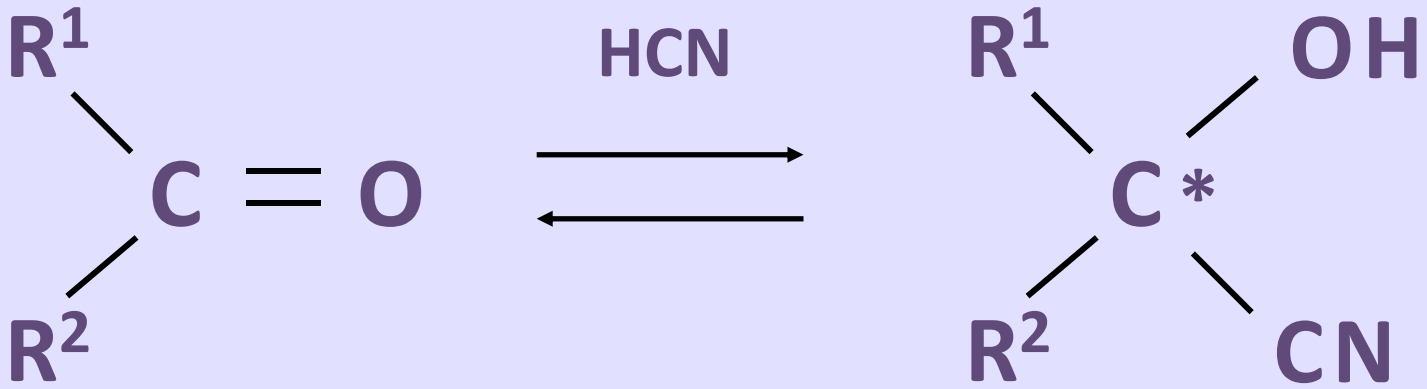
MOL.911

HNL Expression

Hydroxynitrile lyase (Hnl)



S-selective: *Hevea brasiliensis*
R-selective: *Prunus spp.*

(S)-Hnl of *Hevea brasiliensis* and (R)-Hnl of *Prunus amygdalus*

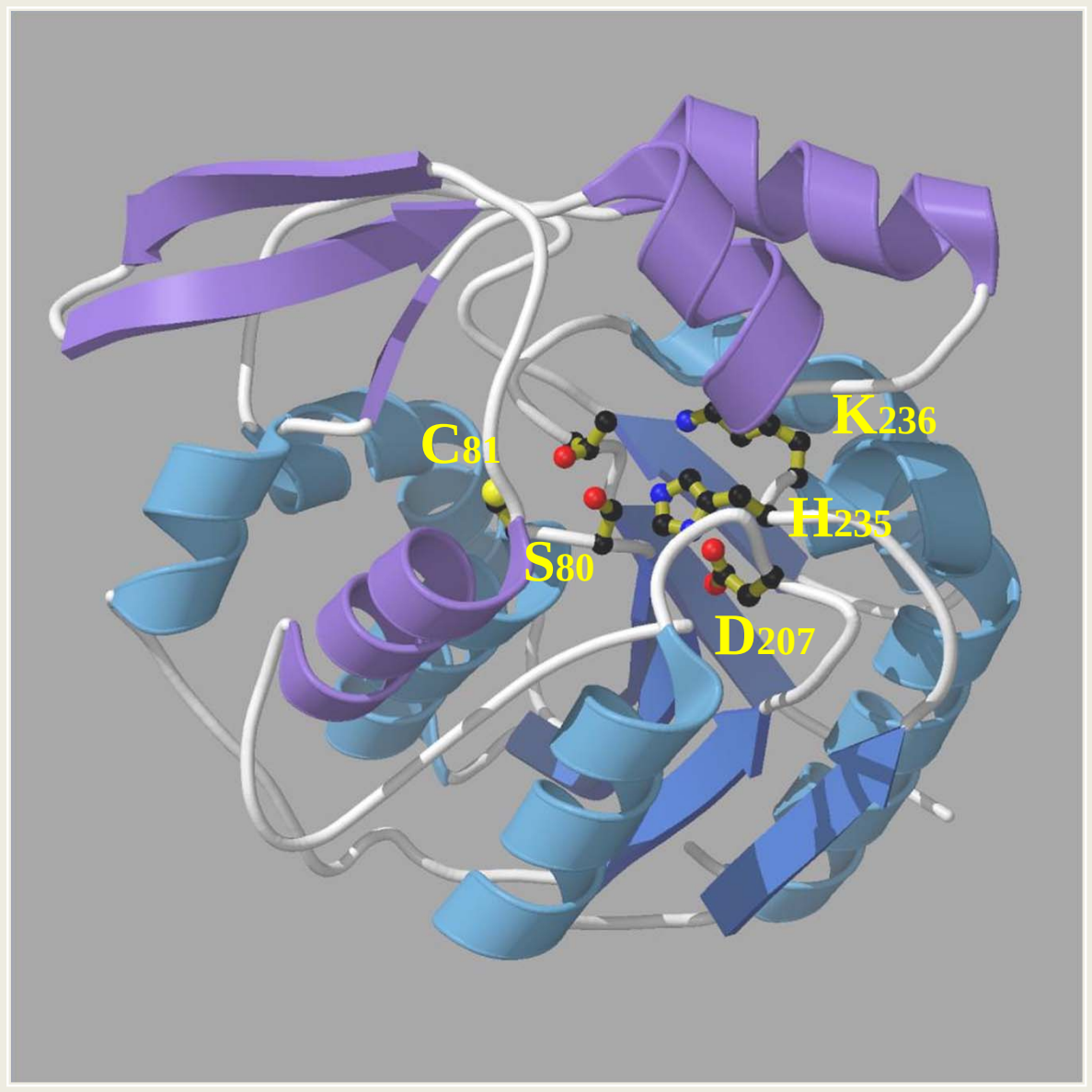
*Hb*_Hnl

- Type II Hnl
- intracellular protein
- 29.2 kDa
- homodimer
- α/β hydrolase fold protein
- catalytic triad
- (S)-selektive

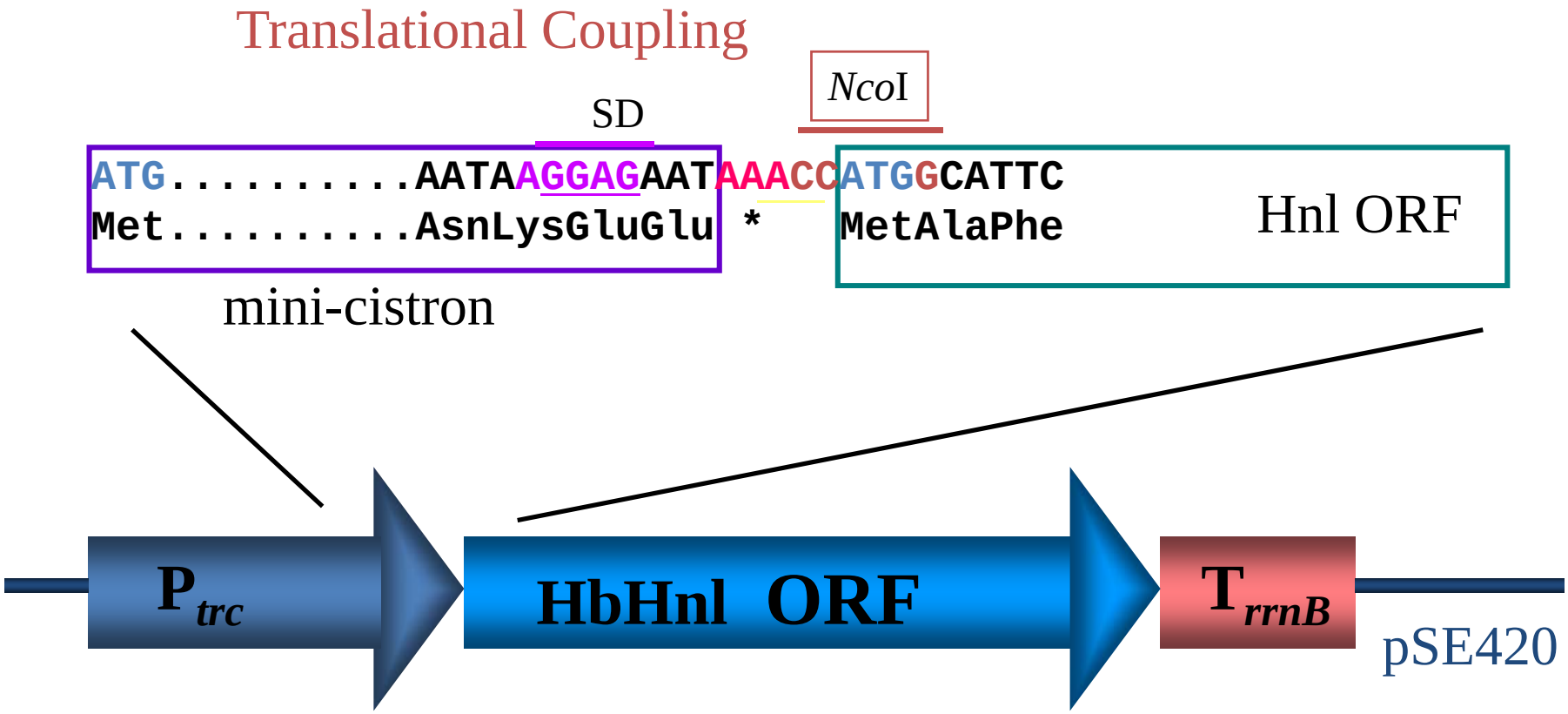
*Pam*_Hnl

- Type I Hnl
- secretory protein
- 61 kDa (57.9 kDa)
- Homology to oxidases
- FAD
- N-glycosylated
- isoenzymes
- (R)-selektive

3-D structure of *Hb*_HNL



Intracellular Hnl Expression in *Escherichia coli*



pHNL-200

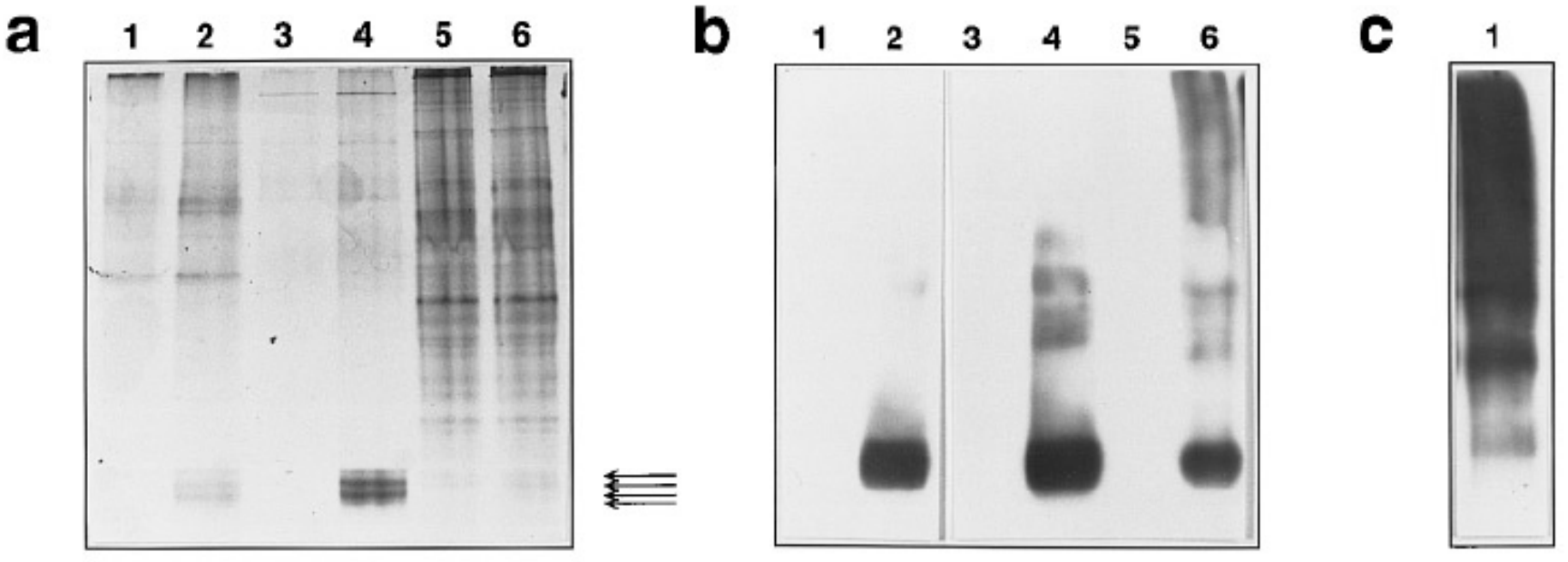


FIG. 2. Native polyacrylamide gel electrophoresis of crude soluble fractions of cell lysates of Hnl expressing transformants. Proteins were electrophoresed in 7.5% gels and stained with Coomassie blue (a) or transferred to membranes for immunostaining using polyclonal anti-Hnl antiserum (b, c). Lanes 1a, b: *S. cerevisiae* W303D/pMA91; lanes 2a, b: *S. cerevisiae* W303D(pHNL-300) (transformant ScW303D-HNL2); lanes 3a, b: *P. pastoris* GS115 transformed with pHIL-D2; lanes 4a, b: *P. pastoris* GS115 transformed with pHNL-400 (PpD1-17); lanes 5a, b: *E. coli* XL1-Blue (pSE420); lanes 6a, b: *E. coli* XL1-Blue (pHNL-200); lane 1c: soluble fraction of refolded inclusion bodies of *E. coli* XL1-Blue/pHNL-200. Hnl-specific bands in (a) are indicated by arrows.

Soluble

Intracellular Hnl Expression in *Saccharomyces cerevisiae* and *Pichia pastoris*

ATTATTCGAACGAGGCCATGGCATTC

*Eco*RI

MetAlaPhe Hnl ORF

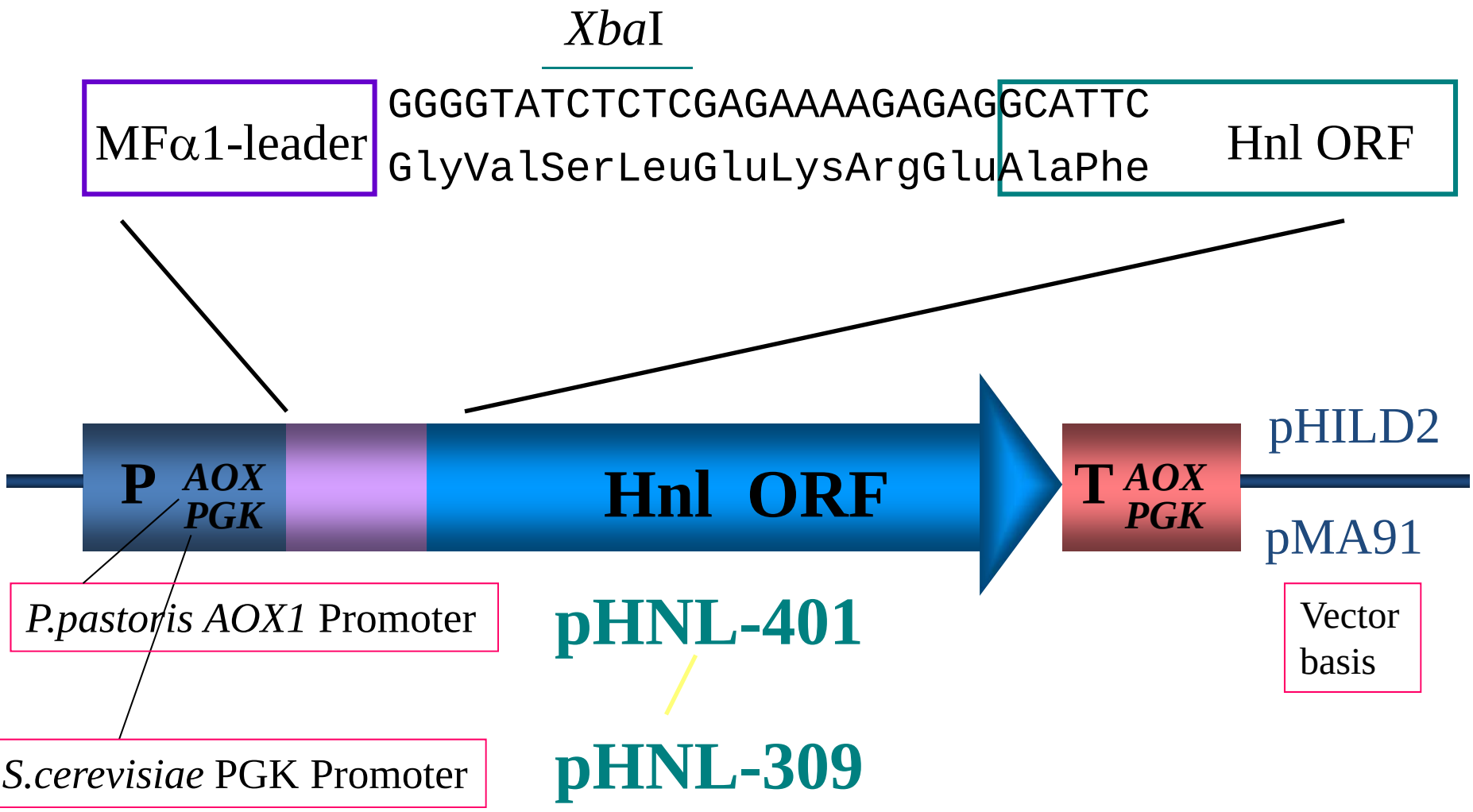
AAAGATCCCCCGGGCTGCAGGAATTCCATGGCATTC

*Bam*HI/*Bgl*II

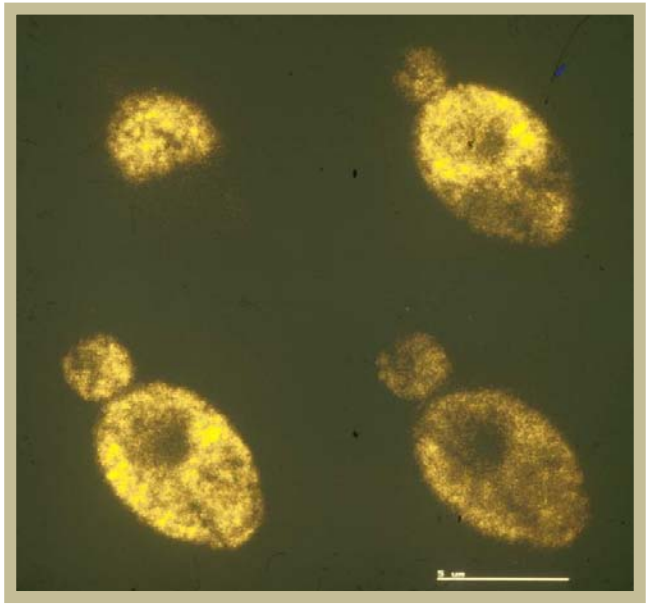
MetAlaPhe Hnl ORF

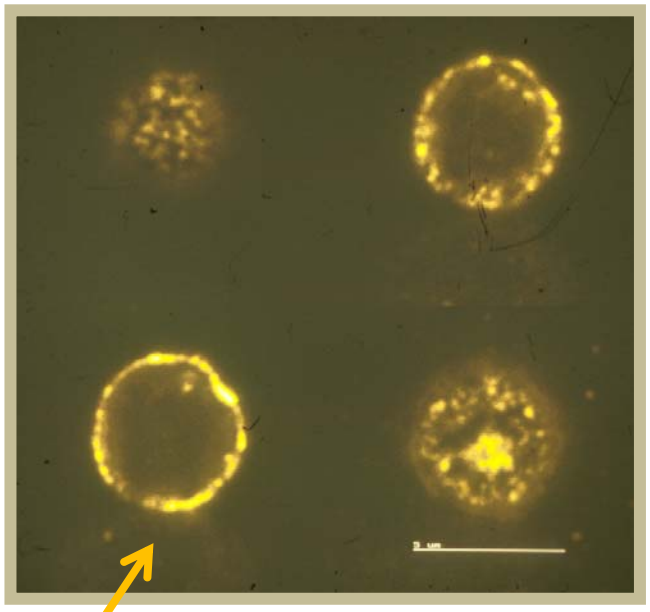
pHNL-400 / pHNL-300

Secretory Hnl Expression in *Saccharomyces cerevisiae* and *Pichia pastoris*



Secretion-targeted *Hb*_Hnl accumulates in the cell periphery



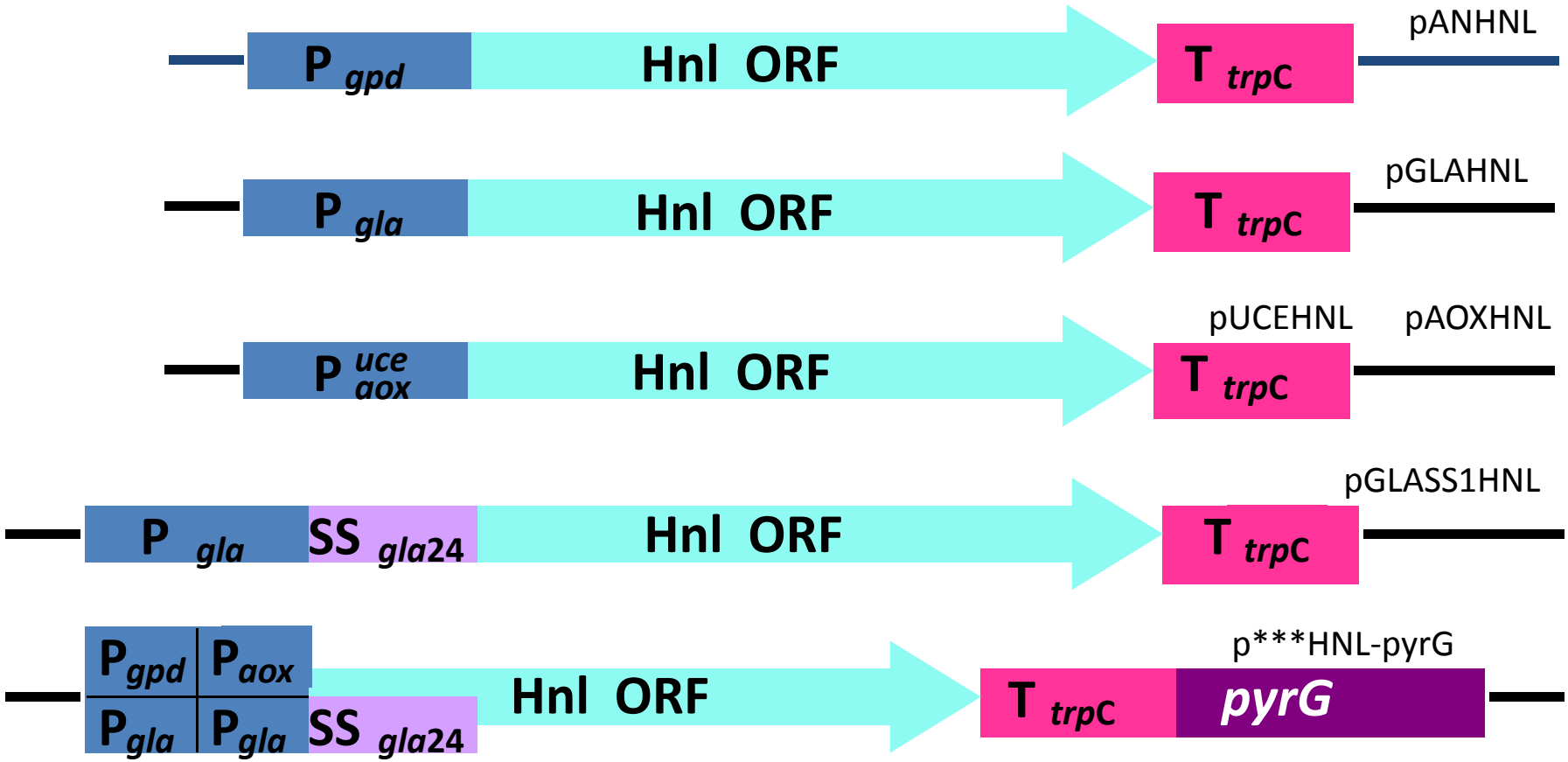


intracellular

secretory

→ Direction of a naturally intracellularly expressed protein into the secretory pathway leads to accumolation in the cell membrane

Hb-Hnl expression in filamentous fungi

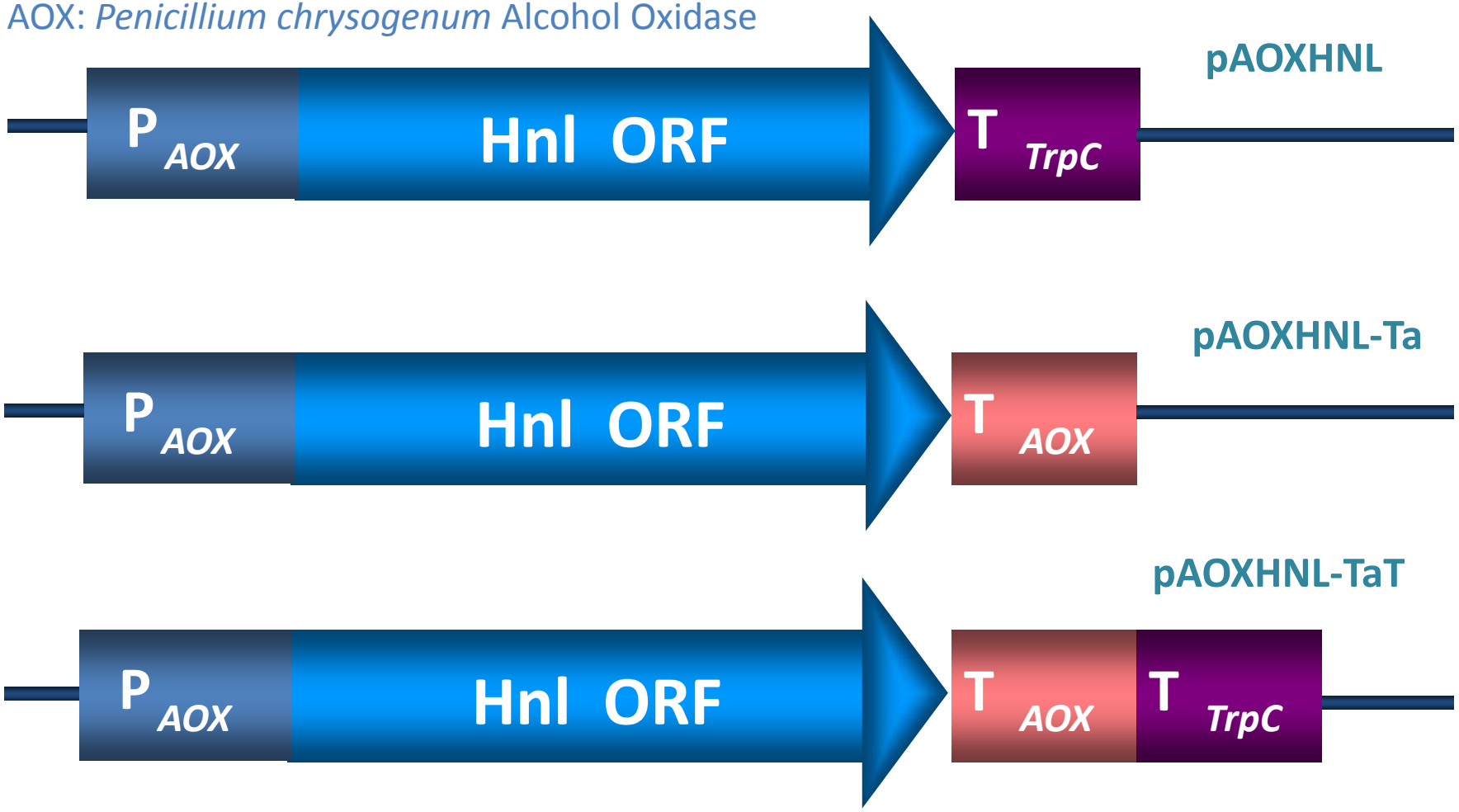


gpd: A.niger glyceraldehydephosphate dehydrogenase
gla: A.awamori glucoamylase
uce: unknown constitutively expressed gene, *P.chrysogenum*
aox: P.chrysogenum alcohol oxidase

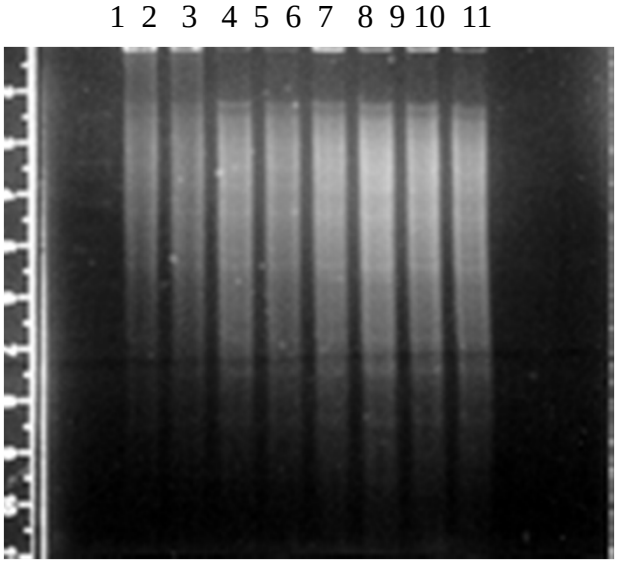
Intracellular Hnl Expression in *Penicillium chrysogenum* under control of P_{AOX}

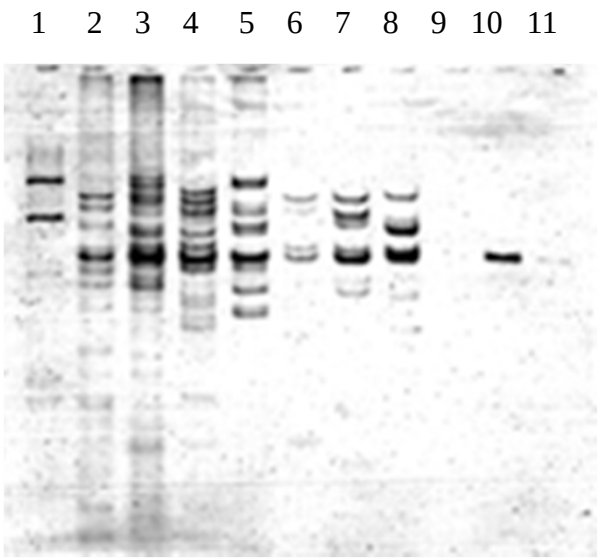
Termination Problem

AOX: *Penicillium chrysogenum* Alcohol Oxidase



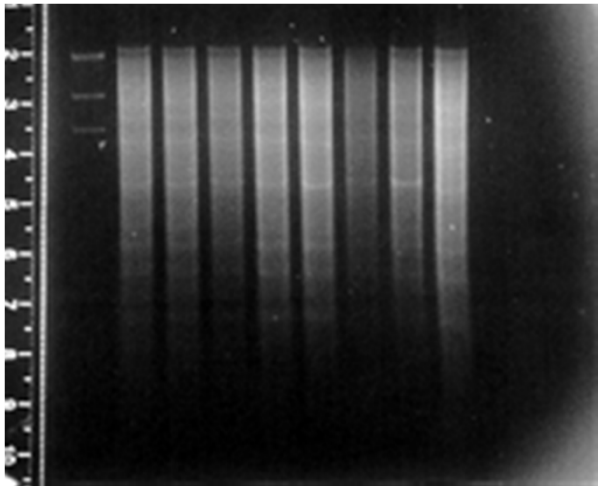
Southern analysis of *Penicillium chrysogenum* P_{AOX} *transformants*

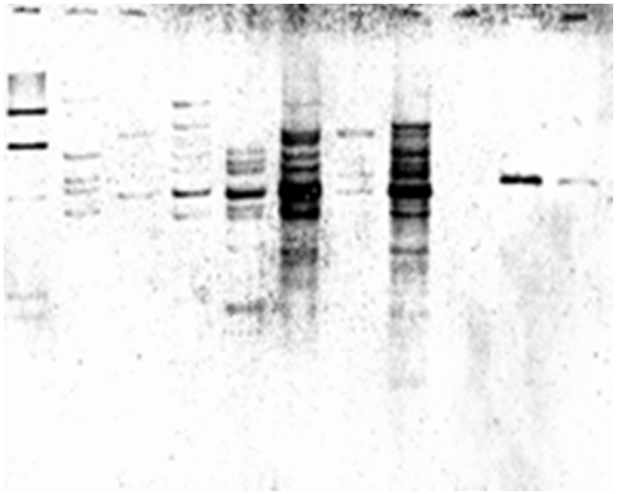




pAOXHNL-TaT

Multiple ectopic integration

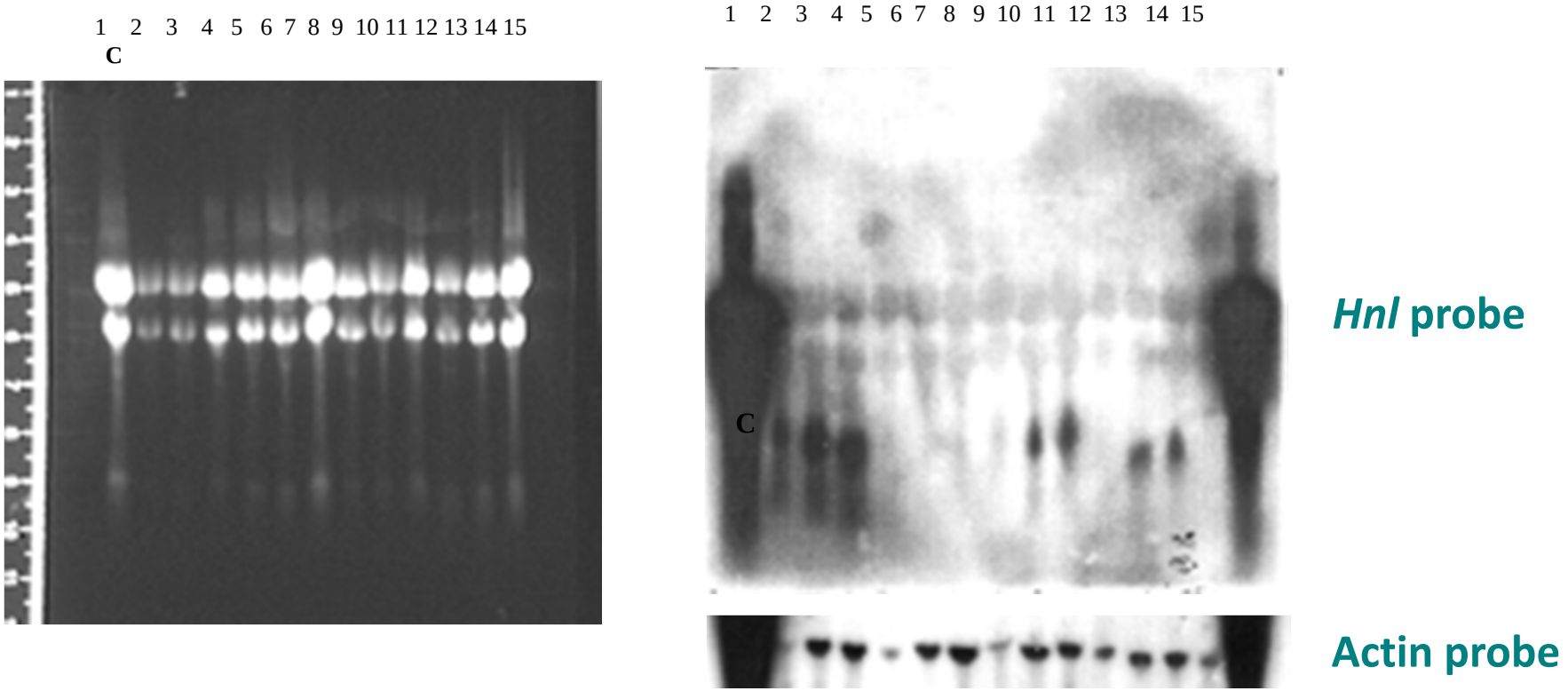




pAOXHNL-Ta

Northern blot analysis of *Penicillium chrysogenum* P_{AOX} *transformants*

pAOXHNL-TaT

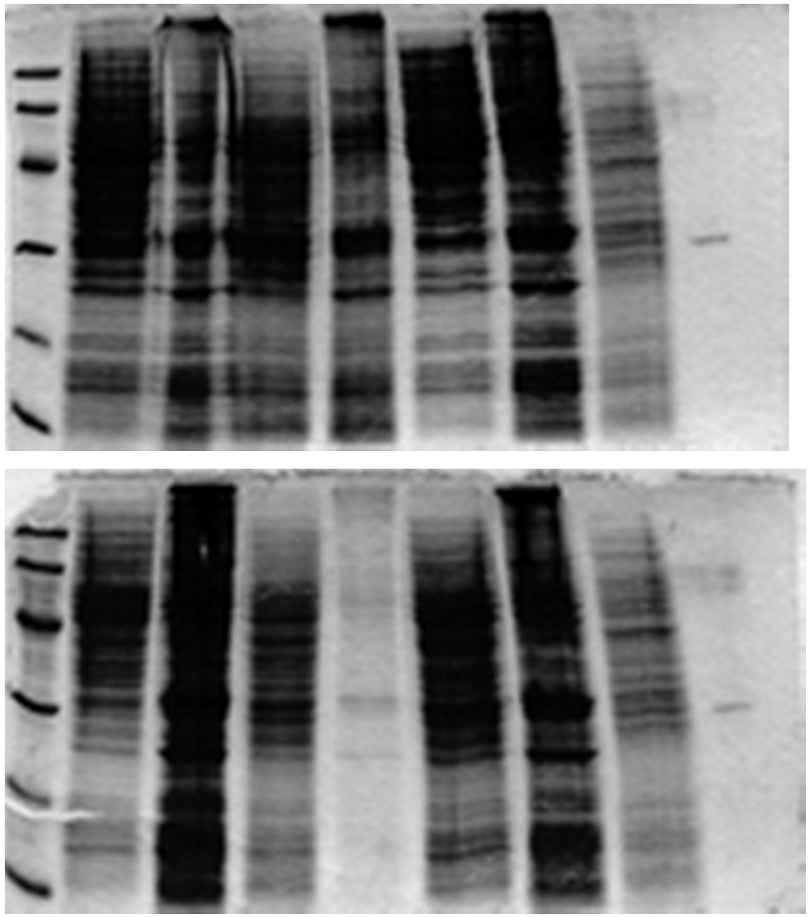


Western blot analysis of *Penicillium chrysogenum* P_{AOX} *transformants*

→ Protease Problems

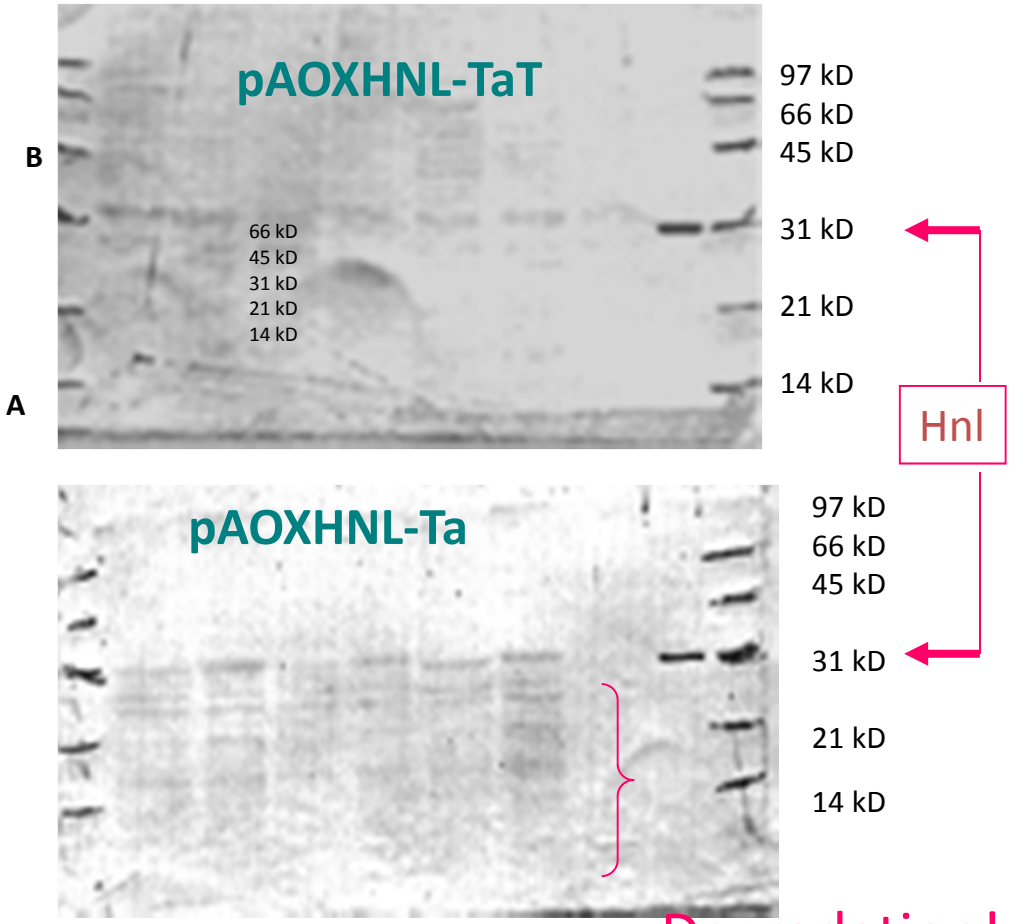
Total cellular proteins - SDS PAGE

1 2 3 4 5 6 7 8 9



Western blot with Hnl Antibody

1 2 3 4 5 6 7 8 9 10



Degradation!

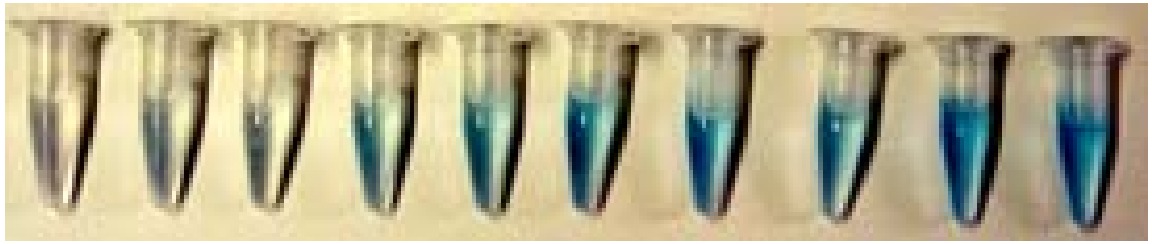
Expression analysis of *Penicillium chrysogenum* P_{AOX} *transformants*

AOX Promoter Activity Test

uidA: Reporter gene,
ß-glucuronidase

***AOX::uidA* fusion**
2% lactose pH 8
Blue colour = cleavage of X-Glucose by ß-glucuronidase

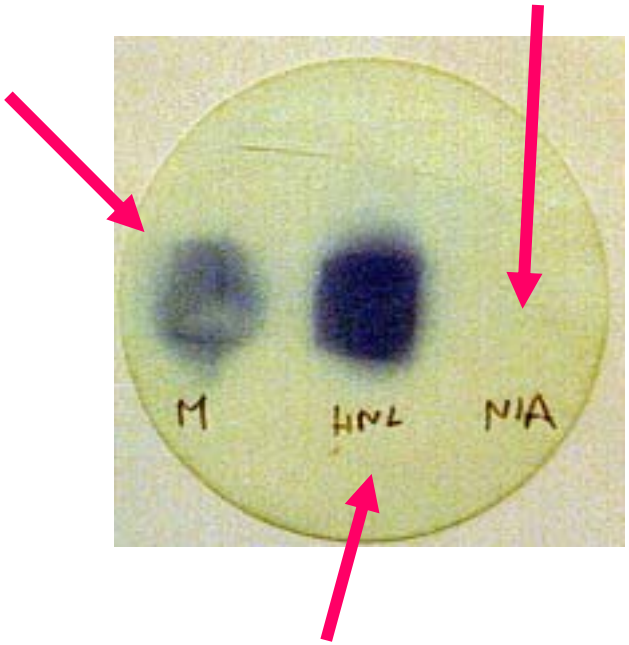
v.l. 2 3 4 5 6 7 8 9 10



Hnl Expression

pAOXHNL-TaT

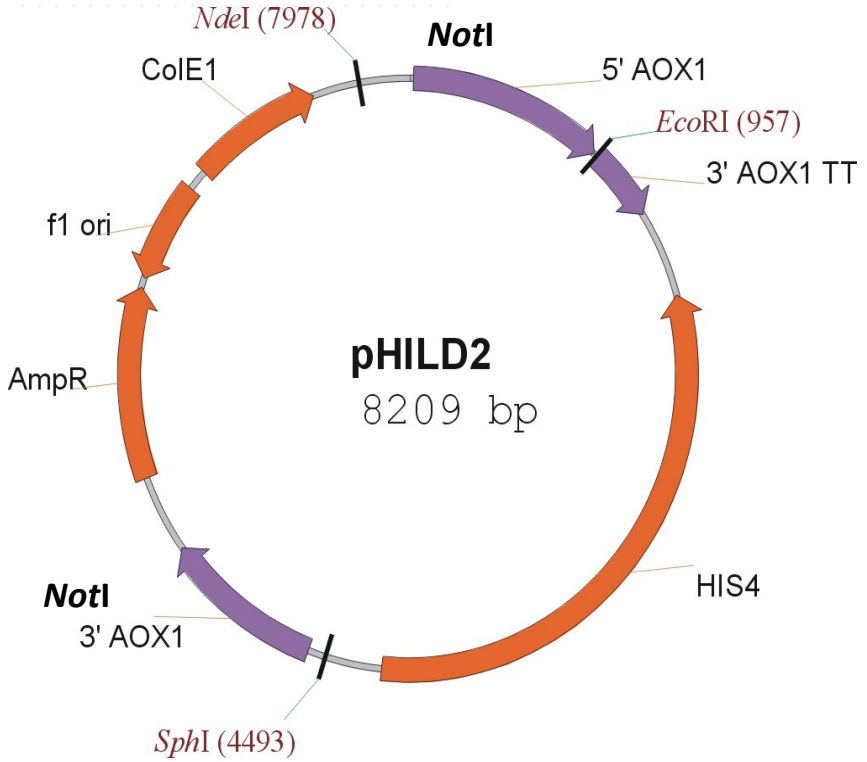
negative control

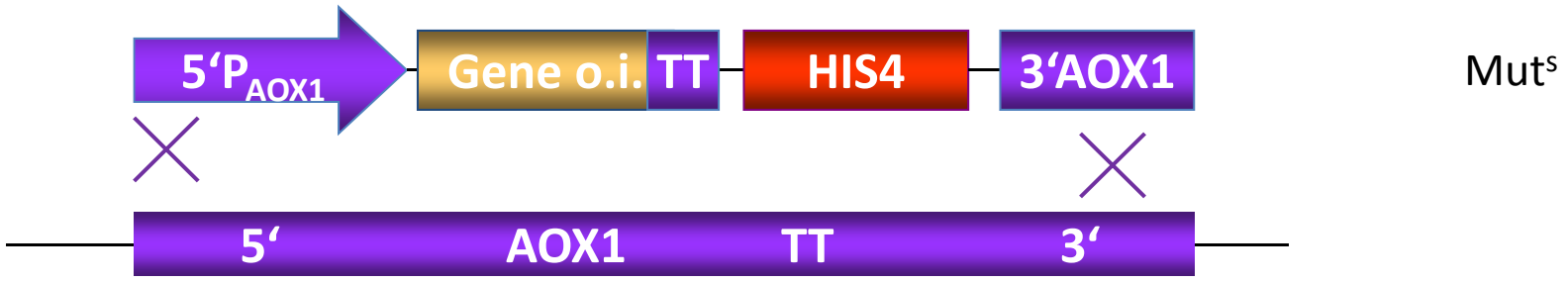


purified HNL

Gene replacement in *Pichia pastoris* at *AOX1*

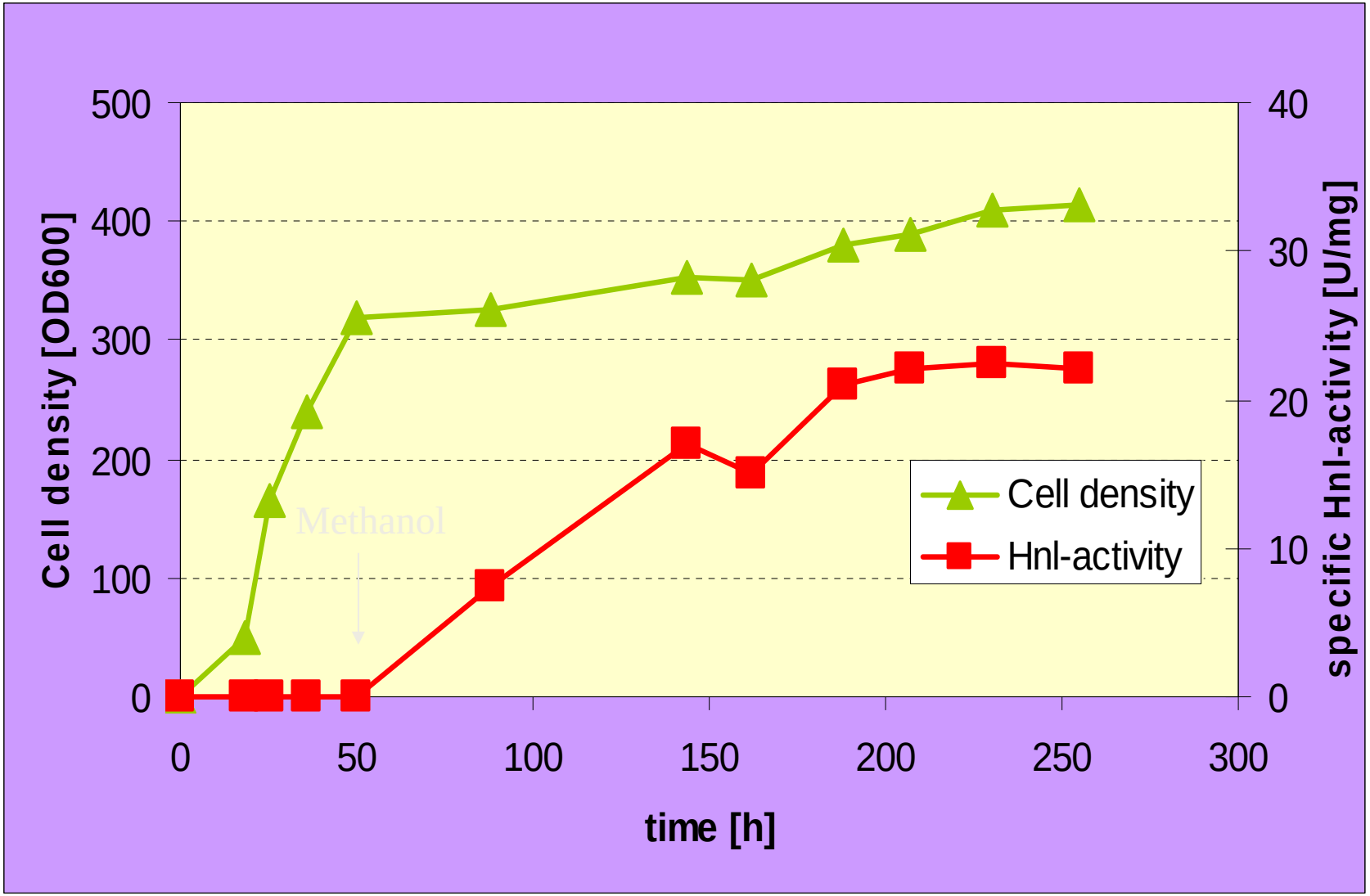
- *AOX1* promoter
- *HIS4* selection marker
- *AOX1* transcription termination signal
- 3' *AOX1* region
- Amp^R
- F1 ori
- ColE1 replicon





Mut^S

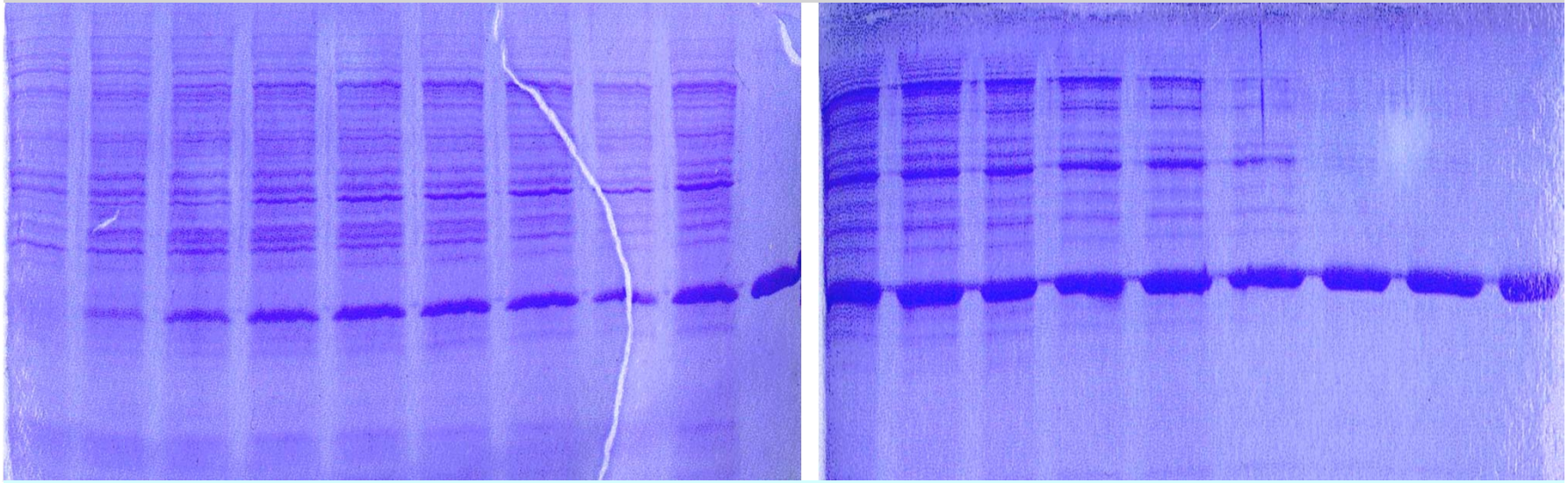
*Pichia pastoris Hb*_Hnl expression strain 1-17
Fed-batch fermentation



*Pichia pastoris Hb*_Hnl expression strain 1-17 Fed-batch fermentation

Fermentation time (hours after induction)

0 15 27 29 49 63 79 87 97 ST 111 119 135 145 151 159 169 194 ST



Soluble proteins in cell extracts

Heterologous Hnl Expression (shake flask experiments)

Construct	Host	cytosol (U/mg)	purified enzyme (U/mg)	total per culture
Hevea brasiliensis		0.42	15-20	0.5 U/g (leaves)
pHNL-200	*E. coli*	0.6	-	0.1 U/ml OD=4
pHNL-300	*S. cerevisiae*	4.6	20	1.2 U/ml OD=4
pHNL-400	*P. pastoris*	15.7	40	6.2 U/ml OD=4
pANHNL	*A. niger*	0.6	-	~ 0.1 U/ml nd

Production of *Hb*_Hnl with *Pichia pastoris* Expression System (Fed-Batch Fermentation)

Cell wet weight	400 g / l
Cell dry weight	100 g / l
Total protein	56 g / l
Hnl protein	23 g / l
Hnl units	1.4×10^6 / l

(S)-Hnl of *Hevea brasiliensis* and (R)-Hnl of *Prunus amygdalus*

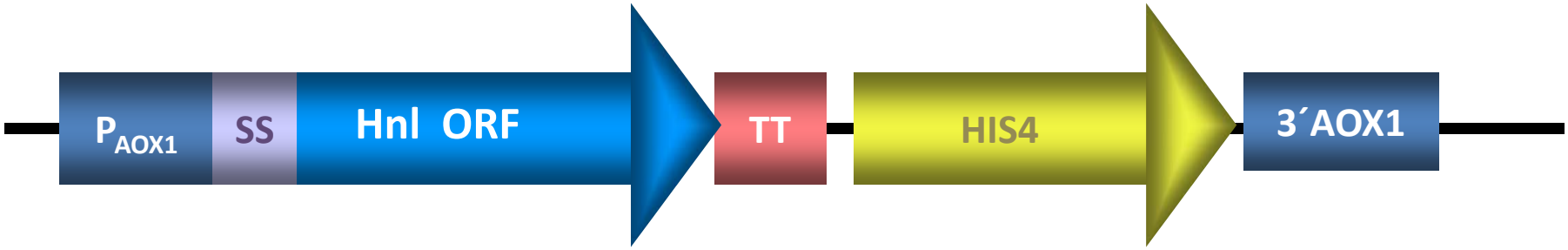
*Hb*_Hnl

- Type II Hnl
- intracellular protein
- 29.2 kDa
- homodimer
- α/β hydrolase fold protein
- catalytic triad
- (S)-selektive

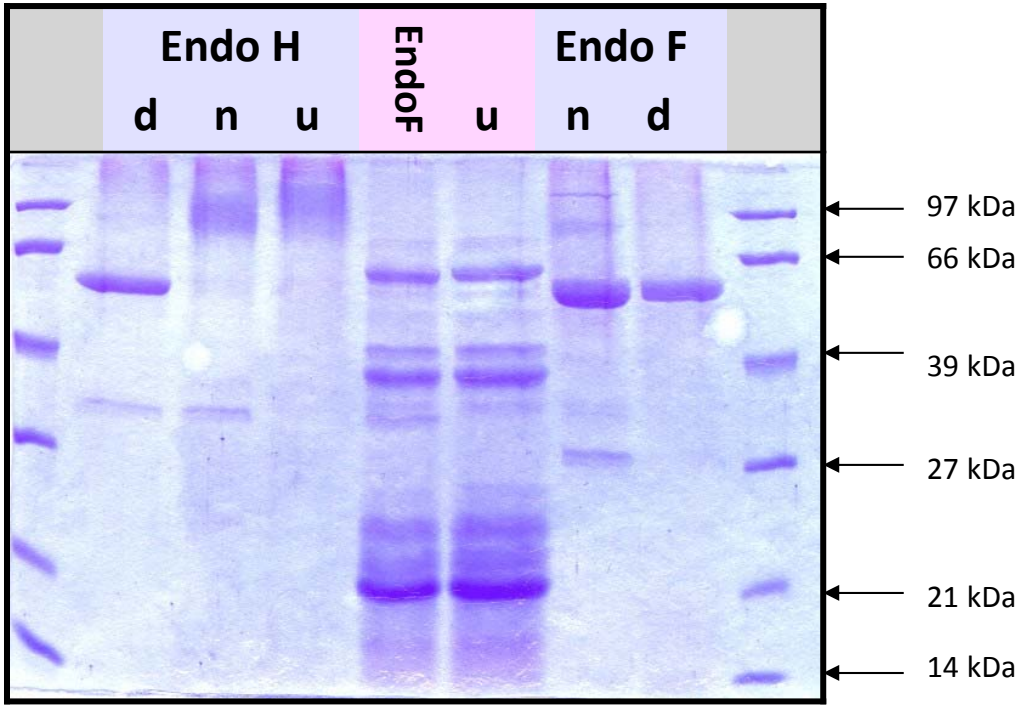
*Pam*_Hnl

- Type I Hnl
- secretory protein
- 61 kDa (57.9 kDa)
- Homology to oxidases
- FAD
- N-glycosylated
- isoenzymes
- (R)-selektive

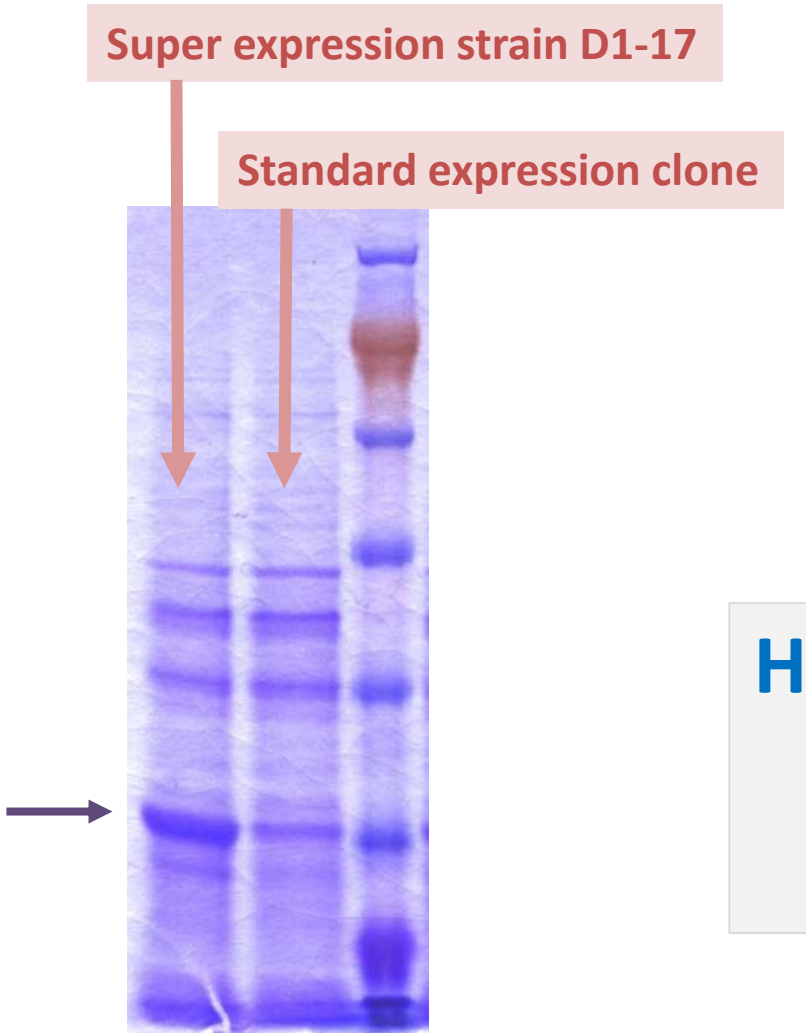
*Pam*_Hnl5: Secretory Expression of *Prunus amygdalus* (R)- Hnl in *Pichia pastoris*



- Host: *Pichia pastoris* GS115
- Alpha factor signal sequence
- Mut^{S} and Mut^{+} Transformants
- Functional secretory expression
- highly glycosylated



High-level Expression Clone D1-17 → ???



Hnl wt
→ great success with expression

Engineered Hnl Proteins
→ do it the same way !

High expression levels were

Not Reproducible !!

High-level Expression Clone D1-17 → ???

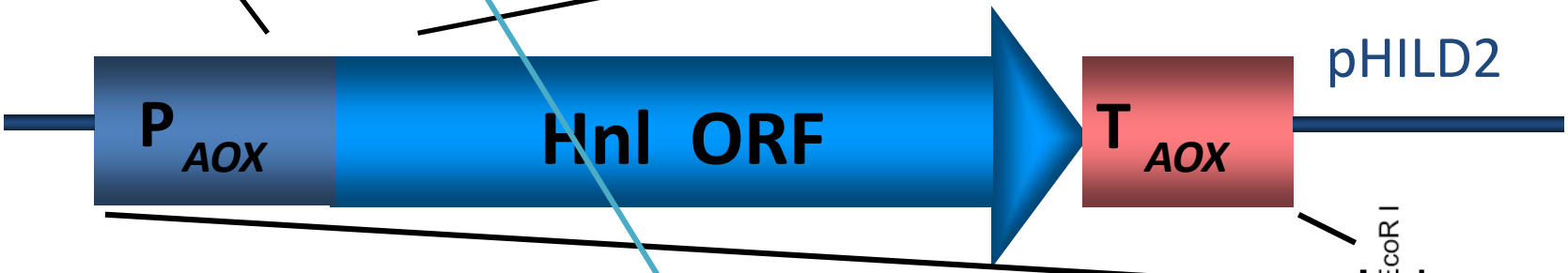
ATTATTCGAACGAGGCCATGGCATTC

Hnl ORF

MetAlaPhe

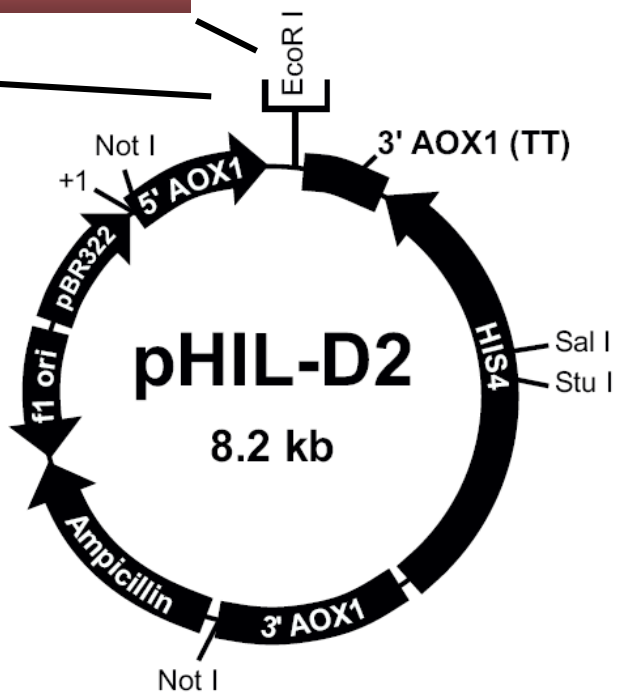
pHNL-400

pHILD2



Intracellular Hnl Expression in *Pichia pastoris*

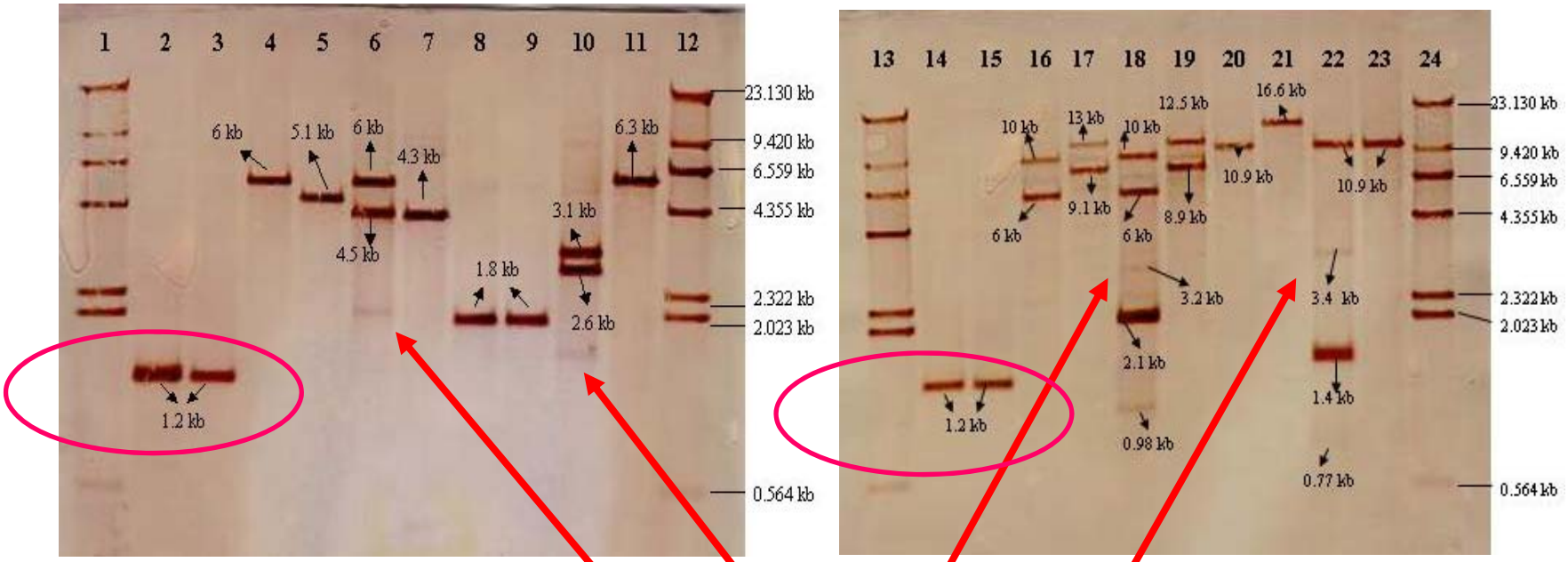
Not the Problem !



Hasslacher et al., Prot.Expr.Purif., 1997

Molecular Analysis of Expression Strain

Southern blotting



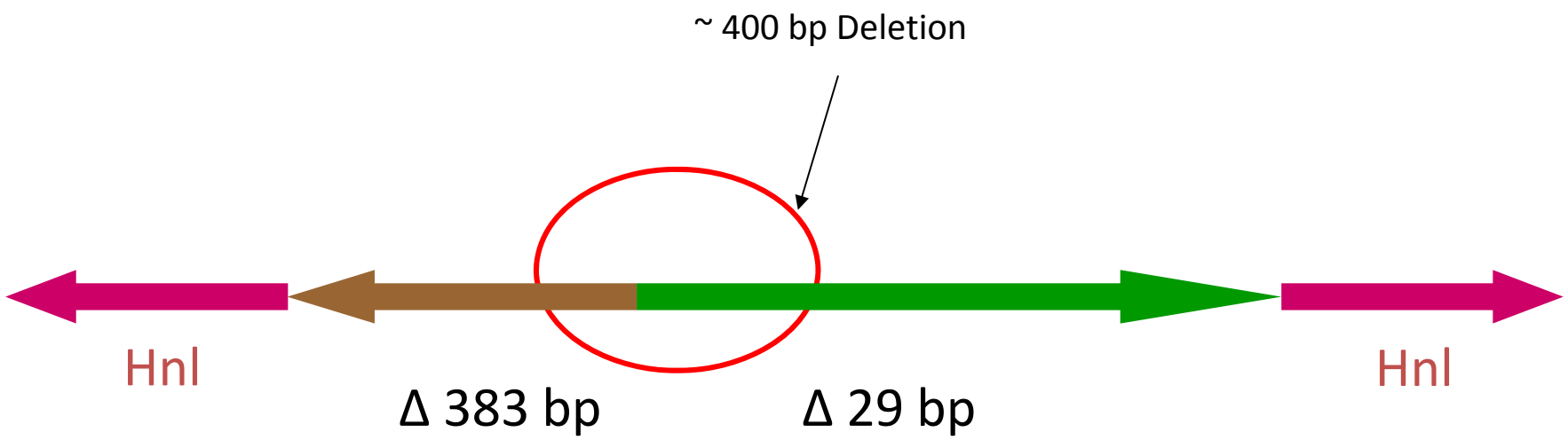
HNL probe

AOX1 Probe

Strange Fragments

→ More than one Copy Integrated → How ??

Molecular Analysis of Expression Strain

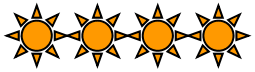
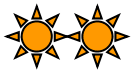
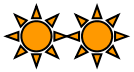
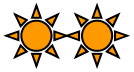
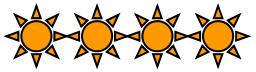


Tandem Integration – head to head (divergent)

P'_{AOX1} P_{AOX1}

3 copies of Hnl,
→ 1 standard Integration in AOX1 Locus
→ 2 truncated, in a head to head oriented AOX1 Promoter Fragments

Expression analysis

	Expression
phhAOX561(-HbHNLwt) 	
phhAOX915(-HbHNLwt) 	
pAOXGRAZlang(-HbHNLwt) 	
pAOXGRAZshort(-HbHNLwt) 	
pAOXGRAZtotal(-HbHNLwt) → single copy 	
D1.17	

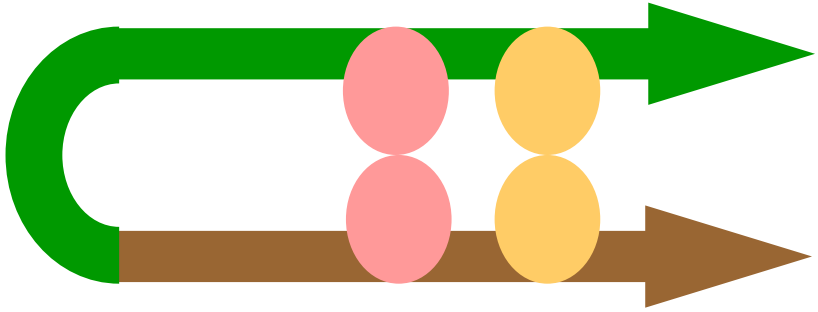
Specific genomic setup

A

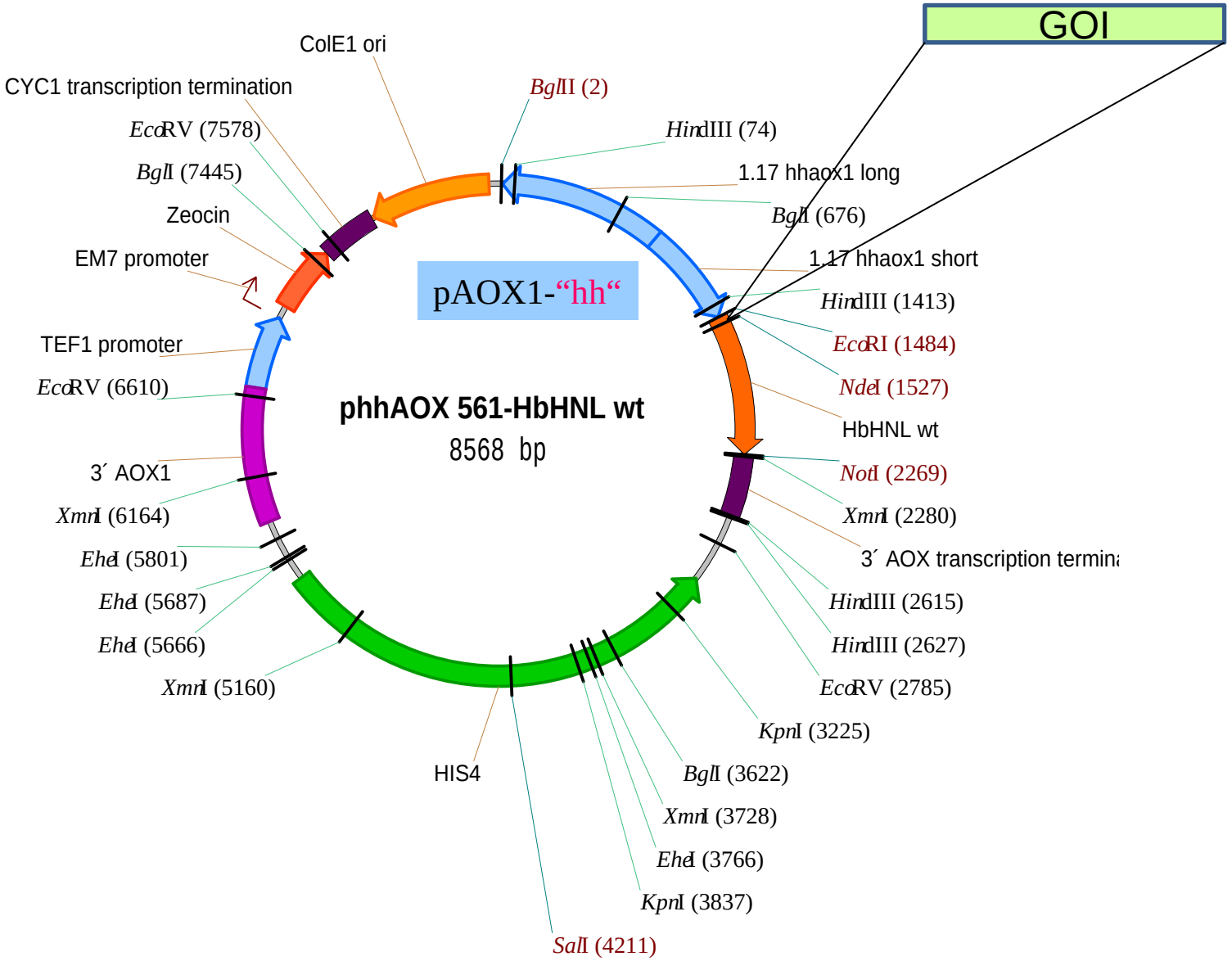
```
                -960                                                -881
                AGATCTAACA TCCAAAGACG AAAGGTTGAA TGAAACCTTT TTGCCATCCG ACATCCACAG
                TCTAGATTGT AGGTTTCTGC TTTCCAACTT ACTTTGGAAA AACGGTAGGC TGTAGGTGTC
                                                                    Hap1
-880                                                                -801
GTCCATTCTC ACACATAAGT GCCAAACGCA ACAGGAGGGG ATACACTAGC AGCAGACCGT TGCAAACGCA GGACCTCCAC
CAGGTAAGAG TGTGTATTCA CGGTTTGCGT TGTCCTCCCC TATGTGATCG TCGTCTGGCA ACGTTTGCGT CCTGGAGGTG
                                                                      HSF
-800                                                                -721
TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA
AGGAGAAGAG GAGTTGTGGG TGAAAACGGT AGCTTTTTGG TCGGGTCAAT AACCCGAACT AACCTCGAGC GAGTAAGGTT
   HSF                                          Hap2/3/4/5 (2x)          abaA
-720                                                                -641
TTCCTTCTAT TAGGCTACTA ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG GCGAGGTTCA TGTTTGTTTA
AAGGAAGATA ATCCGATGAT TGTGGTACTG AAATAATCGG ACAGATAGGA CCGGGGGGAC CGCTCCAAGT ACAAACAAAT
                                                  STRE
-640                                                                -561
TTTCCGAATG CAACAAGCTC CGCATTACAC CCGAACATCA CTCCAGATGA GGGCTTTCTG AGTGTGGGGT CAAATAGTTT
AAAGGCTTAC GTTGTTCGAG GCGTAATGTG GGCTTGTAGT GAGGTCTACT CCCGAAAGAC TCACACCCCA GTTTATCAAA
                              Rap1                                  Adr1
```

Hartner et al., Nucleic Acids Research, 2008, Vol. 36,

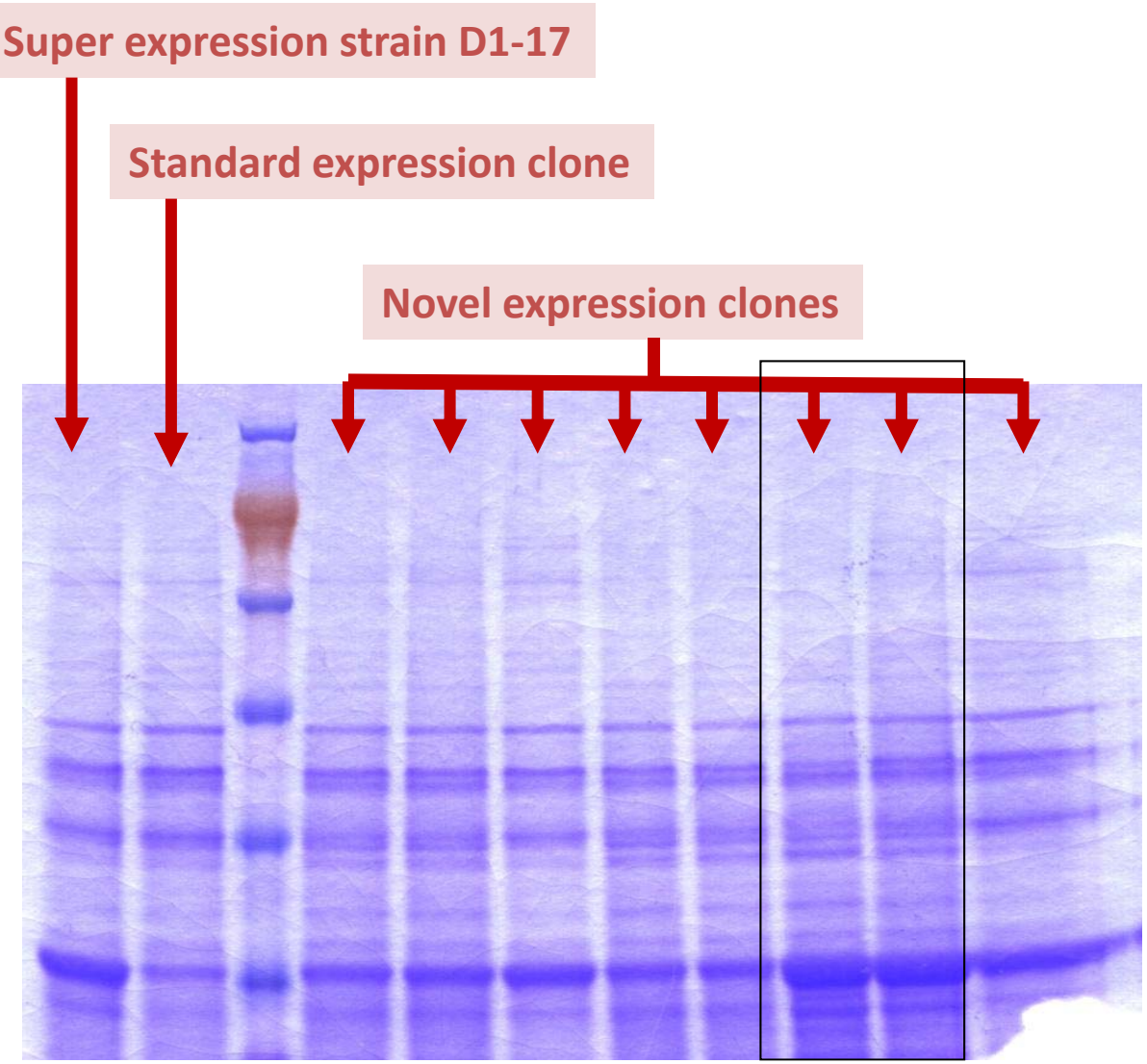
→ Specific complex ??
→ Concerted action of activators ??
→ Repression-active site deleted ??



"hh"- Expression vector



Expression of *Hb*_Hnl mutants in *Pichia pastoris*



Modified
hh-AOX1-based promoter
System

Intracellular Expression

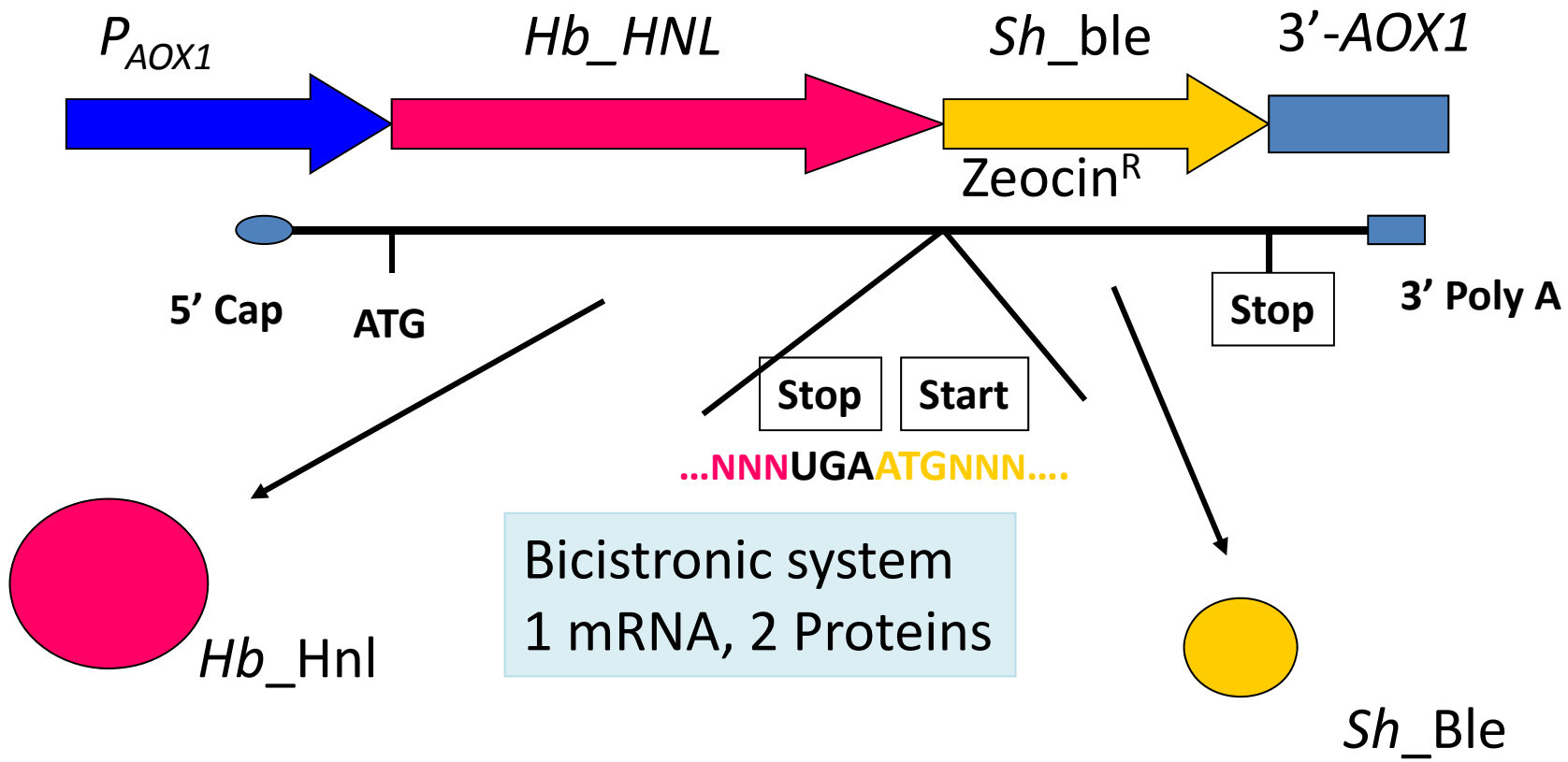
Hnl

Screening for High-level Expression

Screening systems based on principle of **translational coupling**

Well known in Prokayotes

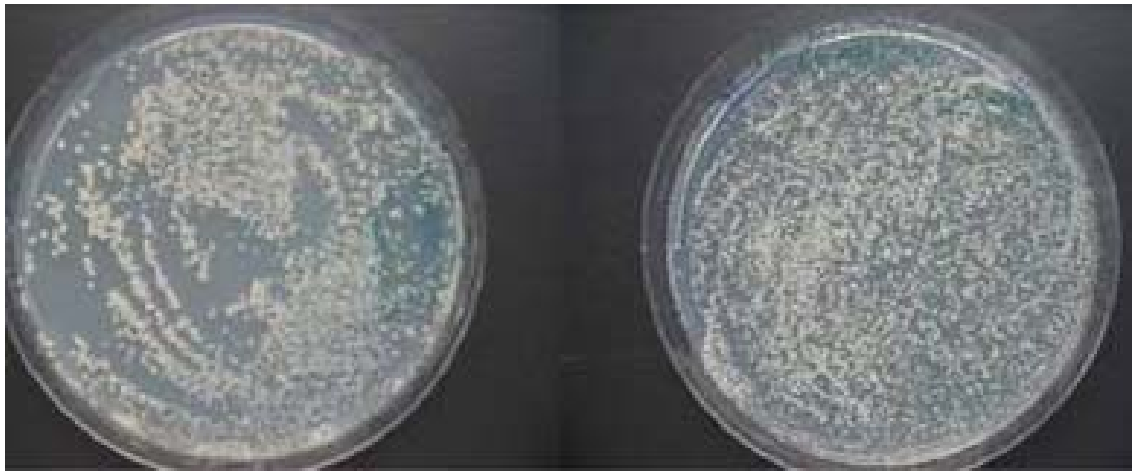
Does it work in Eukaryotes ???



Bicistronic system
1 mRNA, 2 Proteins

Veeresh Juturu, PhD Thesis

Correlating Hnl expression to zeocin resistance

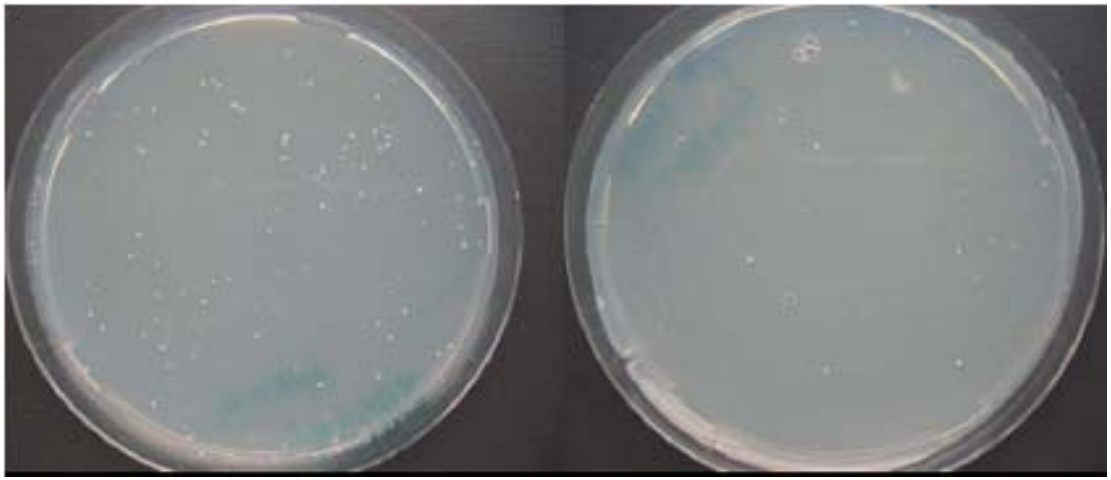


Veeresh Juturu,
PhD Thesis

Increasing
Zeocin
Concentration

MD $4.9x10^4$

BMMS $4.9x10^4$



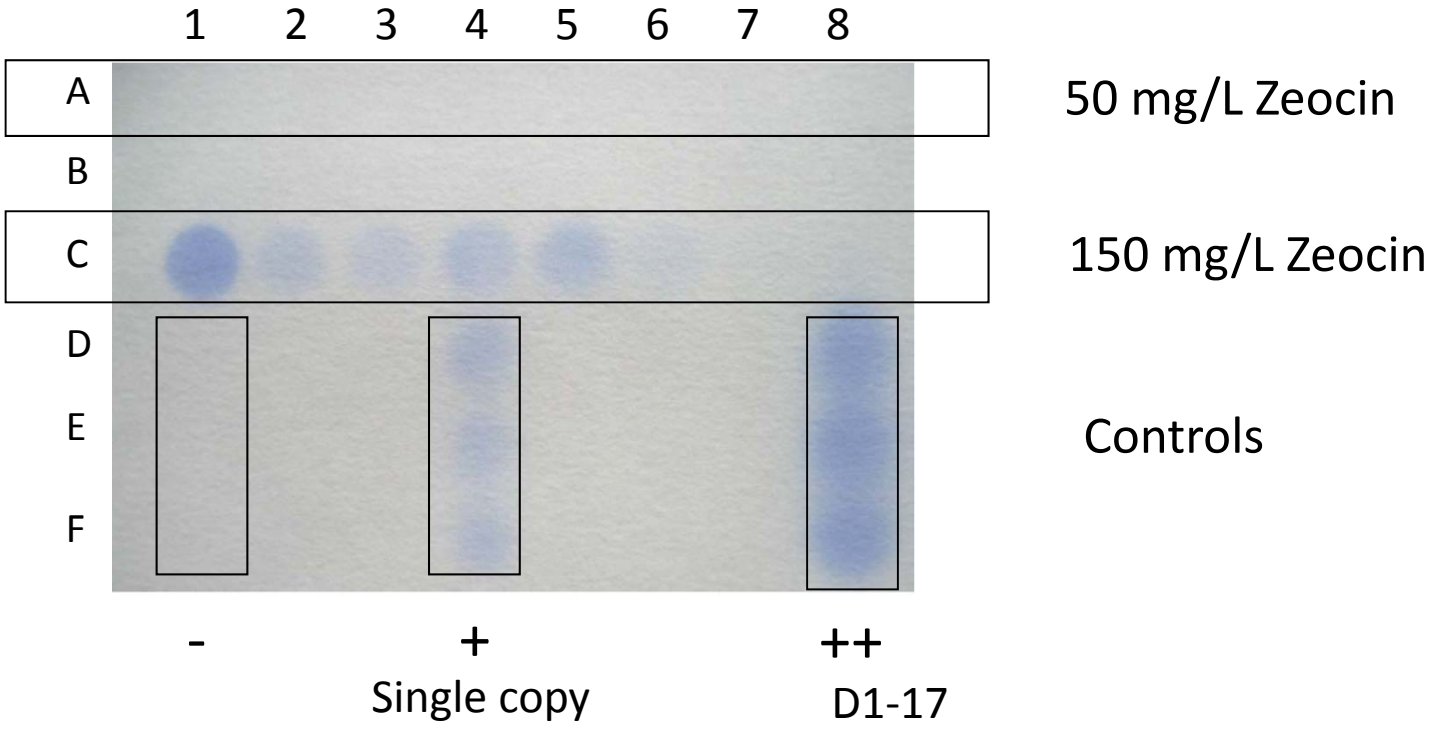
BMMS $4.9x10^4$
50 µg/ml

BMMS $4.9x10^4$
100 µg/ml

BMMS $4.9x10^4$,
150 µg/ml

Hnl expression

Screen for High Resistance → Check for Expression Level



Row A → Clones from BMMS 50 µg/ml Zeo
Row C → Clones from BMMS 150 µg/ml Zeo
Lane 1D-1F → GFP expressing strain (- control)
Lane 4D-4F → *P. pastoris* HNL single copy strain (+ control)
Lane 8D-8F → *P. pastoris* HNL multi copy strain (+ control)