Supporting Information

Biochemical properties and *in planta* effects of NopM, a rhizobial E3 ubiquitin ligase

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Journal of Biological Chemistry

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  1 MNVQRPGLAV GPLFENPESE SSEPGSPAAA ARWVEASTEA EASAASSSQG QIVAAPTAEE
 61 RPWEGRPQEA VSRTRAWREA GDVDEPLDLS FLSLTPLSIP LVSGLRRLNV NNNQLGDLPD
121 TLPGTLLELE ASENRLTRLP DLPAGLQRLN VENNRLTNLP EPLPAALEWL GAGYNQLTRL
181 PEMIPPELIW LGARNNQLTS VPESLLTQLG QWSSIDLENN PLPHGVQTNL VTAMHAAGYA
241 GPQIFLPMGP VELARRPLHE VVADWLEGDL ETVAAWRGFA NEQGARDYAH FLDRLRTTVN
301 YGNDAFRQAV AIGLRQAVAR PQLRAQYFEQ ASGASDSCED RITLTWNGMQ TALLIADVED
361 GVYDGSLHQL LQHGRVMFRL EALDGIARET VNSLRRTDPD ADIDEIEVYL AYQTQLRDTL
421 ELRHVAPDMR FLNVSHVTEE DVARAASSVR ELEARGFGEY VATRWQPWER VMRRIAPASH
481 AAMQEQLIEA MGEEFRSRLD EKLAEHGLTG DADAERVFGA EILNDIARRI KGETMEKVLR
541 GRGLEL
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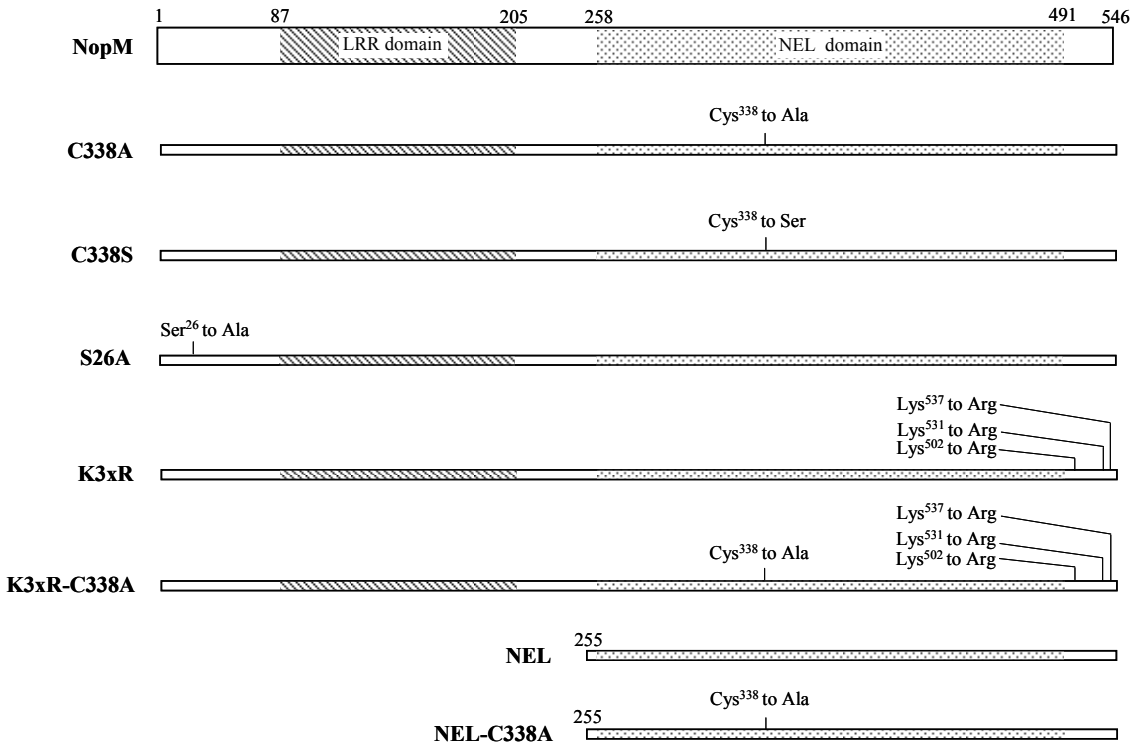
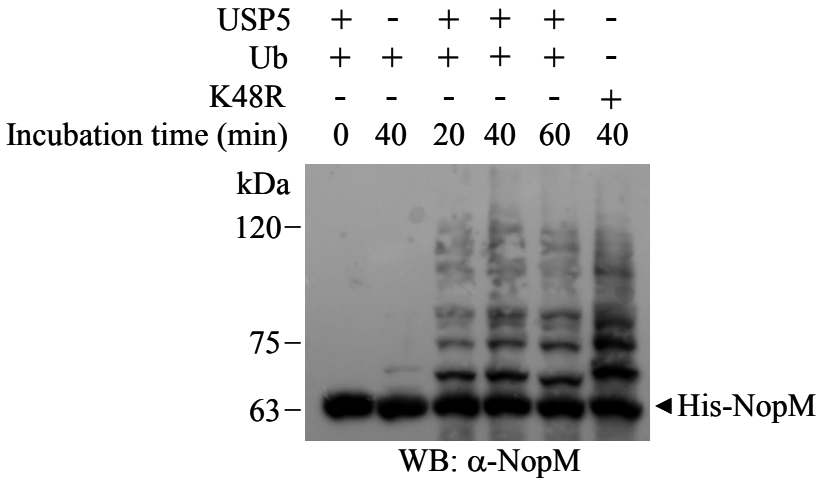
S26: Phosphorylation site identified in this study
C338: Catalytic cysteine
D340: Predicted catalytic acid
D404: Predicted catalytic base
K502, **K**531 and **K**537: Predicted autoubiquitination sites

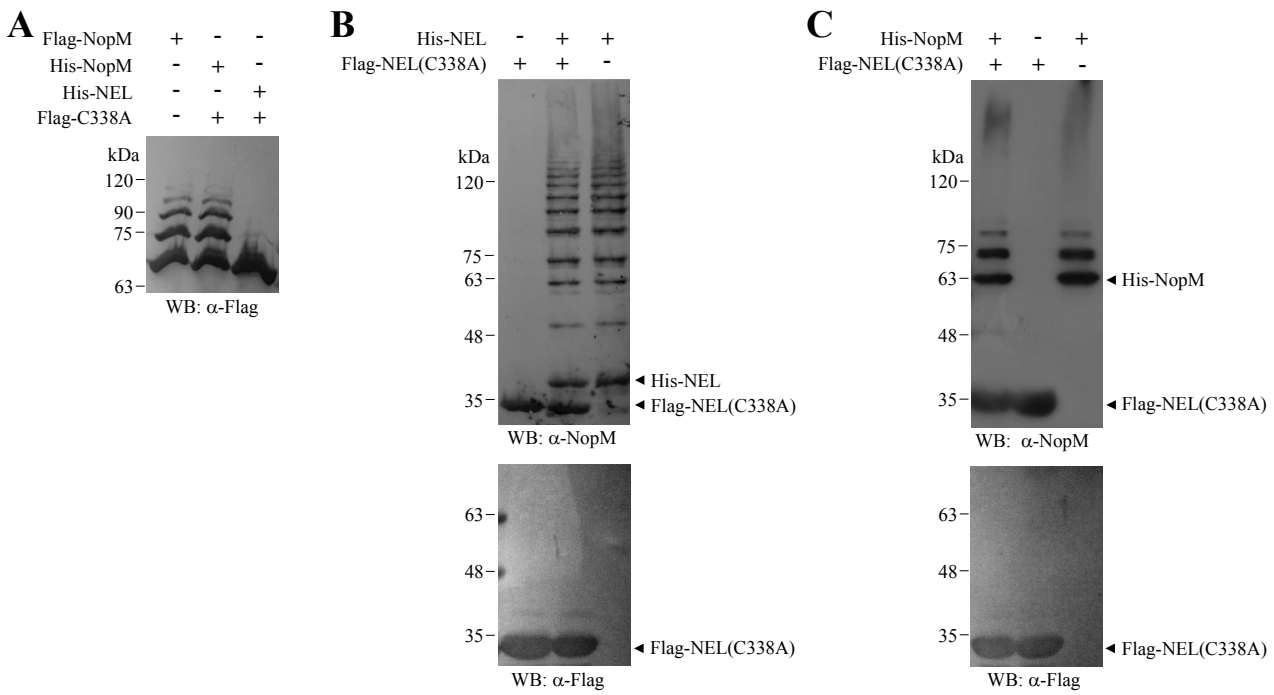
Supplemental Figure S1. Amino acid sequence of NopM (nodulation outer protein M of *Sinorhizobium* sp. NGR234; accession number: NP_443862). S26 was identified as potential phosphorylation site in this study. The C338 residue is required for catalytic activity (Xin et al. 2012, PLoS Pathog. 8(5): e1002707). D340 and D404 are predicted to function as catalytic acid and catalytic base, respectively (Keszei and Sicheri, 2017, Proc. Natl. Acad. Sci. USA 114: 1311-1316). The lysine residues K502, K531 and K537 are predicted autoubiquitination sites.



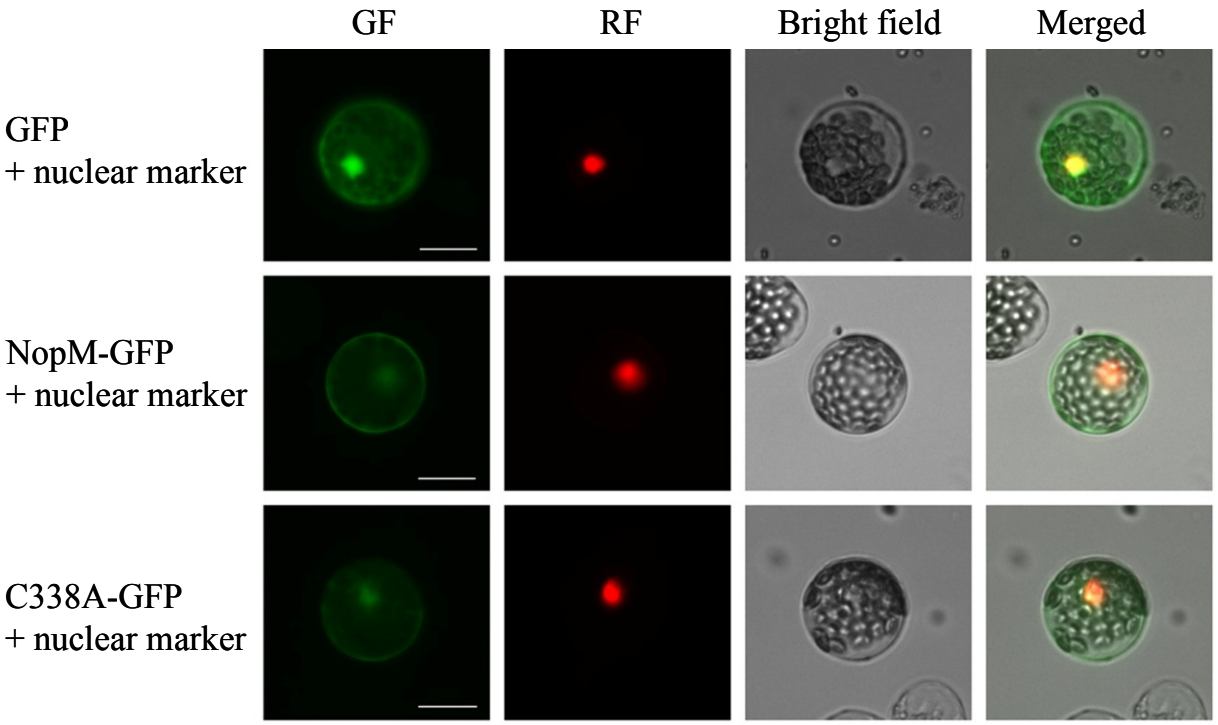
Supplemental Figure S2. Schematic representation of NopM variants used in this study. The LRR domain contains several leucine-rich repeats and the C-terminal NEL (novel E3 ubiquitin ligase domain) domain is necessary for catalytic activity. The C338A variant lacks enzyme activity and the C338S variant likely forms a mono-ubiquitinated conjugate. The S26A variant was used in the phosphorylation experiments. The K3xR variant without lysine residues and the enzymatically inactive form K3xR-C338A were used in autoubiquitination tests. The NEL protein (residues 255-546 with an N-terminal methionine) showed strong enzymatic activity while the corresponding full-length protein NEL-C338A was inactive. Proteins with corresponding tags were expressed in *Escherichia coli.*



Supplemental Figure S3. Effects of USP5 on autoubiquitination of NopM. The USP5 enzyme (0.5 μg) was added to the ubiquitination reaction system containing E1, E2, His-tagged NopM and either ubiquitin (Ub) or the K48R ubiquitin variant. Reactions were performed at 37 ^{o}C for the indicated time. Autoubiquitination of His-NopM was visualized by Western blot analysis with anti-NopM antibodies.



Supplemental Figure S4. Analysis of intermolecular transfer of ubiquitin in autoubiquitination reactions with NEL or NEL-C338A proteins. The ubiquitination reactions with E1, E2 and indicated proteins were performed at 37 ^{o}C for 1.5 h. (A) Reactions with His-NEL and Flag-C338A resulted in no (or very low) intermolecular transfer of ubiquitin. Autoubiquitination reactions with Flag-NopM alone or His-NopM with Flag-C338A were performed for comparison. Flag-C338A was detected on a Western blot with an anti-Flag antibody. (B) Incubation of His-NEL with Flag-NEL(C338A) resulted in no (or very low) intermolecular transfer of ubiquitin. Flag-NEL(C338A) or His-NEL alone were used in control reactions. Reaction products were analyzed on Western blots with anti-NopM or anti-Flag antibodies. (C) Incubation of His-NopM with Flag-NEL(C338A) also resulted in no (or very low) intermolecular transfer of ubiquitin. Control reactions were performed with His-NopM or Flag-NEL(C338A) alone. Western blots were performed with anti-NopM or anti-Flag antibodies.



Supplemental Figure S5. Subcellular localization of NopM in *Arabidopsis* protoplasts. NopM, C338A fused with a C-terminal GFP tag, were transiently expressed in *Arabidopsis* protoplasts. GFP was expressed alone as a control. Co-expressed ARF4-RFP served as a nuclear marker. Protoplasts were microscopically analyzed for green fluorescence (GF), red fluorescence (RF) and under bright field conditions. Bars: 20 μm.

Supplemental Table S1. Strains and plasmids used in this work.

Strains or Plasmids	Description*	Reference or Source
Escherichia coli DH5α	*supE*44 Δ*lac*U169 (Φ80 *lac*ZΔM15) *hsdR*17 *recA*1 *endA1 gyrA*96 *thi-1*, *relA1*	Invitrogen, Carlsbad, CA, USA
Escherichia coli BL21 (DE3)	F^- *ompT hsd*SB (r_B^- m_B^-) *gal dcm* (DE3)	Novagen (Merck Chemicals, Darmstadt, Germany)
Agrobacterium tumefaciens EHA105	A hypervirulent *Agrobacterium tumefaciens* strain (Rif^r)	Hood *et al*., 1993
Agrobacterium rhizogenes LBA9402	Harboring the root-inducing (Ri) plasmid pRil855 (Rif^r)	Hooykaas *et al*., 1977
GFP-expressing *Mesorhizobium loti* MAFF303099	*Mesorhizobium loti* MAFF303099 derivative constitutively expressing GFP (Rif^r)	Kindly provided by Zhong-Ming Zhang, Huazhong Agricultural University, Wuhan, China
pET28a	Expression vector for production of His tagged proteins (Kan^r)	Novagen (Darmstadt, Germany)
pET28a-*nopM*	A pET28a derivative carrying an *EcoR* I-*BamH* I fragment containing the *nopM* coding region amplified from genomic DNA of NGR234 with primers 1 and 2 (Kan^r)	This study

pET28a-NEL	A pET28a derivative carrying an *EcoR* I-*BamH* I fragment encoding the NEL domain amplified from genomic DNA of NGR234 with primers 2 and 45 (Kan^r)	This study
pRT104	Vector containing the cauliflower mosaic virus 35S promoter and a poly-(A) signal (Amp^r)	Töpfer *et al*., 1987
pRT104-*nopM*	A pRT104 derivative carrying an *EcoR* I-*BamH* I fragment containing the *nopM* coding region amplified from genomic DNA of NGR234 with primers 3 and 4 (Amp^r)	This study
pRT104-*nopM*(C338A)	PCR-based site directed mutagenesis of pRT104-*nopM*; amplification with primers 9 and 10 to substitute Cys338 with alanine (C338A) (Amp^r)	This study
pRT104-*nopM*(C338S)	PCR-based site directed mutagenesis of pRT104-*nopM*; amplification with primers 5 and 6 to substitute Cys338 with serine (C338S) (Amp^r)	This study
pRT104-*nopM*(D340N)	PCR-based site directed mutagenesis of pRT104-*nopM*; amplification with primers 7 and 8 to substitute Asp340 with Asn (D340N) (Amp^r)	This study
pRT104-*nopM*(S26A)	PCR-based site directed mutagenesis of pRT104-*nopM*; amplification with primers 46 and 47 to substitute Ser26 with alanine (S26A) (Amp^r)	This study

pRT104-*nopM*(C338A-K502R)	PCR-based site directed mutagenesis of pRT104-*nopM*(C338A); amplification with primers 11 and 12 to substitute Lys502 with arginine (K502R) (Amp^r)	This study
pRT104-*nopM*(C338A-K502&531R)	PCR-based site directed mutagenesis of pRT104-*nopM* (C338A-K502R); amplification with primers 13 and 14 to substitute Lys531 with arginine (K531R) (Amp^r)	This study
pRT104-*nopM*(C338A-K3xR)	PCR-based site directed mutagenesis of pRT104-*nopM* (C338A-K502&531R); amplification with primers 15 and 16 to substitute Lys537 with arginine (K537R) (Amp^r)	This study
pET28a-*nopM*(C338A)	A pET28a derivative carrying an *EcoR* I-*BamH* I fragment containing *nopM*(C338A) coding region amplified from pRT104-*nopM*(C338A) with primers 1 and 2 (Kan^r)	This study
pCAMBIA 1302	Binary vector with a cauliflower mosaic virus (CaMV) 35S promoter and a coding sequence of GFP, (Kan^r)	Cambia, Canberra, Australia
pCAMBIA 1302-*nopM*	A pCAMBIA 1302 derivative carrying a 35S CaMV-*nopM* fragment released from pRT104-*nopM* with *Hin*d III (Kan^r)	This study
pCAMBIA 1302-*nopM*(C338A)	A pCAMBIA 1302 derivative carrying a 35S CaMV-*nopM*(C338A) fragment released from pRT104-*nopM*(C338A) with *Hin*d III (Kan^r)	This study

pCAMBIA 1302-*nopM*(D340N)	A pCAMBIA 1302 derivative carrying a 35S CaMV-*nopM*(D340N) fragment released from pRT104-*nopM*(D340N) with *Hin*d III (Kan^r)	This study
pCAMBIA1302-*nopM*(S26D)	A pCAMBIA 1302 derivative carrying a 35S CaMV-*nopM*(S26D) fragment released from pRT104-*nopM*(S26D) with *Hind* III (Kan^r)	This study
pCAMBIA1302-*nopM*(S26A)	A pCAMBIA 1302 derivative carrying a 35S CaMV–*nopM*(S26A) fragment released from pRT104-*nopM*(S26A) with *Hin*d III (Kan^r)	This study
pCAMBIA 1302-*nopM-gfp*	A pCAMBIA 1302 derivative carrying the *nopM* coding region amplified from pRT104-*nopM* with primers 41 and 42; amplicon inserted with *Spe* I (Kan^r)	This study
pX-DR	Transient expression vector with a coding sequence of DsRed under the control of a CaMV 35S promoter; contains the suicide gene marker *ccdB* inserted between two *Xcm* I restriction sites (Amp^r)	Kindly provided by Guo-Liang Wang (Hunan Agricultural University, Changsha, China) (Chen *et al.,* 2009)
pX-DR-*nopM*	A pX-DR derivative carrying a *nopM* fragment amplified with primers 43 and 44 from genomic DNA of NGR234 and inserted with *Xcm* I (Amp^r)	This study

pSAT1-nEYFP-N1	A bi-molecular fluorescence complementation vector with a multiple cloning site followed by the N-terminal coding region of enhanced YFP; expression under the control of a CaMV 35S promoter (Amp^r)	Kindly provided by Nan Yao, Sun Yat-Sen University, Guangzhou, China (Citovsky *et al*., 2006)
pSAT1-cEYFP-N1	A bi-molecular fluorescence complementation vector with a multiple cloning site followed by the C-terminal coding region of enhanced YFP; expression under the control of a CaMV 35S promoter (Amp^r)	Kindly provided by Nan Yao, Sun Yat-Sen University, Guangzhou, China (Citovsky *et al*., 2006)
pSAT1-nEYFP-N1-*nopM*	A pSAT1-nEYFP-N1 derivative carrying an *Eco*R I-*Bam*H I fragment containing the *nopM* coding region amplified from pRT104-*nopM* with primers 39 and 40 (Amp^r)	This study
pSAT1-cEYFP-N1-*nopM*	A pSAT1-cEYFP-N1 derivative carrying an *Eco*R I-*Bam*H I fragment containing the *nopM* coding region amplified from pRT104-*nopM* with primers 39 and 40 (Amp^r)	This study
pET28a-*nopM*(C338A-K3xR)	A pET28a derivative carrying an *Eco*R I-*Bam*H I fragment containing the *nopM*(C338A-K3xR) sequence amplified from pRT104-*nopM*(C338A-K3xR) with primers 1 and 2 (Kan^r)	This study
pET28a-*nopM*(K3xR)	A pET28a-*nopM*(C338A-K3xR) derivative produced by site directed mutagenesis with primers 52 and 53 to substitute Ala338 with Cys (Kan^r)	This study

pET28a-E1(*AtUBA2*)	A pET28a derivative carrying an *EcoR* I-*Not* I fragment containing the *UBA2* (At5g06460) coding region amplified from cDNA of *A. thaliana* ecotype C24 with primers 19 and 20 (Kan^r)	This study
pET28a-E2(*AtUBC8*)	A pET28a derivative carrying an *EcoR* I-*Hind* III fragment containing the *Ubc8* (At5g53300) coding region amplified from cDNA of *A. thaliana* ecotype C24 with primers 21 and 22 (Kan^r)	This study
pET28a-Ub	A pET28a derivative carrying an *EcoR* I-*BamH* I fragment containing part of the *UBQ14* (At4g02890) coding region amplified from cDNA of *A. thaliana* ecotype C24 with primers 23 and 24 (Kan^r)	This study
pET28a-Ub(K48R)	A pET28a-*Ub* derivative produced by site directed mutagenesis with primers 35 and 36 to substitute Lys48 with Arg (K48R) (Kan^r)	This study
pET28a-Ub(K11R)	A pET28a-*Ub* derivative produced by site directed mutagenesis with primers 27 and 28 to substitute Lys11 with Arg (K11R) (Kan^r)	This study
pET28a-Ub(K6R)	A pET28a-*Ub* derivative produced by site directed mutagenesis with primers 25 and 26 to substitute Lys6 with	This study

	Arg (K6R) (Kan^r)	
pET28a-Ub(K33R)	A pET28a-*Ub* derivative produced by site directed mutagenesis with primers 33 and 34 to substitute Lys33 with Arg (K33R) (Kan^r)	This study
pGEX-4t-1	Prokaryotic expression vector for production of GST fusion proteins (Kan^r)	Amersham Biosciences/GE Healthcare, Buckinghamshire,UK
pGEX-4t-1-*nopM*	A pGEX-4t-1 derivative carrying an *Eco*R I-*Bam*H I fragment containing the *nopM* coding region amplified from genomic DNA of NGR234 with primers 1 and 2 (Amp^r)	This study
pGEX-NtSIPK	pGEX-4T-1 derivative carrying a BamHI-EcoRI fragment containing the coding region of *SIPK* of *Nicotiana tabacum* (Amp^r)	Ge *et al.,* 2016
pGEX-NtMEK2^{DD}	pGEX-4T-1 derivative carrying a SmaI-NotI fragment containing the coding region of *MEK2^{DD}* of *Nicotiana tabacum* (Amp^r)	Ge *et al.,* 2016
pGEX-LjSIP2	pGEX-4T-1 derivative containing the coding region of *SIP2* of *Lotus japonicus* (Amp^r)	Chen *et al.,* 2012

pET28a-*flag-nopM*	A pET28a derivative carrying an *Eco*R I-*Bam*H I fragment containing the *nopM* coding region amplified from genomic DNA of NGR234 with primers 2 and 50 (Kan^r)	This study
pET28a-*flag-nopM*(C338A)	A pET28a derivative carrying an *Eco*R I-*Bam*H I fragment containing the *nopM* coding region amplified from pRT104-*nopM*(C338A) with primers 2 and 50 (Kan^r)	This study
pET28a-Flag-*nopM*(C338S)	A pET28a derivative carrying an *Eco*R I-*Bam*H I fragment containing the *nopM* coding region amplified from pRT104-*nopM*(C338S) with primers 2 and 50 (Kan^r)	This study
pISV2678	Binary vector with a double cauliflower mosaic virus (CaMV) 35S promoter	Kindly provided by Eva Kondorosi, Gif-sur Yvette, France
pISV(RFP)	A pISV2678 derivative containing a 35S CaMV-*RFP*-poly A cassette (Kan^r)	Constructed by Feng Yang (Yang, 2014)
pISV(RFP)-*nopM*	A pISV(RFP) derivative containing a 35S CaMV–*nopM*-poly A cassette (Kan^r); a *Cla* I-*Eco*R I fragment containing the coding region of *nopM* was inserted into pISV(RFP)	Constructed by Feng Yang (Yang, 2014)
pET28a-Flag-NEL(C338A)	A pET28a derivative carrying an *Eco*R I-*Bam*H I fragment containing the *NEL*(C338A) coding region amplified from	This study

	pET28a-*nopM*(C338A) with primers 2 and 51 (Kan^r)	
pISV(RFP)-*nopM*(C338A)	A pISV(RFP) derivative containing a 35S CaMV–*nopM*(C338A)-poly A cassette (Kan^r); a *Cla* I-*Eco*R I fragment containing the coding region of *nopM*(C338A) was inserted into pISV(RFP)	This study

*Amp^r, Kan^r, Rif^r resistance to ampicillin, kanamycin and rifampin, respectively.

References cited in Table S1:

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Supplemental Table S2. Primers used in this work.

No.	Sequence (5' to 3')	Restriction site	Description
1	cgggatcc atgaatgtacaacggcccgg	*Bam*H I	For construction of pET28a-*nopM.*
2	cggaattc tcacagctcaagaccgcgacc	*Eco*R I	
3	ccg gaattc atgaatgtac aacggcccgg	*Eco*R I	For construction of pRT104-*nopM*.
4	cgc ggatcc tcacagctca agaccgcgac	*Bam*H I	
5	ggagctagcgatagctcagaggatcgcatt act		Site-directed mutagenesis; for construction of
6	agtaatgcgatcctctgagctatcgctagc tcc		pRT104-*nopM*(C338S).
7	ctagcgatagctgtgagaatcgcattactttgacc		Site-directed mutagenesis; for construction of
8	ggtcaaagtaatgcgattctcacagctatcgctag		pRT104-*nopM*(D340N).
9	ggagctagcgatagcgctgaggatcgcattact		Site-directed mutagenesis; for construction of
10	agtaatgcgatcctcagcgctatcgctagctcc		pRT104-*nopM*(C338A).
11	ccgcttggacgaacggctcgccgagcacgg		Site-directed mutagenesis; for construction of
12	ccgtgctcggcgagccgttcgtccaagcgg		pRT104-*nopM*(C338A-K502R).
13	cgcccgcaggatccgaggcgagacaatggag		Site-directed mutagenesis; for construction of

14	ctccattgtctcgcctcggatcctgcgggcg		pRT104-*nopM*(C338A-K502R&K531R).
15 16	cgagacaatggagcgggtgcttcggggtcg cgaccccgaagcacccgctccattgtctcg		Site-directed mutagenesis; for construction of pRT104-*nopM*(C338A-K3xR).
17 18	cggaattcatgaatgtacaacggcccgg cgaagctttcacagctcaagaccgcgacc	*Eco*R I *Hin*d III	For construction of pET28a-*nopM*.
19 20	cggaattc atggaaccattcgttgttaagg ataagaatgcggccgc tcaggcgaagtagactgatacg	*Eco*R I *Not* I	For construction of pET28a- E1(*AtUBA2*).
21 22	cggaattc atggcgtcgaagcggatcttg cccaagctt ttagcccatggcatacttctg	*Eco*R I *Hin*d III	For construction of pET28a-E2(*AtUBC10*).
23 24	cgggatcc atgcagatct ttgttaagac cggaattc tcaaccaccacggagcctga	*Bam*H I *Eco*R I	For construction of pET28a-Ub(*AtUBQ14*).
25 26	gcagatctttgttaggactctcaccgg ccggtgagagtcctaacaaagatctgc		Site-directed mutagenesis; for construction of pET28a-Ub(K6R).
27 28	gactctcaccgga aggactatcaccctc gagggtgatagtccttccggtgagagtc		Site-directed mutagenesis; for construction of pET28a-Ub(K11R).
29 30	catcgacaacgttagggccaagatccagg cctggatcttggccctaacgttgtcgatg		Site-directed mutagenesis; for construction of pET28a-Ub(K27R).

31 32	caacgttaaggccaggatccaggataagg ccttatcctggatcctggccttaacgttg		Site-directed mutagenesis; for construction of pET28a-Ub(K29R).
33 34	caagatccaggatagggaaggcattcctc gaggaatgccttccctatcctggatcttg		Site-directed mutagenesis; for construction of pET28a-Ub(K33R).
35 36	gatcttcgctgg gcggcagttggaggatg catcctccaactgccgcccagcgaagatc		Site-directed mutagenesis; for construction of pET28a-Ub(K48R).
37 38	ctacaacatccagagggagtccacacttc gaagtgtggactccctctggatgttgtag		Site-directed mutagenesis; for construction of pET28a-*Ub*(K63R).
39 40	cggaattc atgaatgtacaacggcccgg cgggatccc cagctcaagaccgcgaccc	*Eco*R I *Bam*H I	For construction of pSAT1-nEYFP-N1-*nopM*.
41 42	ggactagt atgaatgtacaacggcccgg ggactagt cagctcaagaccgcgaccc	*Spe* I *Spe* I	For construction of pCAMBIA1302–*nopM-gfp*.
43 44	aatgaatgtacaacggcccgg tcacagctcaagaccgcgaccc		For construction of pX-DR -*nopM*
45	cgggatcc cagga ccgca gatct ttttg	*Bam*H I	Upper primer used for amplification of the NEL domain sequence of *nopM*; for construction of pET28a-NEL.
46	agttcagaac caggggctcc ggccgccgcc		Site-directed mutagenesis; for construction of

47	ggcggcggccg gagcccctggt tctgaact		pRT104-*nopM*(S26A).
48 49	agttcagaac caggggatcc ggccgccgcc ggcggcggc cggatcccctg gttctgaact		Site-directed mutagenesis; for construction of pRT104-*nopM*(S26D).
50	cgggatcc gattacaaggatgacgacgataag atgaatgtacaacggcccgg	*Bam*H I	Upper primer; for construction of pET28a-*flag -nopM*.
51	cgggatcc gattacaaggatgacgacgataag cagga ccgca gatct ttttg	*Bam*H I	Upper primer; for construction of pET28a-*flag -NEL*.
52 53	gctagcgata gctgtgaggt cgcattac gtaatgcgatcctcacagctatcgctagc		Site-directed mutagenesis; for construction of pRT104-*nopM*(K3xR).