glycerol(G) from glucose (S)(g/g).^c Percentage of active hemoglobin on whole soluble cell protein (g/g).

Fig. 6. Heme and hemoglobin production during batch fermentations. (A) Offgas profile: showing that glucose phase finished at around 20 h and ethanol phase started at around 36 h. (B) Heme and porphyrin levels in strains after inoculation for 20 and 36 h respectively. (C) SDS-PAGE analysis of the globin patterns produced at 20 and 36 h respectively. 4, 14, 20 represent strains H3+B/A/A, H3H2H12+B/A/A and pLYC04+pSP-GM1 respectively. a and b represent 20 and 36 h after inoculation. 25 μ g protein loaded.

specific growth rate due to plasmid-encoded recombinant gene expression have been extensively noticed in bacteria, yeast and filamentous fungi (Hoffmann and Rinas, 2004; Pakula et al., 2005; Tyo et al., 2012) all pointing to a higher stress and impairment to cellular process. In the strain H3+B/A/A, a coordinated over production of heme and globin units have been achieved which further assembled to the correct tetramer. This process might result in a flux switching from normal cellular processes to recombinant hemoglobin production, and consequently affect cell performance. The heme and globins production is less effective in the other two recombinant strains thus the normal growth have not been impaired significantly.

The cell growth reached the end of the glucose phase and the beginning of the ethanol phase at around 20–36 h after inoculation respectively (Fig. 6A). Interestingly, we found that the heme level in H3+B/A/A significantly decreased before the ethanol phase started (Fig. 6B) indicating a negative correlation between heme/porphyrin synthesis and respiration. In contrast, in H3H2H12+B/A/A and pLYC04+pSP-GM1, the heme level kept improving after the glucose phase was finished with a decrease in the free porphyrin level suggesting that porphyrin synthesized in the glucose phase were gradually converted into heme. The correlation between the heme levels and the hemoglobin production was also measured by SDS-PAGE (Fig. 6C). Clearly, the level of hemoglobin produced in the glucose phase is higher than in the beginning of the ethanol phase (about 1.3 fold) for H3+B/A/A, confirming that efficient heterologous hemoglobin production requires a low rate of respiration, as it occurs during the glucose consumption phase in the fermentation process.

4. Conclusion

Previous studies have reported successful expression of recombinant human hemoglobin in the yeast *S. cerevisiae* (Adachi et al., 1992; Coghlan et al., 1992; Martin de Llano et al., 1993; Ogden et al., 1994; Wagenbach et al., 1991). In this study, yeast heme

synthesis pathway was engineered for the first time to facilitate the synthesis of recombinant hemoglobin, resulting in an increase of 1.3 times (H3+B/A/A against pLYC04+B/A/A) in hemoglobin production. This work has generated a plasmid platform for further improving heterologous hemoglobin production in yeast. Further improvement might be achieved by engineering the heme membrane transport and/or the ion trans-membrane pathway (e.g. ferric reductase and permease-oxidase complex required for iron uptake in *S. cerevisiae*) (Dancis et al., 1990; Ginzburg et al., 2009; Graves et al., 2008; Stearman et al., 1996; Villarreal et al., 2008). In addition to hemoglobin, a similar strategy can be applied to enhance the production of other hemoproteins, e.g. cytochromes, catalases and P450 enzymes which are powerful catalysts for synthesis of drug metabolites (Jung et al., 2011). This work is further demonstrating the possibility for efficient production of complex proteins (e.g. multimer with a prosthetic group), as we have demonstrated that the production of a prosthetic group can be increased through metabolic engineering and hence eliminate the need for exogenous supplementation with expensive precursors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2013.10.010>.

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High-level secretion of biologically active aprotinin from the yeast *Pichia pastoris*

Thomas Vedvick, Richard G. Buckholz, Michael Engel, Mary Urcan, Jennifer Kinney,
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SUMMARY

A synthetic gene encoding aprotinin (bovine pancreatic trypsin inhibitor) was fused to the *Saccharomyces cerevisiae* prepro alpha mating factor leader sequence at the dibasic amino acid processing site. *Pichia pastoris* strains were developed to express one or multiple copies of a methanol-inducible expression cassette containing the gene fusion. *P. pastoris* containing a single copy of the vector secreted approximately 150 mg/l of immunoreactive protein. A construct bearing five copies of the expression cassette secreted 930 mg/l of aprotinin. The purified aprotinin molecule was equipotent with the native molecule in a trypsin inhibition assay. Protein sequence analysis showed that the alpha factor-aprotinin fusion was not processed at the basic amino acid residues Lys-Arg. Instead, recombinant aprotinin had additional N-terminal amino acids derived from prepro alpha factor. The N-terminal extension was variably 11 or 4 amino acids. Inclusion of the spacer DNA sequence encoding Glu and Ala between aprotinin and the Lys-Arg processing site led to the secretion of a biologically active aprotinin containing only a Glu-Ala N-terminal extension.

INTRODUCTION

Aprotinin is a 58-amino acid peptide which has trypsin inhibitory activity. The molecule has a molecular weight of 6512 Da and contains three disulfide bonds. Aprotinin is a trypsin-specific member of the Kunitz-type protease inhibitor family [12]. It has been purified from bovine lung, spleen, pancreas and other organs. As a protease inhibitor, aprotinin forms a tight complex ($K_i = 6 \times 10^{-14}$) with the active site of trypsin. A lysine residue at position 15 is implicated in providing the trypsin-like specificity [16].

Aprotinin is used as a human therapeutic, marketed under the name TrasylolTM, for treatment of hemorrhagic shock. Although of bovine origin, aprotinin is well-tolerated in humans, presumably because of its identity with human Kunitz-type inhibitors [5]. This protease inhibitor is also used in mammalian cell culture media to stabilize the desired product and also to potentiate media growth factor activity by diminishing proteolysis, and as

a reagent in the recovery and purification of proteins produced in cell culture [7].

This paper describes the expression of aprotinin in the yeast *Pichia pastoris*, a yeast developed for industrial scale production of proteins via recombinant DNA technology. The high cell density fermentations and methanol-regulated expression cassettes that characterize the *Pichia* technology have been described previously [2]. This expression system is characterized by high productivity and excellent reproducibility both at laboratory and pilot plant scale [2,4]. *Pichia* has been used to express both intracellular and secretory proteins [2,4].

In these experiments, we characterize the efficacy of the *Saccharomyces cerevisiae* prepro alpha factor secretion signal in the secretion of aprotinin. Prepro alpha factor is an 85-amino acid peptide that, in many cases, can facilitate the secretion of peptides fused directly to the paired basic amino acids at the C-terminus of the leader sequence. A membrane-bound endopeptidase can then cleave the mature peptide from the precursor allowing efficient secretion [6]. This work defines some aspects of the limitations of the prepro alpha factor leader sequence. Spatial relationships between the mature aprotinin and the dibasic amino acid processing site play a key role in the efficiency of processing.

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MATERIALS AND METHODS

Vectors, recombinant strain construction and growth

The aprotinin DNA sequence was generated from a back-translation of the protein sequence using codons from highly expressed *Pichia* genes [11]. Oligonucleotides were annealed, ligated together, and products were separated via agarose gel electrophoresis. A band of the correct size was eluted from the gel and subcloned into M13mp18. The sequence was verified by dideoxy sequencing [14].

The aprotinin coding sequence was fused to the prepro alpha factor sequence by primer-directed mutagenesis creating pAPR104 [17]. The prepro alpha factor-aprotinin fusion was then subcloned as an *EcoRI* fragment into pAO815, a *Pichia pastoris* integrative expression vector, to generate pAPR205 (Fig. 1A). This plasmid contains the aprotinin fusion gene under the control of the methanol responsive *AOX1* promoter and transcription terminator. The wild-type *Pichia HIS4* gene is also present for selection in yeast. A vector containing five copies of the aprotinin expression cassette was generated by an in vitro amplification scheme [1] and is called pAPR501 (Fig. 1A). The vector pAPR904, containing the Glu-Ala residues immediately upstream of the aprotinin, was generated by primer-directed mutagenesis of a subclone of pAPR205 in M13mp19 (pAPR305). This mutagenized gene was subsequently subcloned into pAO815 to create pAPR894.

Pichia pastoris strain GS115 (*his4*) [2] was separately transformed with plasmids pAPR205, pAPR501 and pAPR894 using the lithium chloride method [9]. Transformants were analysed via Southern blot analysis to ascertain both expression cassette copy number and locus of integration. The *Pichia* strains used in these experiments are listed in Table I.

Pichia pastoris strains were grown in 1-l fermentors essentially as described by Digani et al. [4]. The fermentation protocol for foreign protein expression in *Pichia* contained three steps. Cell mass was accumulated (in the absence of aprotinin expression) by growth on excess glycerol. A limited glycerol feed was used to increase cell mass and derepress the *AOX1* promoter followed by growth in a methanol fed-batch mode to fully induce expression.

Measurement of aprotinin activity

The biological activity of aprotinin was measured in a trypsin inhibition assay using benzoyl L-arginine p-nitroanilide as substrate. A 1-ml reaction contained 50 mM Tris · HCl pH 8.0, 10 mM MgCl₂, 10 mM CaCl₂, 20 enzyme units of trypsin and either from 0 to 22.4 µg/ml standard (Sigma Chemical Co., St. Louis, MO) or an unknown concentration of recombinant aprotinin. The

reaction was incubated for 30 min at ambient temperature. A 50-µl aliquot of the enzyme-inhibitor complex was then added to 20 mM substrate in a 1-ml total volume reaction at pH 8.0 and the change in absorbance at 410 nm was recorded for 5 min. A standard curve was generated by measuring residual trypsin activity for different quantities of aprotinin standard and trypsin-inhibitory aprotinin activity of the recombinant material was determined.

Radioimmunoassay

Aprotinin was iodinated using the chloramine T procedure [13]. Rabbit antisera was raised by injection of aprotinin with Freund's adjuvant. Reaction products were separated on a Bond-elute column, followed by HPLC fractionation on a C18 (Vydac) column, using a 0–100% CH₃CN gradient in 0.1% trifluoroacetic acid. A 1:20000 dilution of antisera, incubated 20 h at 4 °C in the presence of 10⁴ cpm of ¹²⁵I-aprotinin and various concentrations of unlabelled aprotinin in 50 mM sodium phosphate pH 7.4, 25 mM EDTA, 0.1 M NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.1% sodium azide, was subsequently precipitated with Pansorbin (1/40). After addition of 2.0 ml of wash buffer (0.9% NaCl, 5 mM EDTA, 2% Triton X-100) the tubes were centrifuged, decanted, and ¹²⁵I-aprotinin was measured in a gamma counter. A dilution series using unlabelled aprotinin gave an ED₅₀ of approximately 0.5 ng, with a linear range of 0.1 to 1.0 ng.

Protein sequencing

To determine the amino terminal peptide sequence of recombinant aprotinin, clarified broth samples were separated by SDS-PAGE in a tricine gel system [15]. Subsequently, proteins in the gel were transferred electrophoretically onto Immobilon, a polyvinylidene difluoride membrane, at 200 mA constant current for 60 min in 10 mM CAPS buffer, pH 11, containing 10% methanol. After the electrotransfer was complete, the membrane was stained with Coomassie Blue (0.1% Coomassie Blue 250, 50% methanol). The aprotinin band was excised from the blot and subjected to sequential Edman degradation on the Applied Biosystems 470 gas phase sequencer.

RESULTS

The strains listed in Table I were analysed by Southern blotting to determine expression cassette copy number and locus of integration. Single copy vectors, all integrated at the *AOX1* locus, were chosen for study. One strain, G + APR205S10, had a fortuitous double integration of the plasmid at *AOX1*, and is consequently, a two-expression cassette copy strain. The five-copy strain G + APR501S3 was integrated at the *HIS4* locus. Sub-

TABLE 1

Aprotinin expression recombinant strains

Gene	Recombinant strain designation	Expression cassette copy number	Locus of integration
Aprotinin	G + APR205S5	1	<i>AOX1</i>
Aprotinin	G + APR501S3	5	<i>HIS4</i>
EA-Aprotinin	G + APR894S12	1	<i>AOX1</i>
Aprotinin	G + APR205S10	2	<i>AOX1</i>

sequently, these strains were grown in 1-l fermentors to characterize aprotinin expression. Aprotinin levels were measured in cell-free broth by RIA at the end of the fermentation (Table 2). Single-copy strains consistently produced approx. 150 mg of aprotinin/l, whereas a five-copy strain synthesized almost 1 g/l. Each of these fermentations was characterized by robust cell growth with final cell yields between 300 and 400 g/l (wet weight). These results suggest that aprotinin production was not toxic to *Pichia pastoris*. The increase in expression level seen between the one- and five-copy strains also suggested that cells were tolerant of aprotinin at high levels (around 1 g/l). The expression levels measured by RIA correlated well with the values measured by the enzyme inhibition assay, indicating that recombinant aprotinin is correctly folded and fully active (Table 2).

Biochemical characterization of the aprotinin peptide using high-resolution Tricine gels (followed by silver staining) indicated that aprotinin produced by the single-copy strain G + APR205S5 was slightly larger than the authentic aprotinin standard. The molecule produced by the two-copy strain G + APR205S10 was considerably larger (data not shown), and no authentic aprotinin was detected in the broth. These results were verified by protein sequence analysis (Table 3).

G + APR205S5, a single-copy strain, produced a peptide containing four amino acids of prepro alpha factor

TABLE 2

Aprotinin yields from four recombinant strains

Recombinant strain designation	Yeast cells (g/l wet weight)	Immunoreactive aprotinin (mg/l)	Active aprotinin (mg/l)
G + APR205S5	320	143	143
G + APR501S3	441	930	825
G + APR894S12	302	143	83
G + APR205S10	304	320	324

TABLE 3

N-terminal sequence of aprotinin produced by various recombinant strains

Recombinant strain designation	N-terminal sequence
G + APR205S5	LDKRRPDFC
G + APR205S10	AKEEGVSLDKRRPDFC
G + APR984S12	EARPDFC

appended to the N-terminus of aprotinin. The strain G + APR205S10, a two-copy strain, contained an 11-amino acid extension of aprotinin. In both cases, aprotinin was not processed at the paired basic amino acid residues at the C-terminus of prepro alpha factor (Fig. 1B). Time course analysis of aprotinin expression in strain G + APR205S5 revealed that the 11-amino acid extension was the first aprotinin species to appear in the broth. The molecule was subsequently degraded to the four-amino acid extension presumably by media proteases. There was no evidence of unprocessed material in the fermentation broth.

To further analyse the expression of this improperly processed aprotinin, we inserted the coding sequence for the dipeptide Glu-Ala between the Lys-Arg processing sites and the first amino acid of aprotinin. It was hypothesized that this modification would serve to separate the basic amino acids from the target peptide and introduce a potential exopeptidase processing site analogous to the dipeptidyl amino peptidase activity encoded by the *Saccharomyces cerevisiae* *STE13* gene [10]. The aprotinin analog gene was inserted into an expression vector, which was used to create a *Pichia* transformant, G + APR894S12.

Protein sequence analysis of the fermentor broth indicated that a single species was present which contained the N-terminal sequence Glu-Ala-Arg-Pro-Asp, i.e., the unprocessed Glu-Ala spacer followed by mature aprotinin. There was no indication that the authentic sequence was present.

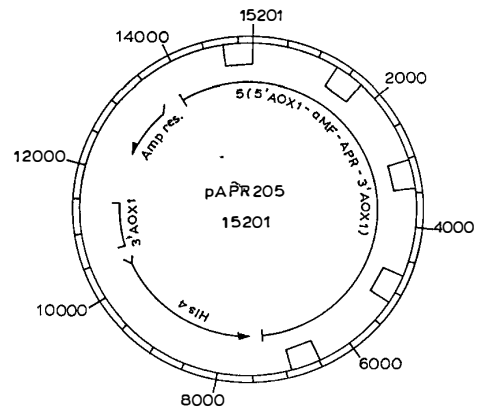
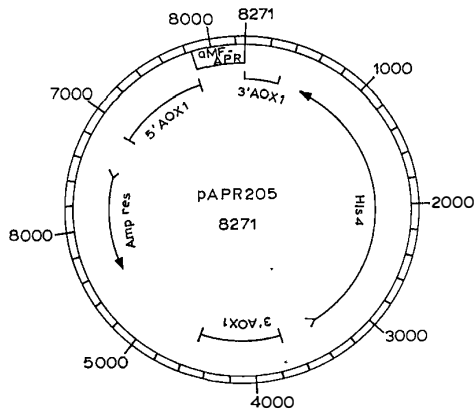
DISCUSSION

We have demonstrated that aprotinin-like peptides, bearing various N-terminal extensions can be produced in *Pichia pastoris* as secreted products. High level of expression is related to three factors: high-level transcription of the *AOX1* promoter [3], efficient translation of the heterologous mRNA [2,4], and efficient processing and secretion of the aprotinin peptide.

The proportional increase in aprotinin expression seen

200

'A'



'B'

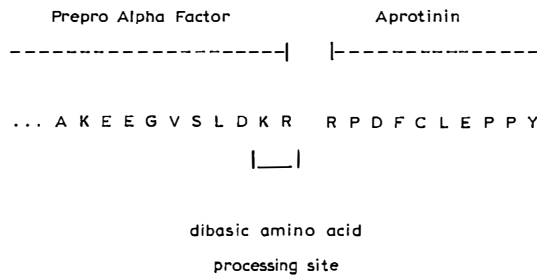


Fig. 1. **A.** Plasmid maps of the single-copy vector pAPR205 and multi-copy vector pAPR501. *AOX1* 5' and 3' regulatory regions are highlighted. The boxed areas are coding sequences flanked by the regulatory regions. The selectable marker, the *Pichia HIS4* gene is delineated by an arrow. **B.** Amino acid sequence at the junction of the prepro alpha factor leader sequence and the aprotinin peptide.

by increasing the copy number five-fold, was consistent with efficient secretion, since increased synthesis of the alpha factor-aprotinin fusion resulted in a direct increase in secreted aprotinin. The strain bearing five expression cassettes results in levels approaching 1 g/l. We anticipate that further increases in copy number would result in even higher levels of aprotinin.

One unexpected result of this research was the inability of the dibasic amino acid processing enzyme to cleave the precursor at the appropriate Lys-Arg residues. Previous studies have demonstrated the existence of this processing enzyme in *Pichia*, e.g., accurate processing of epidermal growth factor (EGF) from an alpha factor EGF fusion (R. Siegel, in preparation). Therefore, the resulting 11-amino acid extension seen in this work probably arises from another endoproteolytic activity located in the secretory pathway. The +11 form was the first aprotinin

species that was detectable in broth. In some cases, it was degraded to a four-amino acid extension (+4 form) by proteases present in the fermentor broth. The lack of proteolysis at the Lys-Arg processing site was remedied by placing a Glu-Ala encoding spacer in the gene between Lys-Arg and the mature aprotinin. In this gene construct, complete cleavage was seen at Lys-Arg, implying that steric factors derived from the aprotinin portion of the fusion to alpha factor were responsible for inhibiting processing. By moving the basic amino acids away from the N-terminus of aprotinin, efficient proteolysis at Lys-Arg was achieved.

In the case of G + APR894S12, the Glu-Ala extension, there was no removal of the Glu-Ala residues (Table 3). Strains containing two and three Glu-Ala pairs at the N-terminus showed no evidence of Glu-Ala removal (data not shown). One possible conclusion is that a membrane-

bound or secretory dipeptidyl amino peptidase is not present in *Pichia*. Alternatively, these enzymes, which are serine proteases, may be inhibited by aprotinin, resulting in the inability to remove the N-terminal Glu-Ala dipeptide [8].

We observed full anti-trypsin activity with the *Pichia*-derived aprotinin present in the crude broth and also with purified recombinant aprotinin in both G+APR205S5 and G+APR894S12. Apparently the +11, +4, and Glu-Ala extensions of aprotinin did not interfere with biological activity. Although these aprotinin analogs may not be acceptable for all human pharmaceutical applications they may find an excellent niche in industrial use. Further, high yields and simplicity of production may result in considerable cost reduction in mammalian cell culture applications.

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Karl-Heinz Engel, Gary R. Takeoka,
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Safety Issues

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Chapter 1

Foods and Food Ingredients Produced via Recombinant DNA Techniques

An Overview

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Commercial products resulting from recombinant DNA techniques in agricultural biotechnology have just entered or are about to enter the market. Some of the progress in improving nutrition and acceptability of foods as well as improving herbicide, insect, and virus resistance of plants are discussed. Enzymes and microorganisms can now be more precisely altered to optimize their roles in food production. Genetic modification of animals is much more complex than that of microorganisms and plants, nevertheless, some interesting progress is being made. The applications of genetic engineering in food production are evoking positive and negative reactions. The concepts presently being developed to assess the safety of foods derived from modern biotechnology are outlined. The aspects involved in creating a framework for regulatory oversight on genetically modified foods are also discussed.

The use of microorganisms in the production of foods and beverages, such as bread, cheese, wine, or beer has a long tradition dating back to ancient days. Industrial biotechnology has its roots in the classical production of fermented foods. Therefore, it is a logical consequence that the application of the so-called "modern biotechnology" does not remain limited to areas such as medicine and pharmaceuticals but is increasingly applied in the production of foods and food ingredients. Developments in genetic engineering have created new dimensions in classical biotechnology. By using recombinant DNA techniques, it has become possible to direct the movements of specific and useful segments of genetic material between unrelated organisms, thereby crossing the barriers between plants, animals, and microorganisms. The scope of "modern biotechnology" goes beyond the traditional area of food fermentation processes. It is now possible to make specific genetic modifications in plants and animals that introduce traits or substances that could not be introduced by traditional methods.

After fascinating developments in recent years on the level of basic research, the applications of recombinant DNA techniques in agricultural biotechnology are now heading towards attractive businesses. Commercial products have just entered or are about to enter the market. The spectrum of applications is comprised of genetically modified crops, foods produced from genetically modified microorganisms, as well as food ingredients/additives obtained from genetically modified organisms.

Plants

The enormous advances in genetic engineering of plants are due to progress in both cellular and molecular biology. The first practical technique for introduction of genetic material into plants was based on the use of vectors from *Agrobacterium tumefaciens*. Due to the narrow host range of this soil bacterium, the scope of the method had been limited to dicotyledonous plants. The progress in cell biology enabling the recovery of intact and fertile plants from single cell and protoplast cultures stimulated research activities in the area of direct DNA transfer. Because it is possible to introduce foreign DNA into protoplasts after electroporation or polyethyleneglycol treatment, the range of plants accessible to genetic engineering was increased significantly. A further decisive breakthrough was achieved by the development of the biolistics approach involving bombardment of the plant tissue with DNA-coated metal particles. It is now possible to engineer almost all important legumes and cereals (1-5).

Genetic engineering offers the possibility not only to add new traits to an organism but also to down-regulate the activities of specific endogenous genes. A versatile and useful method for such a blocking of decisive steps in metabolic pathways is the so-called "antisense technique" (6). The methodology is based on the introduction of an oligonucleotide which is transcribed into messenger-RNA (m-RNA) consisting of sequences complementary to the m-DNA produced through the transcription of the endogenous target gene. Due to the interaction (hybridization) of "sense" and "antisense" RNA, the translation of the corresponding enzyme is strongly reduced. The first genetically modified crop approved for food use, the Flavr-Savr™ tomato, is a prominent example of the commercial exploitation of the "antisense" technology (7).

Analogous to the goals of conventional breeding programs, recombinant DNA techniques are applied to improve (i) agronomic characteristics of crops, such as yield and resistance to diseases and pests, (ii) processing parameters, e.g., optimum solids levels or increased shelf life, and (iii) food quality, including factors such as aroma, taste, and nutritional value. Currently, the majority of commercial applications of plant genetic engineering aim at increasing the yield of food crops by influencing their tolerances to specific herbicides and their resistance to insects and diseases.

Herbicide tolerance. One of the agronomically and commercially important applications, and at the same time an application of genetic engineering strongly opposed by consumer advocates, is the attempt to increase crop yields by increasing the tolerance of plants to particular herbicides. Strategies applied to achieve this goal demonstrate the diversity of tools offered by recombinant DNA

techniques: (i) increasing the level of the target enzyme for the herbicide by overexpression, (ii) decreasing the sensitivity of the target enzyme by introducing a gene coding for an enzyme resistant to the herbicide either naturally (from a microbial source) or through specific mutation, and (iii) introducing a gene encoding an enzyme metabolizing and thus detoxifying the herbicide (8, 9). Soybeans tolerant to the herbicide glyphosate, oilseed rape tolerant to phosphinotricin, and cotton exhibiting tolerance to bromoxynil are examples of genetically modified crops presently entering the market (3, 10).

Insect resistance. The use of the bacterium *Bacillus thuringiensis* (*B.t.*) to control plant insect pests has long been known (11). The insecticidal properties are due to the biosynthesis of different toxic crystal proteins. These pro-toxins are proteolytically cleaved in the midgut of the insects, bind to specific membrane receptors and finally lead to the death of the insect. The different strains of *B.t.* produce specific toxins differing in their activity against various classes of insects, such as the larval stages of *Lepidoptera* (moths and butterflies), *Diptera* (flies), and *Coleoptera* (beetles). By means of recombinant DNA techniques it is possible to create plants expressing the *B.t.* δ -endotoxins, thus making them resistant to insect damage. This concept has been applied successfully in commercially important crops, such as cotton, potato, tomato, and corn (3).

Virus resistance. Agronomically important results have been achieved by creating plants resistant to viral infections. The most successful approach, the coat protein mediated protection, is based on the long known observation that infection of a plant with a mild strain of a virus protected it from subsequent infection of a more virulent strain. By means of recombinant DNA techniques it has been possible to express the viral coat protein in plants, thus protecting them against viral diseases. From the first demonstration that the expression of the coat protein gene of the tobacco mosaic virus in tobacco conferred resistance to infection by this virus (12), this approach has been successfully applied in a wide spectrum of plant species (13).

Plants can also be genetically engineered to express proteins which increase their resistance to attacks by fungi or bacteria (14). The increasing understanding of the molecular basis for the phenomenon of "systemic acquired resistance" (15) offers promising applications of recombinant DNA techniques. The strategy involving the expression of bactericidal enzymes from heterologous sources in plants is exemplified by the bacteriophage T4 encoded lysozyme reported to give rise to increased resistance of potatoes to *Erwinia carotovora* (16). Transgenic tobacco expressing a stilbene synthase from peanut was more resistant to infections of *Botrytis cinerea* (17).

Food Quality. Texture, taste, and aroma belong to the major criteria determining the acceptance of a food by consumers. For tomatoes recombinant DNA techniques have proven to be suitable to improve these important attributes. By introducing either the "antisense" (7) or a truncated "sense" (18) polygalacturonase gene, the biosynthesis of this cell wall enzyme, responsible for the breakdown of pectin during ripening, is slowed down. In terms of processing

characteristics, this results in optimum solids levels and viscosity. The reduced softening process can also be used either to increase the shelf life of the tomato or to improve its flavor properties by allowing the fruit to remain longer on the vine.

The other approach applied to improve the flavor characteristics of tomatoes is to interfere directly with the metabolism of ethylene, the compound which triggers fruit ripening and aroma formation. By applying the "antisense" technology it is possible to inhibit either the 1-amino-cyclopropane-1-carboxylate (ACC) synthase, the rate-limiting enzyme in the biosynthetic pathway of ethylene (19) or the ACC oxidase, the enzyme involved in the conversion of ACC to ethylene (20).

Potential future strategies to influence flavor properties of a plant have been demonstrated by results of the genetic transformation of a scented *Pelargonium* species, referred to as "lemon geranium". Transformation by means of *Agrobacterium rhizogenes* increased the production of essential oil and significantly changed the distribution of monoterpene alcohols (21).

Nutritional Quality. An area which will attract increasing attention in the near future is the improvement of the nutritional value of foods by means of recombinant DNA techniques. Pioneering examples for changing content and composition of the macronutrients (fats, protein and carbohydrates) have already been described. The direction of fatty acid biosyntheses in favor of medium-chain fatty acids can be achieved by expression of a 12:0-acyl-carrier protein thioesterase in transgenic oilseed plants (22). Increasing the level of sulfur-containing amino acids in soybean by introducing a gene from Brazil nut exemplifies the strategy to improve the balance of essential amino acids in important crops (23). However, this project also demonstrates potential limits, such as the influence of the genetic modification on the allergenicity of the host plant (24). The modification of carbohydrate metabolism by recombinant DNA techniques has been demonstrated for starch in potatoes. The composition of this biopolymer, i.e. the ratio of amylose to amylopectin, as well as its amount in the tubers can be influenced (25,26).

Plants are increasingly being considered as "bioreactors" for production of industrially or pharmacologically important substances, such as proteins (27). Compared to microorganisms the eukaryotic plant cells offer the advantage that the post-translational processing necessary for many valuable proteins can be accomplished. Correctly processed human serum albumin could be obtained from transgenic potatoes (28).

Microorganisms

Microorganisms have played an important role in food production for millennia. The transition from the empirical use of microorganisms to an understanding of the underlying scientific principles was initiated by the pioneering discoveries of Pasteur in the middle of the last century. With the increase of knowledge there have always been attempts to optimize and standardize the microorganisms used in order to meet the requirements of food production. The modern food industry can make use of a spectrum of well-defined "starter-

cultures" for the production of fermented foods (29). They are the result of mutagenesis and selection techniques based on classical bacteriological and genetic methods. By means of genetic engineering, the properties of microorganisms can be changed more precisely (30). Major goals are optimization of the production process, improvement of product quality, and safety (hygienic 'status), and enlargement of product diversity.

Strategies applied are based on (i) increasing the copy number of the gene of interest, (ii) coupling genes with strong promoters or other regulatory sequences, and (iii) expressing genes from other sources in microorganisms which are safe for use in food production (GRAS) and which are easily handled under commercial fermentation conditions. There is a broad array of recombinant microorganisms available for industrial and agricultural applications (31).

Because of its importance both as a model organism in basic research and as a production microorganism in the baking and brewing industry, the yeast *Saccharomyces cerevisiae* has attracted considerable attention among molecular biologists (32, 33). In the United Kingdom genetically modified baker's yeast with increased activities of maltase and maltosepermease (34) and a genetically modified amylolytic brewer's yeast (35) have been reviewed for food use.

Lactic acid bacteria play an outstanding role in food fermentation (36). Accordingly, many examples for their genetic engineering have been reported (37, 38). The construction of safe, so-called "food-grade", vectors has been especially and extensively studied with these microorganisms (39).

Enzymes

For many important processes in food production, enzymes rather than intact microorganisms are employed. These biocatalysts possess outstanding properties, such as substrate specificity, regioselectivity, and enantioselectivity. In particular hydrolases which do not require coenzyme regeneration are increasingly being used commercially. Amylases, pectinases or cellulases are applied in the starch and baking industry as well as in fruit juice production. Lipases are employed to modify the properties of triglycerides by hydrolysis, esterification, and interesterification (40).

Enzymes are isolated from animal, plant, and microbial sources. Pure culture fermentations of selected strains of microorganisms are used to obtain enzyme preparations at industrial scale. Genetic engineering offers the possibility to increase the yield of the desired enzyme by introducing multiple copies of the corresponding gene into the production organism or by influencing the regulatory sequences. A major strategy is to introduce the gene encoding the enzyme in safe and efficient microorganisms. Yeasts have been proven to be ideal expression systems for heterologous proteins (41).

One of the most prominent and pioneering examples for an enzyme obtained from genetically modified microorganisms is the milk-clotting protease, chymosin, the first food ingredient produced via recombinant DNA techniques which has been cleared for food use (42).

In the future, applications of recombinant DNA techniques will not be limited to the production of enzymes, which are structurally and functionally identical to their traditional counterparts. Increasing emphasis will be placed on

"protein engineering". This allows for the change of the DNA sequence and subsequently the corresponding amino acid at specific positions, thus designing enzymes with new and optimized properties. Adaptation of enzymes to specific conditions, such as pH and temperature, becomes possible. The use of designed proteases and lipases in the detergent industry indicates the future potential of this strategy in food production.

Food Ingredients

Biotechnological fermentation and biotransformation processes have been used for the production of food ingredients, such as vitamins, amino acids, organic acids, or sweeteners (43, 44). By using the strategies described above for the production of enzymes, recombinant DNA techniques can be applied in order to increase the yield and to improve the recovery and the purification of single compounds from fermentation broths (45).

Because of consumers' demand for "natural" flavor, there has been increasing application of biotechnological methods such as fermentations and biotransformations of corresponding precursors in the production of flavor compounds in recent years (46, 47). Therefore, flavor compounds are ideal examples for food ingredients being suitable for the application of recombinant DNA techniques in the course of the production process.

Animals

The commercial use of recombinant bovine somatotropin (rBST), a growth hormone produced from a genetically modified microorganism, is an example for the *indirect* application of recombinant DNA techniques in farm animal production. In contrast to the progress achieved in genetic engineering of microorganisms and plants, *direct* application resulting in transgenic animals will require more years before it reaches the stage of commercialization. Genetic modification of animals is a much more complex task than that of microorganisms or plants (48). Goals of genetic engineering of animals focus on improved growth and on increased resistance to specific diseases (49). Most of the progress has been made with transgenic fish because the efficiency of integration of DNA into fish is much higher than for mammals (50, 51). Goals such as increased production efficiency and improved growth rate have been achieved by introducing mammalian and fish growth hormone genes. The transfer of the antifreeze protein gene from winter flounder to salmon is an example of improving the tolerance of abiotic stresses.

A promising field is the so-called "gene farming", the production of pharmaceutically or nutritionally important proteins in the mammary glands of transgenic animals (52).

Safety Assessment

The outstanding and fast development of genetic engineering and the accelerating transition from basic research to commercial applications provoke both enthusiastic and very strong negative reactions. Today, it is acceptable to produce life-saving pharmaceuticals by means of genetically modified microorganisms. However, the application of this technology in the production of foods is still the subject of controversial discussion.

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The issues being raised can be divided into three major categories: (i) the fact that this technique touches the fundamentals of life by changing the genetic information of organisms provokes ethical concerns; (ii) the release of viable genetically modified organisms into the environment raises ecological issues; (iii) concerns are expressed whether unexpected or unintentional effects will occur as a result of the genetic modification of crops or microorganisms and whether new substances introduced into food will be safe.

National and international organizations are involved in establishing principles for safety evaluations of foods and food ingredients produced via recombinant DNA techniques. The concept of "substantial equivalence" as developed by the OECD (53) is widely agreed upon. It involves the comparison of a food or food ingredient developed by modern biotechnology to its traditional counterpart. If substantial equivalence can be established, the new food or food component can be treated in a similar manner with respect to safety (54).

Current evaluation procedures of genetically modified foods and food ingredients pay particular attention to the safety implications of (i) intentional changes, (ii) any unintentional changes arising from the genetic modification, (iii) the stability of the genetically modified organism under the intended conditions of use; and (iv) the likelihood of genetic transfer.

There is a general consensus that the applicability of classical toxicological assessment procedures developed for single chemical substances, such as pesticides or food additives, is limited. Safety and wholesomeness studies with whole foods have to be carefully designed in order to avoid nutritional imbalances causing artifacts and uninterpretable results.

Particular attention has been paid to the safety evaluation of marker genes and their respective expression products. Marker genes are needed to identify and select cells which have been successfully transformed at an early stage of the genetic modification process. The most important ones are those conferring resistance to antibiotics and tolerance to herbicides, respectively (10). The safety assessment of the antibiotic resistance marker genes has been the subject of detailed investigations (55); international organizations (10), and national regulatory authorities (56) have discussed this issue.

Another concern being raised is whether there is an increased potential for allergenicity of transgenic foods due to the transfer of new proteins. The above mentioned transfer of a gene from Brazil nut to soybean demonstrates that there are methods available to assess the allergenic potential of proteins derived from sources to which consumers have reacted and for which serum is available (24). However, at present the potential allergenicity of proteins that are derived from sources that are not recognized as allergens cannot be predicted.

Regulatory Aspects

There is a relatively broad consensus about the general scientific principles underlying the safety evaluation of foods and food ingredients produced via recombinant DNA techniques. However, the philosophies about regulatory oversight and the need for legislative restrictions vary in different countries. There are two major approaches: one is based on the assumption that the

application of a certain technology, such as genetic engineering, bears the potential for specific risks and therefore requires a corresponding oversight; the second approach primarily focusses on the final product and its safety rather than on the technology applied. These philosophies are reflected in different regulatory frameworks set up around the world, and some of the world leaders discuss their regulatory policies in this book.

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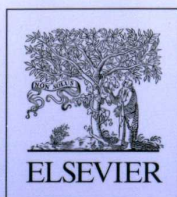
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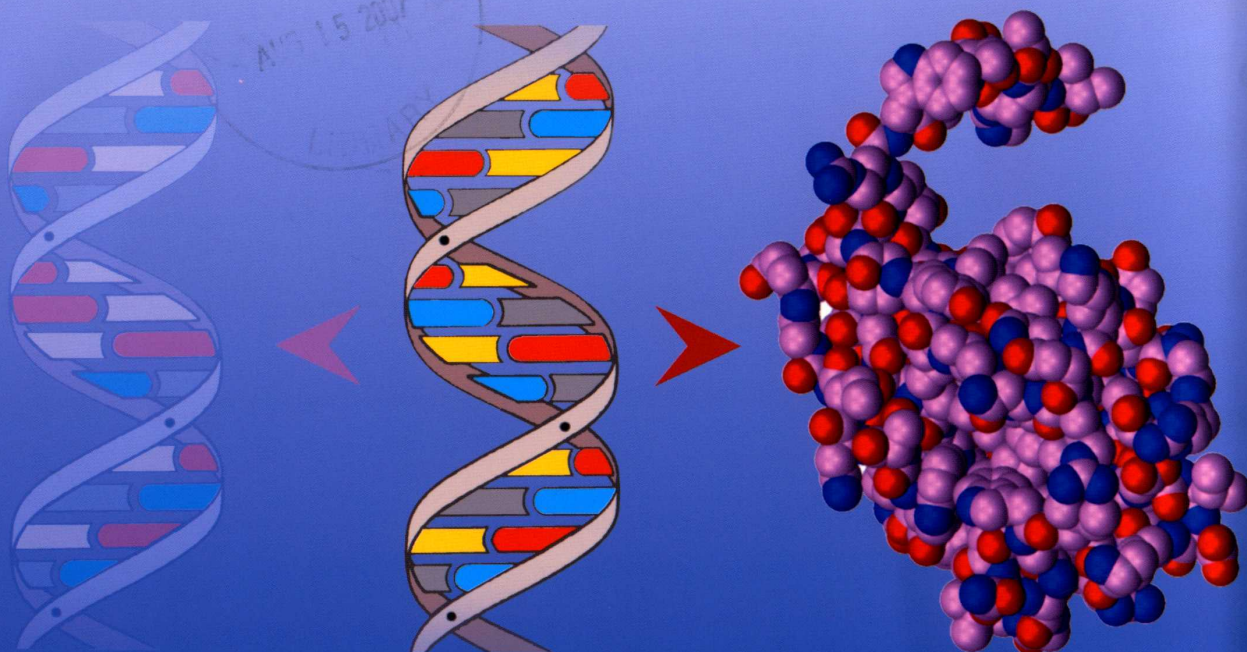


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Special Issue

Devoted to the XIVth International Conference
on Dioxygen Binding and Sensing Proteins,
Naples, Italy, 3-7 September 2006

Luc Moens, Martino Bolognesi, Guido di Prisco and Cinzia Verde (Organizers and Editors)

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Review

Plant hemoglobins: What we know six decades after their discovery[☆]

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Abstract

This review describes contributions to the study of plant hemoglobins (Hbs) from a historical perspective with emphasis on non-symbiotic Hbs (nsHbs). Plant Hbs were first identified in soybean root nodules, are known as leghemoglobins (Lbs) and have been characterized in detail. It is widely accepted that a function of Lbs in nodules is to facilitate the diffusion of O₂ to bacteroids. For many years Hbs could not be identified in plants other than N₂-fixing legumes, however in the 1980s a Hb was isolated from the nodules of the non-legume dicot plant *Parasponia*, a *hb* gene was cloned from the non-nodulating *Trema*, and Hbs were detected in nodules of actinorhizal plants. Gene expression analysis showed that *Trema* Hb transcripts exist in non-symbiotic roots. In the 1990s nsHb sequences were also identified in monocot and primitive (bryophyte) plants. In addition to Lbs and nsHbs, Hb sequences that are similar to microbial truncated (2/2) Hbs were also detected in plants. Plant nsHbs have been characterized in detail. These proteins have very high O₂-affinities because of an extremely low O₂-dissociation constant. Analysis of rice Hb1 showed that distal His coordinates heme Fe and stabilizes bound O₂; this means that O₂ is not released easily from oxygenated nsHbs. Non-symbiotic *hb* genes are expressed in specific plant tissues, and overexpress in organs of stressed plants. These observations suggest that nsHbs have functions additional to O₂-transport, such as to modulate levels of ATP and NO.

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Keywords: History; Leghemoglobin; Non-symbiotic; Truncated

1. Introduction

Hemoglobins (Hbs) are hemoproteins that reversibly bind O₂. The most common function of Hbs is associated with the transport of O₂, however Hbs also bind other gaseous ligands, such as NO (Cooper, 1999; Poole and Hughes, 2000; Moller and Skibsted, 2002; Dordas et al., 2003b; Milani et al., 2003; Frey and Kalio, 2005), and organic molecules (Ollesch et al., 1999; Bonamore et al., 2003; D'Angelo et al., 2004; Rinaldi et al., 2006), which suggests that they are multifunctional proteins in living organisms. Phylogenomic analysis showed that Hbs are widespread in organisms, as they have been

identified in genomes of archaeobacteria, eubacteria and eukaryotes, including plants (Vinogradov et al., 2005, 2006). In plants three types of Hbs have been identified: symbiotic, non-symbiotic (nsHb) and truncated (2/2) Hbs (tHbs) (Ross et al., 2002). This review describes contributions to the study of plant Hbs from a historical perspective with emphasis on nsHbs. The authors refer interested readers to reviews by Appleby (1992), Fuchsmann (1992), Arredondo-Peter et al. (1998), Hill (1998), Ross et al. (2002) and Kundu et al. (2003) for detailed discussion and relevant references on the structure, function and molecular biology of plant Hbs.

2. The discovery of plant Hbs

Plant Hbs were first identified by Kubo (1939). This author analyzed the red pigment of soybean root nodules. Nodules form in legumes after root infection by soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium* and *Sinorhizobium* (collectively known as rhizobia). Nodules

Abbreviations: Hb, hemoglobin; HGT, horizontal gene transfer; Lb, leghemoglobin; Mb, myoglobin; nsHb, non-symbiotic hemoglobin; NO, nitric oxide; tHb, truncated (2/2) hemoglobin.

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are red in color and are the plant organs where nitrogen fixation (by symbiotic rhizobia) occurs. Spectral examination of red pigment isolated by Kubo showed absorption bands that are characteristic of deoxy-ferrous, O₂-ferrous, CO-ferrous, and ferric Hbs. Kubo also crystallized hemin from this pigment and showed that crystals are identical to those of hemin from horse Hb. From this evidence Kubo concluded that the red pigment isolated from soybean root nodules was a hemeprotein.

To identify the physiological role of this hemeprotein in nodules, Kubo incubated nodule bacteria with it and observed stimulation of bacterial O₂-consumption. From this observation he concluded that the physiological role of the hemeprotein in nodules is to stimulate the assimilation and transport of O₂. The nodule hemeprotein discovered by Kubo has been characterized in detail by others. Biochemical, biophysical and molecular biology analyses have shown that Kubo's hemeprotein is a plant Hb with similar (*i.e.* structural) properties to animal Hbs (Fig. 1). Kubo's plant Hb was named as leghemoglobin (Lb) by Virtanen and Laine (1946) and is also known as plant symbiotic Hb.

3. The discovery of the function of Lb in root nodules

The role of Lb in N₂-fixing nodules was elucidated by Wittenberg, Bergersen, Appleby and Turner in 1974 (Wittenberg et al., 1974). These authors observed that the addition of Lb to suspensions of bacteroids enhanced the rate of O₂-uptake and nitrogenase activity (measured as the reduction of acetylene to ethylene), and promoted the formation of ethylene with increasing air O₂ concentrations. Ferric Lb, ferric horseradish peroxidase and ferric cytochrome *c* peroxidase were ineffective, however twelve O₂-binding proteins other than Lb also increased O₂-uptake and

acetylene reduction by bacteroids. From these observations the authors concluded that oxyLb and other O₂-binding proteins facilitated the diffusion of O₂ to bacteroids in suspension, and that the function of Lb in nodules is to facilitate the diffusion of O₂ to bacteroids at an internal concentration too low to inhibit or destroy their O₂-sensitive nitrogenase. This concept of high O₂-flux at low free O₂ in the bacteroid vicinity is now generally accepted.

4. The hypothesis for the origin of Lbs by horizontal gene transfer

For many years Hbs were identified in N₂-fixing legumes, but not elsewhere in the plant kingdom. Early attempts to detect Hbs in non-legumes using biochemical and molecular biology approaches were unsuccessful, so plant Hbs were apparently restricted to N₂-fixing legumes, and thus its origin was a mystery. In 1982 Jeffreys hypothesized that plant Hbs originated by a unique act of horizontal gene transfer (HGT) from a phytophagous insect to a primitive legume *via* a viral vector (Jeffreys, 1982). For this hypothesis to be correct the gene structure of insect *hb* and plant *lb* genes should be identical. Leghemoglobins are encoded by genes that are interrupted by three introns (Jensen et al., 1981; Hyldig-Nielsen et al., 1982). After Jeffreys' HGT hypothesis it was revealed that *hb* gene from the insect *Chironomus* contains no introns (in Appleby et al., 1988), thus the HGT hypothesis for the origin of plant Hbs was discarded, and the vertical evolution hypothesis of plant Hbs was favored. It is now known that other insect *hb* genes do contain introns, some in the exact positions found for legume *lbs* (Kloek et al., 1993; Hankeln et al., 2002; Hoogewijs et al., 2004); even so, other evidence in favor of vertical rather than horizontal evolution of plant *hb* genes soon emerged (see Section 5). An implication of the vertical evolution hypothesis is that Hbs might exist in all (legume and non-legume) land plants.

5. The vertical evolution of plant Hbs: discovery of plant non-symbiotic Hbs

A discovery that supported the vertical evolution hypothesis of plant Hbs was the identification of a Hb in the N₂-fixing root nodules of the non-legume dicot plant *Parasponia* (Appleby et al., 1983). *Parasponia* is a member of the Ulmaceae family, and the only non-legume known to be nodulated by *Rhizobium* strains. *Parasponia* Hb was extracted under anaerobic conditions and final purification was achieved by preparative isoelectric focusing, which produced a single major component of oxyHb (with pI of 6.28), and small amount of ferric Hb (with pI of 6.67). The absorption spectra of (deoxy-ferrous, O₂-ferrous and CO-ferrous) *Parasponia* Hb are similar to those of Lbs and animal Hbs and kinetic constants (O₂-affinity and O₂-association and -dissociation constants) are also similar in *Parasponia* Hb and soybean and other legume Lbs (Gibson et al., 1989). This evidence suggested that the function of Hb in *Parasponia* nodules is similar to function of Lb in legume nodules, *i.e.* to facilitate the diffusion of O₂ to bacterioids.

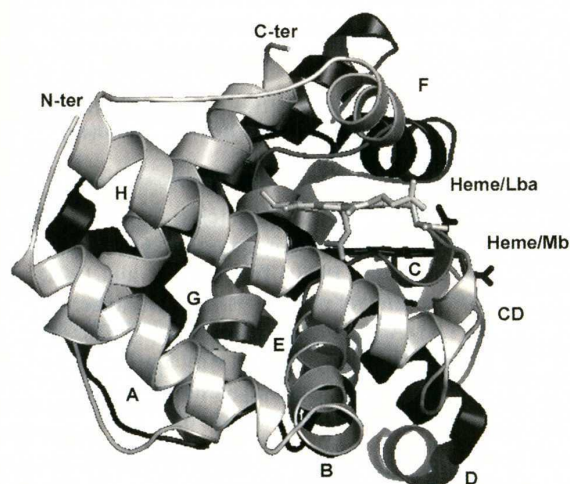


Fig. 1. Tertiary structure of Kubo's nodule hemeprotein (soybean leghemoglobin *a*) (gray) overlaid on the structure of sperm whale myoglobin (Mb) (black). Coordinates for the Lba and Mb were obtained from the Brookhaven Protein Database using the identification numbers 1BIN and 2MYC, respectively, and structures were displayed using the PyMol program (<http://www.pymol.org/funding.html>). Helices (and CD loop in Lba) are shown with the A–H letters.

During the 1980s a cDNA probe for *Parasponia* Hb was used to detect *hb* sequences in *Trema* and *Celtis*. *Trema* and *Celtis* are non-nodulating (ulmacean) plants closely related to *Parasponia*. Southern blot analysis revealed the existence of Hb-like sequences in *Trema* and (possibly) *Celtis* DNAs (Appleby et al., 1988; Boguz et al., 1988). DNA cloning and sequencing showed that *Trema* sequences have all the characteristics of a functional plant *hb* gene: the *Trema hb* gene is interrupted by three introns and exon sequences are 93% similar to *Parasponia hb*. Also, Northern blot analysis using the *Parasponia* Hb cDNA probe showed that Hb transcripts exist in *Trema* roots and in *Parasponia* roots and nodules, but not in *Trema* and *Parasponia* leaves. Western blot analysis using anti-*Parasponia* Hb antibodies showed that mRNA is translated into monomeric and dimeric Hbs in *Trema* roots and *Parasponia* roots and nodules, respectively (Boguz et al., 1988).

During the same decade Tjepkema detected high concentrations of Hb-like proteins in nodule extracts of actinorhizal plants (which are nodulated by the actinomycete *Frankia*), such as *Casuarina cunninghamiana* and *Myrica gale*, and low concentrations in nodules of *Comptonia peregrina*, *Alnus rubra* and *Eleagnus angustifolia* (Tjepkema, 1983). A few years later after Tjepkema's report Hbs were purified and characterized from the actinorhizal *Casuarina glauca* (Fleming et al., 1987), *Alnus glutinosa* (Suharjo and Tjepkema, 1995), and *M. gale* (Pathirana and Tjepkema, 1995) root nodules. The spectral properties of actinorhizal Hbs are similar to those of Lbs and non-plant Hbs.

The detection of Hbs in *Parasponia*, *Trema* and actinorhizal plants suggested that plant Hbs are widespread in land plants and that plant *hb* genes vertically evolved from a common ancestor. Also, the detection of Hb transcripts in *Parasponia* and *Trema* roots showed that *hb* genes express and Hbs probably function in non-symbiotic plant organs.

cDNAs coding for monocot (barley and rice) nsHbs were cloned and sequenced in the 1990s. The first monocot Hb cDNA was cloned from barley (Taylor et al., 1994). The deduced amino acid sequence of barley Hb is 71% similar to *Parasponia* Hb. Southern blot analysis using the barley Hb cDNA probe showed that a single copy (or a very low copy number) of the *hb* gene exists in barley and that *hb*-like sequences exist in other monocot species, such as rye, maize and wheat. In 1997 Arredondo-Peter et al. reported the cloning and characterization of *hb1* and 2 genes from rice (Arredondo-Peter et al., 1997). Southern blot analysis showed that at least three copies of the *hb* gene exist in rice, however recent work showed that five copies of the *hb* gene exist in the rice genome (Lira-Ruan et al., 2002; Bustos-Rivera et al. manuscript in preparation). Sequence comparison showed that the predicted Hb1 and 2 proteins are 93% similar to each other, and that the rice Hbs are 68 to 82% similar to other non-symbiotic Hbs and about 50% similar to symbiotic Hbs (Arredondo-Peter et al., 1997).

Recently, Hb sequences were identified in very many evolved and primitive plants (Table 1), mostly because of numerous plant genome sequencing programs. For example, nsHb sequences have been identified in monocots such as maize

and teosinte (Aréchaga-Ocampo et al., 2001) and wheat (Larsen, 2003), dicots such as soybean (Andersson et al., 1996), *Arabidopsis* (Trevaskis et al., 1997), chicory (Hendriks et al., 1998) and tomato (Wang et al., 2003), and bryophytes such as the moss *Physcomitrella patens* (Arredondo-Peter et al., 2000). The existence of nsHbs in the genome of primitive bryophytes suggests that the ancestor of land plant Hbs existed when the land was colonized by bryophytes, about 550 million years ago, and the existence of Hbs in primitive and evolved plants (Table 1) shows that Hbs are widespread in land plants. Moreover, *nshb* genes from the bryophytes *Marchantia polymorpha*, *Ceratodon purpureus* and *Physcomitrella patens* (GenBank accession numbers AY026341, EF028054 and EF028055, respectively) are interrupted by three introns in identical positions to introns of known plant *nshb* and *lb* genes. This evidence shows that *nshb* and *lb* genes evolved from an ancestral plant *hb* gene that was interrupted by three introns.

In addition to Lbs and nsHbs, Hb sequences that are similar to those of microbial truncated (2/2) Hbs (the reader is referred to reviews by Pesce et al. (2000) and Wittenberg et al. (2002) for a detailed description of the "truncated (2/2) folding" of globins) were detected in primitive and evolved plants (Table 1) (Watts et al., 2001). Sequence alignment showed that plant 2/2-like Hbs are highly conserved (not shown), and phylogenetic analysis showed that plant 2/2-like Hbs and Lbs and nsHbs evolved through different lineages (Fig. 2). Phylogenomic analysis by Vinogradov et al. (2006) revealed that plant 2/2-like Hbs are more similar to bacterial 2/2 Hbs than to plant (symbiotic and non-symbiotic) Hbs, which suggested that plant 2/2-like Hbs originated by a HGT event from a bacterial (2/2 *hb* gene) donor. Expression analysis showed that 2/2-like *hb* genes express in vegetative and embryonic organs and in organs from stressed plants (Hunt et al., 2001; Watts et al., 2001), however the function of 2/2-like Hbs in plant organs is not yet known, although kinetic properties of a recombinant *Arabidopsis* 2/2-like Hb suggest that these proteins may function as O₂-carriers (Watts et al., 2001).

6. Non-symbiotic (class 1) Hbs: hexacoordinate proteins with very high O₂-affinity

A distinctive characteristic of plant (class 1) nsHbs is that the affinity of these proteins for O₂ is very high (Duff et al., 1997; Arredondo-Peter et al., 1997; Trevaskis et al., 1997). For example, the O₂-affinity of rice Hb1 is 78-times higher than that from soybean Lba (Arredondo-Peter et al., 1997). This high O₂-affinity of rice Hb1 is mainly due to a very low O₂-dissociation constant ($k_{off}=0.038\text{ s}^{-1}$), which suggests that O₂ is stabilized after binding to Hb1. Spectral analysis of deoxy-ferrous rice Hb1 showed that a distal ligand coordinates to Fe: the unliganded ferrous state of Hb1 exhibits peaks at 526 and 556 nm, which is similar to the absorption spectrum of (ferrous) cytochrome *b* where the heme Fe is hexacoordinate (Smith, 1978; Weiss and Ziganke, 1978). Histidine was identified as the distal ligand to heme Fe in wild type rice Hb1 by comparison with its mutant Hb1 H74L (in which distal His H74 was replaced by Leu). Absorbance spectra of mutant Hb1 showed no

Table 1
Hemoglobin sequences identified in land plants

Organism	Hb ^a (accession number ^b)
<i>I. Seed plants</i>	
<i>a) Angiosperms</i>	
Monocotyledonous	
<i>Hordeum vulgare</i> (barley)	nsHb-1 (U94968), tHb (AF376063)
<i>Oryza sativa</i> (rice)	nsHb1-1 (U76029), nsHb2-1 (U76031), nsHb3-1 (AF335504), nsHb4-1 (AF335504), tHb (BAD32857)
<i>Saccharum</i> spp. (sugarcane)	nsHb-1 (C0373680)
<i>Triticum aestivum</i> (wheat)	nsHb-1 (AY151390), tHb (AY151391)
<i>Zea mays</i> ssp. <i>mays</i> (maize)	nsHb-1 (AY005818), nsHb-2 (DQ171946)
<i>Z. mays</i> ssp. <i>parviglumis</i> (teosinte)	nsHb-1 (AF291052)
Dicotyledonous (legumes)	
<i>Astragalus sinicus</i>	symHb (DQ199647)
<i>Canavalia lineata</i> (bay bean)	symHb (UD9671)
<i>Glycine max</i> (soybean)	symHb (V00453), nsHb-1 (U47143), tHb (AA548191)
<i>Lotus japonicus</i>	symHb (AB238218), nsHb-1 (AB238220)
<i>Lupinus luteus</i> (yellow lupine)	symHb (Y00401)
<i>Phaseolus vulgaris</i> (kidney bean)	symHb (P02234)
<i>Pisum sativum</i> (pea)	symHb (P02233)
<i>Psophocarpus tetragonolobus</i> (winged bean)	symHb (X65874)
<i>Medicago sativa</i> (alfalfa)	symHb (M32883), nsHb-1 (AF172172)
<i>M. truncatula</i> (barrel medic)	symHb (X57733), tHb (ABE93362)
<i>Sesbania rostrata</i>	symHb (M23313)
<i>Vicia faba</i> (broad bean)	symHb (Z54159)
<i>V. sativa</i> (common vetch)	symHb (Y00229)
<i>Vigna unguiculata</i> (cowpea)	symHb I (U33206), symHb II (U33207)
Dicotyledonous (no-legumes)	
<i>Alnus firma</i>	nsHb-1 (AB221344)
<i>Arabidopsis thaliana</i>	nsHb-1 (NM_127165), nsHb-2 (NM_111887), tHb (NM_119421)
<i>Beta vulgaris</i> (sugar beet)	nsHb-2 (BE590299)
<i>Brassica napus</i> (canola)	nsHb-2 (AY026337)
<i>Casuarina glauca</i> (swamp oak)	nsHb-2 (X77694), nsHb-1 (X53950)
<i>Cichorium intybus</i> × <i>C. endivia</i> (chicory)	nsHb1-2 (CAA07547), nsHb2-2 (AJ277797)
<i>Citrus unshiu</i> (satsuma orange)	nsHb-1 (AY026338)
<i>Datisca glomerata</i> (durango root)	tHb (CAD33536)
<i>Euryale ferox</i> (fox nut)	nsHb1-2 (AY281295), nsHb2-2 (AY281296)
<i>Gossypium hirsutum</i> (cotton)	nsHb-1 (AY899302), nsHb-2 (AY026340)
<i>Lycopersicon esculentum</i> (tomato)	nsHb-1 (AY026343), nsHb-2 (AY026344)
<i>Malus domestica</i> (apple)	nsHb-1 (AY224132)
<i>Myrica gale</i>	nsHb-1 (EF405885)
<i>Nicotiana tabacum</i> (tobacco)	nsHb-1 (BQ842804)

Table 1 (continued)

Organism	Hb ^a (accession number ^b)
<i>I. Seed plants</i>	
<i>a) Angiosperms</i>	
Dicotyledonous (no-legumes)	
<i>Parasponia andersonii</i>	nsHb-1 (U27194)
<i>P. rigida</i>	nsHb-1 (P68169)
<i>Pyrus communis</i> (pear)	nsHb-1 (AY224133)
<i>Raphanus sativus</i> (horseradish)	nsHb-1 (AY286331)
<i>Solanum tuberosum</i> (potato)	nsHb2-1 (AY151389)
<i>Trema orientalis</i>	nsHb-1 (AF027215)
<i>T. tomentosa</i>	nsHb-1 (P07803)
<i>T. virgata</i>	nsHb-1 (AJ131349)
<i>b) Gymnosperms</i>	
<i>Pinus tadea</i> (pine)	tHb (BQ291271)
<i>II. Bryophytes</i>	
<i>a) Mosses</i>	
<i>Physcomitrella patens</i>	nsHb (AF218049)
<i>Ceratodon purpureus</i>	nsHb (AF309562)
<i>b) Liverworts</i>	
<i>Marchantia polymorpha</i> (liverwort)	nsHb (AY026341)

^ansHb, non-symbiotic Hb; nsHb-1, non-symbiotic Hb (class 1) with high O₂-affinity; nsHb-2, non-symbiotic Hb (class 2) with moderate O₂-affinity and/or sequence similarity to symbiotic Hbs; symHb, symbiotic Hb (including Lbs) with moderate O₂-affinity, which is specifically localized in nodules of N₂-fixing plants; tHb, 2/2-like plant Hb.

^bAccession numbers in the GenBank database.

evidence of His coordination. Also, the substitution of H74 by L in Hb1 resulted in an increase of O₂-association and -dissociation constants. A conclusion from these observations was that distal His hexacoordinates to Fe and stabilizes bound O₂ in oxygenated Hb1 (Arredondo-Peter et al., 1997; Hargrove et al., 2000; Goodman and Hargrove, 2001).

7. Localization of nsHbs and expression of *nshb* genes in plant organs

Use of anti-rice Hb1 antibodies and Western blot showed that Hbs are localized in embryonic (coleoptiles, seminal roots and embryos) and (young and mature) vegetative (leaves and roots) rice organs. These observations showed that nsHbs are synthesized in different rice organs and during plant development (Lira-Ruan et al., 2001). However, analysis by confocal microscopy revealed that nsHbs are localized in the cytoplasm of differentiated and differentiating cell types of the developing rice seedling, such as the aleurone, scutellum, root cap cells, sclerenchyma, and tracheary elements. The immunolocalization of nsHbs in the vasculature of root, mesocotyl and leaf tissues, specifically in the xylem, suggests that nsHbs play roles in the metabolism accompanying the differentiation of the xylem (Ross et al., 2001).

Also, high levels of nsHb transcripts and proteins have been detected in organs and tissues of stressed plants. For example, *nshb* genes overexpress in barley aleurones subjected to microaerobiosis (<5% O₂) or sustained anoxia (up to 12 h) and in roots of flooded barley plants (Taylor et al., 1994). In

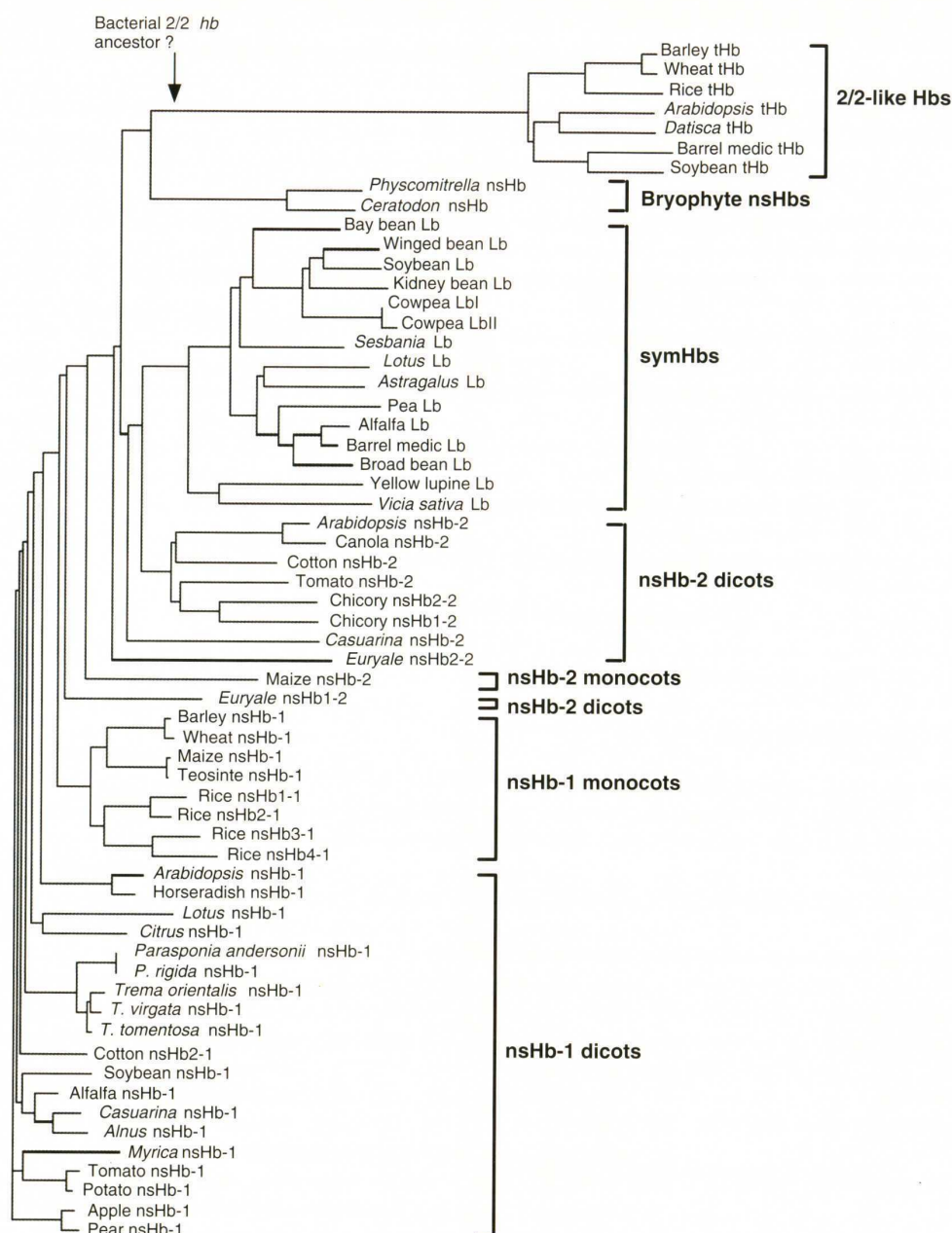


Fig. 2. Cluster (phenetic) analysis of selected plant Hbs. Full Hb sequences (partial or incomplete Hb sequences were not included in this analysis) were obtained from the GenBank database using the accession numbers shown in Table 1. Hb sequences were aligned and phenogram was constructed using the Clustal W program (Thompson et al., 1997). The possible origin of plant 2/2-like Hbs by HGT is indicated according to Vinogradov et al. (2006). Abbreviations are the same as in Table 1. *Parasponia* Hbs were classified as nsHb-1 because they cluster with high O_2 -affinity (class 1) nsHbs, however *Parasponia* Hbs are intermediate because they are localized in nodules and non-symbiotic roots and their O_2 -affinity is similar to that of symHbs (Lbs).

etiolated rice levels of Hbs increased 2.4-fold compared to control plants grown under a normal light/dark regimen (Lira-Ruan et al., 2001), and levels of nsHb transcripts increased in microaerobic (0.1% O_2) and osmotically (1% sucrose) stressed *Arabidopsis* roots (Trevaskis et al., 1997). These observations suggest that nsHbs take part of the plant response to some stress conditions.

8. Multiple functions of plant nsHbs?

The kinetic constants (see Section 6) and localization of nsHbs in metabolically active tissues as well as the over-expression of *nshb* genes in stressed plants (see Section 7) suggest that nsHbs have functions other than or additional to O_2 transport. In 1998 it was proposed that nsHbs may play three

major roles in plant metabolism (Arredondo-Peter et al., 1998): 1) bind gaseous ligands, such as O₂, NO and CO, to modulate cell metabolism through interaction with other cell molecules, which could modify the rate constants for ligand binding; 2) bind small organic molecules, such as fatty acids, for transport and synthesis processes; and 3) scavenge O₂ based on the extremely tight binding of O₂ or interact with flavoproteins (for example with a ferric Hb reductase) for electron transfer.

Work aimed at the elucidation of nsHb function suggests that these proteins play roles in plant cells by maintaining levels of ATP and modulating levels of NO. Experiments with maize cells transformed with sense and antisense barley Hb cDNA (Hb⁺ and Hb⁻ cells, respectively) showed that ATP levels decreased about 5, 40 and 65% when Hb⁺, native (untransformed) and Hb⁻ maize cells were subjected to microaerobiosis, respectively (Sowa et al., 1998). Thus, it was concluded that a function of nsHbs is to maintain the energy status of cells under low O₂-concentrations. Also, work from the same laboratory showed that substantial amounts of NO were produced in alfalfa root cultures maintained under hypoxic (3% O₂) conditions, and that the amount of NO accumulated in alfalfa cells underexpressing Hb (Hb⁻ lines) was 2.5-fold higher than that in overexpressing barley Hb (Hb⁺ lines) (Dordas et al., 2003a). It has been claimed that *Arabidopsis* nsHb1 scavenges NO and that it reduces levels of NO under hypoxic stress (Perazzolli et al., 2004). From these and others' observations it was concluded that a function of nsHbs is to modulate levels of NO (Dordas et al., 2003a; Perazzolli et al., 2004; Igamberdiev et al., 2005; Perazzolli et al., 2006) and, directly or indirectly, regulate a number of NO-dependent processes in the plant cell.

9. Concluding remarks and future directions

Plant Hbs were described for the first time more than six decades ago. Since then substantial amounts of information about their structure, function and evolution have accumulated. However, there is still plenty of work to be done for a complete characterization and understanding of these plant proteins. For example, it is widely accepted that a function of Lb in nodules is to facilitate the diffusion of O₂ to the respiring bacteroids, however the mechanism for O₂-delivery from oxygenated Lb to the peribacteroid membrane is not yet known. It is well documented that Hbs interact with proteins (Low, 1986; Nagata et al., 1995; Park et al., 2002) and membrane lipids (Bonamore et al., 2003; Wakasugi et al., 2004; Rinaldi et al., 2006), thus it is of interest to evaluate whether or not Lb interacts with peribacteroid membranes and if this interaction promotes O₂-delivery from oxygenated Lb. Also, primitive *nshb* genes could be selected within a particular plant family to help develop primitive symbiosis. Therefore, it is of interest to analyze primitive legumes to identify the ancestral *nshb/lb* that evolved into a functional symbiotic Hb.

Non-symbiotic Hbs have been characterized in detail. For example, *in vitro* analysis showed that O₂-affinity of (class 1) nsHbs is very high, mostly due to a very low O₂-dissociation constant. Thus, it is of interest to evaluate whether or not the O₂-affinity of nsHbs is also high *in vivo*, otherwise it is possible that nsHbs function as O₂-carriers or -sensors in plant tissues (Appleby et al., 1988). It is known that *nshb* genes (over)express

in specific plant tissues (including organs from plants subjected to stress conditions), however details about the regulation of *nshb* gene expression are still not known. This information is essential to understand the role that nsHbs play in the physiology of a plant cell. A number of potential promoters have been identified upstream of the rice *hb1–4* genes (Lira-Ruan et al., 2002), thus it is likely that the regulation of *nshb* genes is complex. However, it has been shown that the expression of an *Arabidopsis nshb* gene is enhanced by the addition of the cytokinin 2iP (6-[γ - γ -dimethylallylamino]purine) (Hunt et al., 2001) and that a cytokinin-regulated element modulates the expression of rice *hb2* (Ross et al., 2004). This data shows that the expression of *nshb* genes is mediated by plant hormones, thus it is of interest to identify plant hormones that affect the synthesis of nsHbs and the role that these proteins play in the physiological/metabolic processes that are hormonally modulated.

Finally, compared to Lbs and nsHbs, little is known about the function of plant 2/2-like Hbs. However, it was postulated that these proteins originated (by HGT) from a bacterial tHb (Vinogradov et al., 2006), so it is likely that properties of plant 2/2-like Hbs and some bacterial tHbs are similar. Several lines of evidence suggest that a function of microbial tHbs is to detoxify NO (Ouellet et al., 2002; Milani et al., 2003; Giangiacomo et al., 2005; Sarma et al., 2005), thus it is of interest to evaluate whether or not plant 2/2-like Hbs participate in the metabolism of NO in plant cells. For example, (reduced) plant 2/2-like Hbs may bind O₂ and NO, dioxygenate NO and generate NO₃⁻ and ferric Hb. For this process, it is of interest to identify mechanisms that reduce ferric to ferrous plant 2/2-like Hb (which will subsequently bind O₂ for dioxygenation of NO), as has been documented for the reduction of ferric to ferrous Lbs (Lee and Klucas, 1984; Saari and Klucas, 1984, 1987; Klucas et al., 1988; Becana and Klucas, 1990; Ji et al., 1991).

In summary, the diversity of interactions of plant Hbs with molecules suggests that Hbs are multifunctional proteins that are essential for the functioning of plant cells. If this is true, Hbs have played an important role during the evolution and adaptation of plants to land environment.

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