Functional evaluation of individual mutations of identified amino acids in PobR

8.2 Beimeng Zhang

Site-directed mutagenesis of the PobR CDS

Site-directed mutagenesis PCR (50 μL×3)

Reaction system:

247spm	126spm	122spm
247spm-F: 1 μL	126spm-F: 1 μL	122spm-F: 1 μL
247spm-R: 1 μL	126spm-R: 1 μL	122spm-R: 1 μL
pYB1a-PobR-eGFP-Cmr: 1 μL	pYB1a-PobR-eGFP-Cmr: 1 μL	pYB1a-PobR-eGFP-Cmr: 1 μL
2×HF Mix: 25 μL	2×HF Mix: 25 μL	2×HF Mix: 25 μL
DDW: 22 μL	DDW: 22 μL	DDW: 22 μL

Reaction procedure

98℃ 5 min

98℃ 30 s
60℃ 30 s
72℃ 3 min
} ×24

72℃ 5 min

16℃ 1 h

Agarose gel electrophoresis assay showed correct bands, the concentration of the product after purified:

247spm (PCR): 32.416 ng/μL

126spm (PCR): 91.342 ng/μL

122spm (PCR): 33.478 ng/μL

8.3 Shuning Guo

Site-directed mutagenesis of the PobR CDS

1. DpnI digestion

Reaction system:

Spm247	Spm126	Spm122
DpnI: 0.2 μL	DpnI: 0.2 μL	DpnI: 0.2 μL
10×Cutsmart: 1 μL	10×Cutsmart: 1 μL	10×Cutsmart: 1 μL
247spm (PCR) (32 ng/μL)：8.8 μL	126spm (PCR) (91 ng/μL): 4.4 μL	122spm (PCR) (33 ng/μL): 8.8 μL
DDW: 0	DDW: 4.4 μL	DDW: 0

Reaction procedure: 37℃ 2 h

70℃ 20 min

2. Transformation

System:

	Spm247	Spm126	Spm122

Competent cells	DH5α: 100 μL	DH5α: 100 μL	DH5α: 100 μL
Digestion product	10 μL (282 ng)	10 μL (400.4 ng)	10 μL (290.4 ng)
plate	Ampicillin (50 μL/mL)	Ampicillin (50 μL/mL)	Ampicillin (50 μL/mL)

Culture at 37℃ for 12 h.

8.4 Chuyao Xu

Site-directed mutagenesis of the PobR CDS

Picked 3 clones from every plate and inoculated into 5 mL LB medium and culture at 37℃ for 8 h.

Then, 700 μL culture were used for storage at -20℃ and other culture were store at 4℃ for further experiments.

8.5 Baitong Shen

Site-directed mutagenesis of the PobR CDS

Extracted plasmid DNA from the culture at 4℃ and send them to sequencing.

8.7 Xinlu Liu

Site-directed mutagenesis of the PobR CDS

1. Sequencing result:

Spm126/spm122 have expected mutation on the plasmids, spm247 does not.

2. Site-directed mutagenesis PCR (50 μL×1)

Reaction system:

247spm
247spm-F: 1 μL
247spm-R: 1 μL
pYB1a-PobR-eGFP-Cmr: 1 μL
2×HF Mix: 25 μL
DDW: 22 μL

Reaction procedure

98℃ 5 min

98℃ 30 s
60℃ 30 s ×24
72℃ 3 min

72℃ 5 min

16℃ 1 h

Agarose gel electrophoresis assay showed correct bands, the concentration of the product after purified:

247spm (PCR): 38 ng/μL

3. Transformation

System:

	Spm247
Competent cells	DH5α: 100 μL
Digestion product	1 μL (38 ng)

plate	Ampicillin (50 μL/mL)

Culture at 37℃ for 12 h.

8.8 Shuning Guo

Site-directed mutagenesis of the PobR CDS

Picked 2 clones from the plate and inoculated into 5mL LB medium and culture at 37℃ for 8 h.

Then, 700 μL culture were used for storage at -20℃ and other culture were store at 4℃ for further experiments.

8.9 Beimeng Zhang

Site-directed mutagenesis of the PobR CDS

Extracted plasmid DNA from the culture at 4℃ and send them to sequencing.

8.10 Baitong Shen

Site-directed mutagenesis of the PobR CDS

1. Sequencing result:

Both the two clones do not have expected mutation on the plasmids.

2. DpnI digestion

Reaction system:

Spm247
DpnI: 0.2 μL
10×Cutsmart: 1 μL
247spm (PCR) (38 ng/μL): 8.8 μL
DDW: 0

Reaction procedure:

37℃ 2 h

70℃ 20 min

8.16 Xinlu Liu

Site-directed mutagenesis of the PobR CDS

1. The second-round site-directed mutagenesis PCR (50 μL×3)

Reaction system:

125spm	124spm	16spm
Spm125-F: 1 μL	Spm126-F: 1 μL	Spm16-F: 1 μL
Spm125-R: 1 μL	Spm126-R: 1 μL	Spm16-R: 1 μL
pYB1a-PobR-eGFP-Cmr: 1 μL	pYB1a-PobR-eGFP-Cmr: 1 μL	pYB1a-PobR-eGFP-Cmr: 1 μL
2×HF Mix: 25 μL	2×HF Mix: 25 μL	2×HF Mix: 25 μL
DDW: 22 μL	DDW: 22 μL	DDW: 22 μL

Reaction procedure:
98℃ 5 min
98℃ 30 s
60℃ 30 s ×24
72℃ 3 min
72℃ 5 min
16℃ 1 h
Agarose gel electrophoresis assay showed correct bands, the concentration of the product after purified:
Spm16 (PCR): 55 ng/μL
Spm124 (PCR): 29 ng/μL
Spm125 (PCR): 29 ng/μL
2. DpnI digestion
Reaction system:

Spm125	Spm124	Spm16
DpnI: 0.2 μL	DpnI: 0.2 μL	DpnI: 0.2 μL
10×Cutsmart: 1 μL	10×Cutsmart: 1 μL	10×Cutsmart: 1 μL
Spm125 (PCR) (29 ng/μL): 8.8 μL	Spm124 (PCR) (29 ng/μL): 8.8 μL	Spm16 (PCR) (55 ng/μL): 7.3 μL
DDW: 0	DDW: 0	DDW: 1.5 μL

8.17 Chuyao Xu

Site-directed mutagenesis of the PobR CDS
Transformation
System:

	Spm125	Spm124	Spm16	Spm247
Competent cells	DH5α: 100 μL	DH5α: 100 μL	DH5α: 100 μL	DH5α: 100 μL
Digestion product	10 μL (255 ng)	10 μL (255 ng)	10 μL (401 ng)	10 μL (334.4 ng)
plate	Ampicillin (50 μL/mL)	Ampicillin (50 μL/mL)	Ampicillin (50 μL/mL)	Ampicillin (50 μL/mL)

Culture at 37℃ for 12 h.

8.18 Shuning Guo

Site-directed mutagenesis of the PobR CDS
Picked 2 clones from each of the plate and inoculated into 5 mL LB medium and culture at 37℃ for 8 h.
Then, 700 μL culture were used for storage at -20℃ and other culture were store at 4℃ for further experiments.

8.20 Beimeng Zhang

Site-directed mutagenesis of the PobR CDS

Sequencing result:
Two clones of spm125 have expected mutation on the plasmids, spm247/16/124 do not.

8.21 Baitong Shen

Site-directed mutagenesis of the PobR CDS

DpnI digestion

Reaction system:

Spm247	Spm124	Spm16
DpnI: 0.2 μL	DpnI: 0.2 μL	DpnI: 0.2 μL
10×Cutsmart: 1 μL	10×Cutsmart: 1 μL	10×Cutsmart: 1 μL
Spm247 (PCR) (38 ng/μL): 1.3 μL	Spm124 (PCR) (29 ng/μL): 1.7 μL	Spm16 (PCR) (55 ng/μL): 1 μL
DDW: 7.5 μL	DDW: 7.1 μL	DDW: 7.8 μL

Reaction procedure:

37℃ 2 h

70℃ 20 min

8.22 Xinlu Liu

Site-directed mutagenesis of the PobR CDS

Transformation

System:

	Spm247	Spm124	Spm16
Competent cells	DH5α: 100 μL	DH5α: 100 μL	DH5α: 100 μL
Digestion product	10 μL (50 ng)	10 μL (49.3 ng)	10 μL (55 ng)
plate	Ampicillin (50 μL/mL)	Ampicillin (50 μL/mL)	Ampicillin (50 μL/mL)

Culture at 37℃ for 12 h.

8.23 Shuning Guo

Site-directed mutagenesis of the PobR CDS

Picked 2 clones from each of the plate and inoculated into 5mL LB medium and culture at 37℃ for 8h.

Then, 700 μL culture were used for storage at -20℃ and other culture were store at 4℃ for further experiments.

8.24 Beimeng Zhang

Site-directed mutagenesis of the PobR CDS

DNA sequencing result showed that clones named as spm124-1/spm124-2/spm16-1/spm247-2 have expected mutation on the plasmids, spm16-2 does not have mutation on its plasmid, spm247-1 has two mutation sites on its plasmid.

8.30 Baitong Shen

Fluorescence re-screening of single amino acid mutants

We used re-screening method to detected the induction intensity of HMA for five PobR mutants with single amino acid mutation (Figure1).

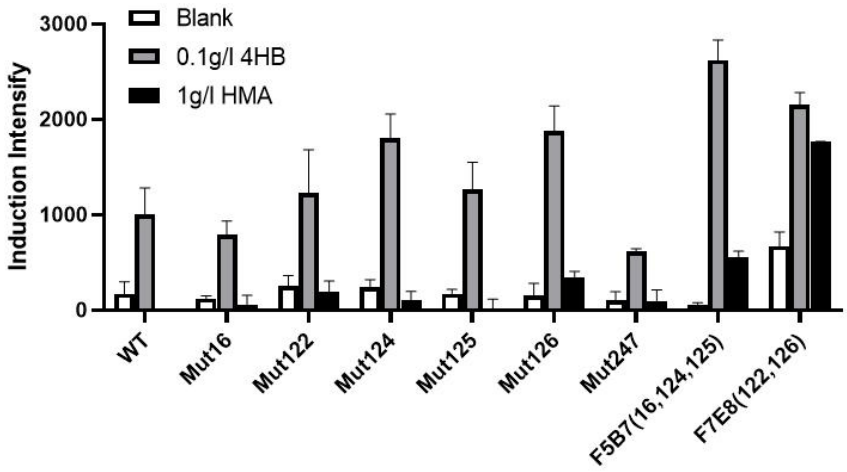

Figure 1 The induction intensity of HMA for five PobR mutants with single amino acid mutation

After analyzing the result, E124G, E126V showed a higher IB than $PobR^{WT}$; E126V might influence the HMA sensitivity of PobR. What needs to be emphasized is that all five single amino acid mutants did not increase the IB or IA to the level of double (P5-B7) or triple mutants (P7-E8). This result supported that the increased induction level of P5-B7 and P7-E8 is caused by the synergistic effect of multiple amino acid mutations.