The Nep1-like protein family of *Magnaporthe oryzae* is dispensable for the infection of rice plants

Ya-Li Fang[1], You-Liang Peng[1,2] and Jun Fan[1*]

[1] Ministry of Agriculture Key Laboratory of Plant Pathology, China Agricultural University, Beijing 100193, China

[2]State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100193, China

[*] corresponding: jfan@cau.edu.cn

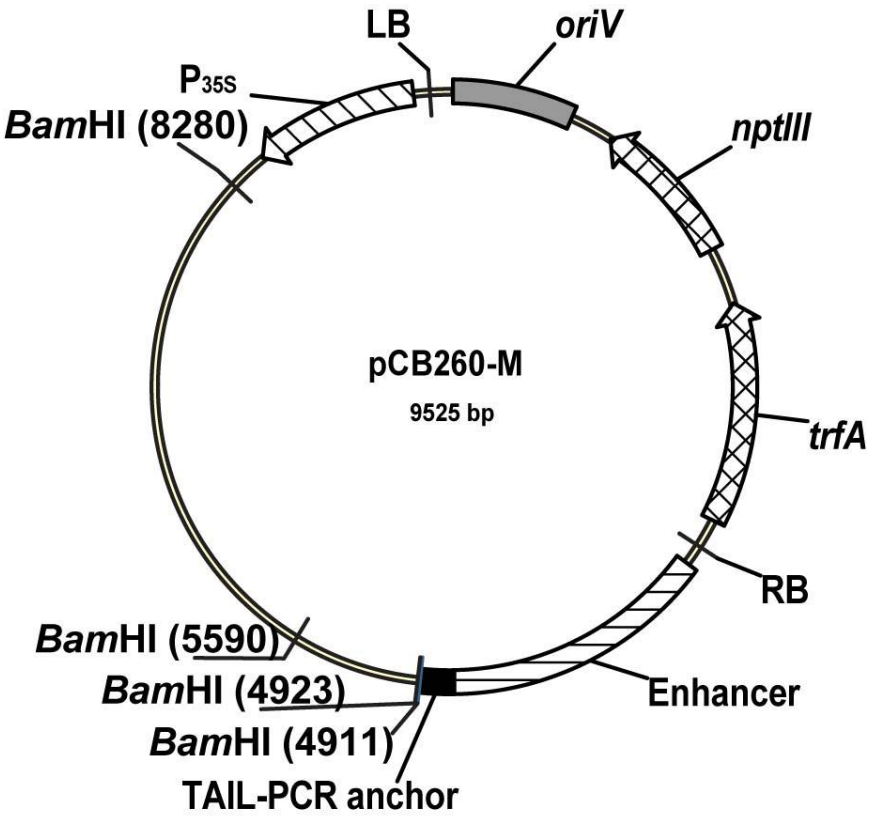

Fig. S1. Schematic diagram of pCB260-M. P_{35S}, 35S promoter of cauliflower mosaic virus; LB and RB, left and right border of T-DNA; *oriV*, replication origin from RK2; *nptIII*, neomycin phosphotransferase for kanamycin resistance; *trfA*, the replication protein gene of RK2; Enhancer, 4×35S enhancer repeats.

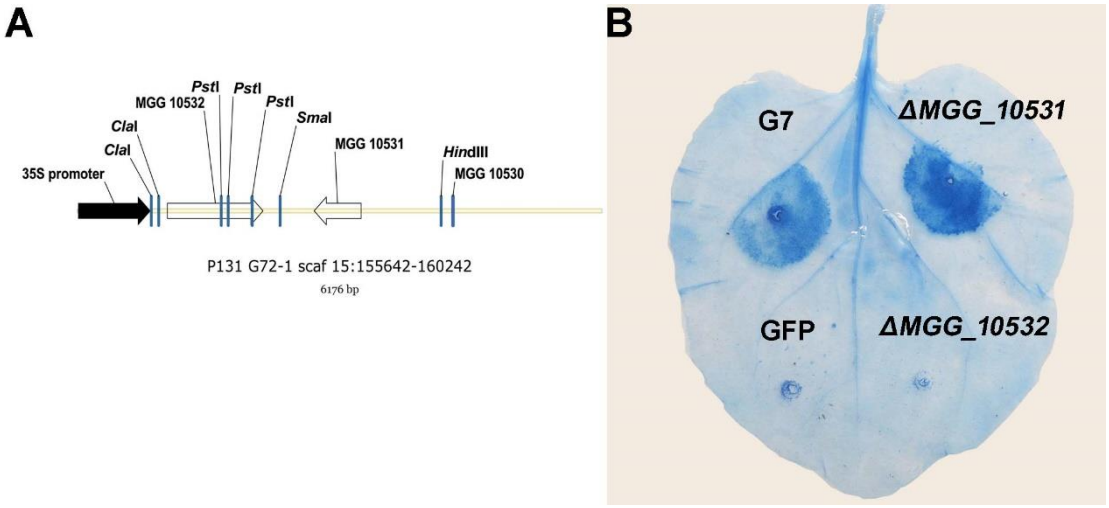

Fig. S2. *MGG_10532* of G7 clone triggers cell death by *Agro*-mediated transient expression in *Nicotiana benthamiana*. (A) Diagram of the genomic region from *Magnaporthe oryzae* in G7 clone. (B) Cell death-inducing activity of G7 clone is abolished in Δ*MGG_10532* but not in Δ*MGG_10531*clone. Leaves were stained with trypan blue at 72 hours post inoculation, and destained subsequently before the photograph was taken.

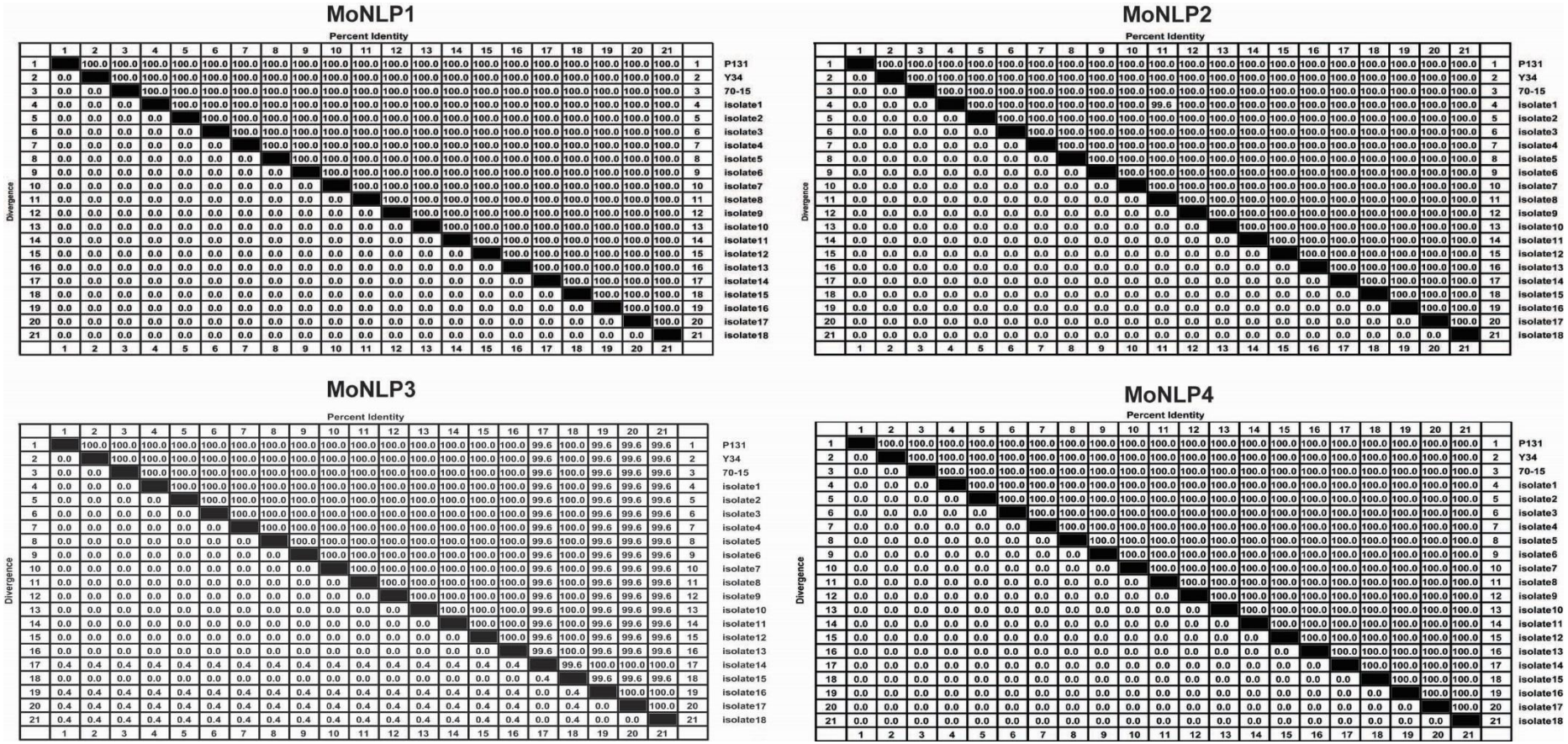

Fig. S3. The four necrosis- and ethylene-inducing protein (Nep1)-like proteins (NLP) from *M. oryzae* are conserved among lab strains and field isolates. The amino acid sequences of twenty-one isolates collected from Heilongjiang, Hunan, Liaoning, Guangzhou and Hubei province were analyzed using Cluster W method. The percentage of amino acid sequence identity and divergence was analyzed using DNA Star software.

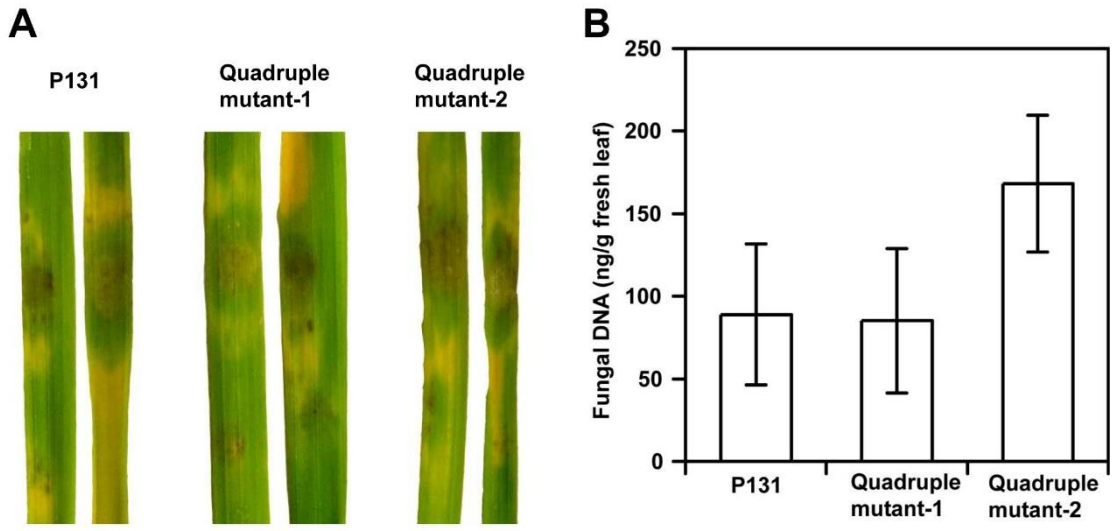

Fig. S4. The *MoNLP* family is dispensable for virulence of *M. oryzae* on rice plants. (A) Disease symptoms caused by wild type and two mutant fungal strains are similar on rice leaves. Susceptible rice seedlings (cv. LTH) were inoculated with 5 µl conidial suspensions (2.5×10^5 conidia ml^{-1}) and incubated in moist plates at room temperature. Leaves were photographed at 3 days post inoculation. (B) The fungal growth of two independent quadruple mutants is similar to that of wild type during infection. Samples were collected and weighted at 72 hpi, and DNA was extracted for qPCR assay of the fungal *Actin*. The fungal growth was quantitatively determined by the amount of fungal DNA in fresh leaves. Data shown in (B) are means±sd (n=3). No significant difference was detected by statistical analysis (Student's t test, $P<0.05$)

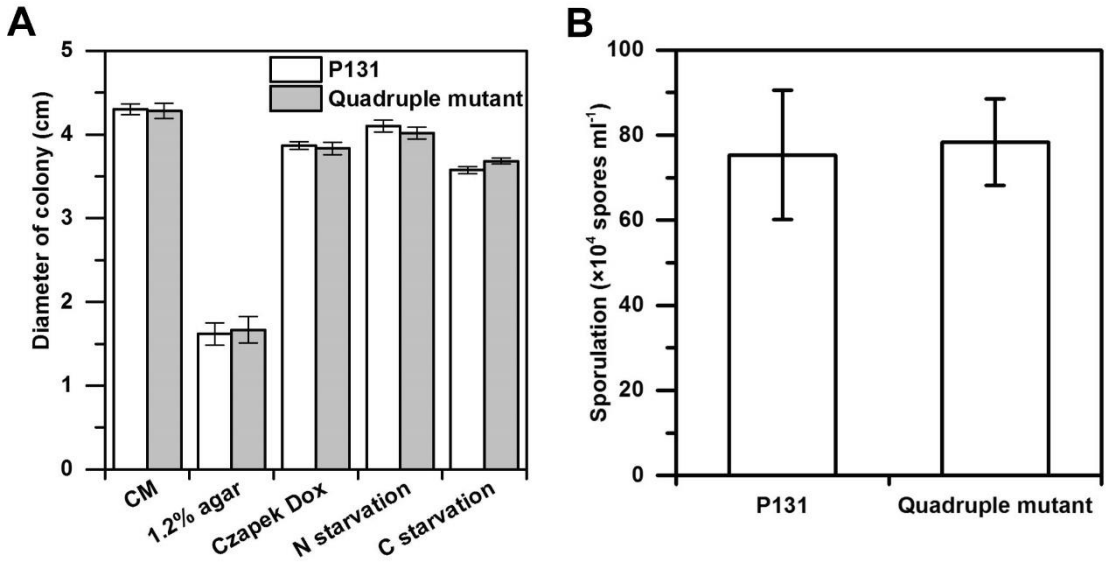

Fig. S5. Deletion of the *MoNLP* family does not affect the growth and sporulation of *M. oryzae*. (A) The colony diameters of wild type and quadruple mutant were measured after 6 days of incubation on CM medium, 1.2% agar, Czapek-Dox medium, and N or C starvation medium at 28°C. (B) A comparison of levels of conidia production by P131 and quadruple mutant. Data shown in (A) and (B) are means±sd (n=3). No significant difference was detected by statistical analysis (Student's t test, $P<0.05$)

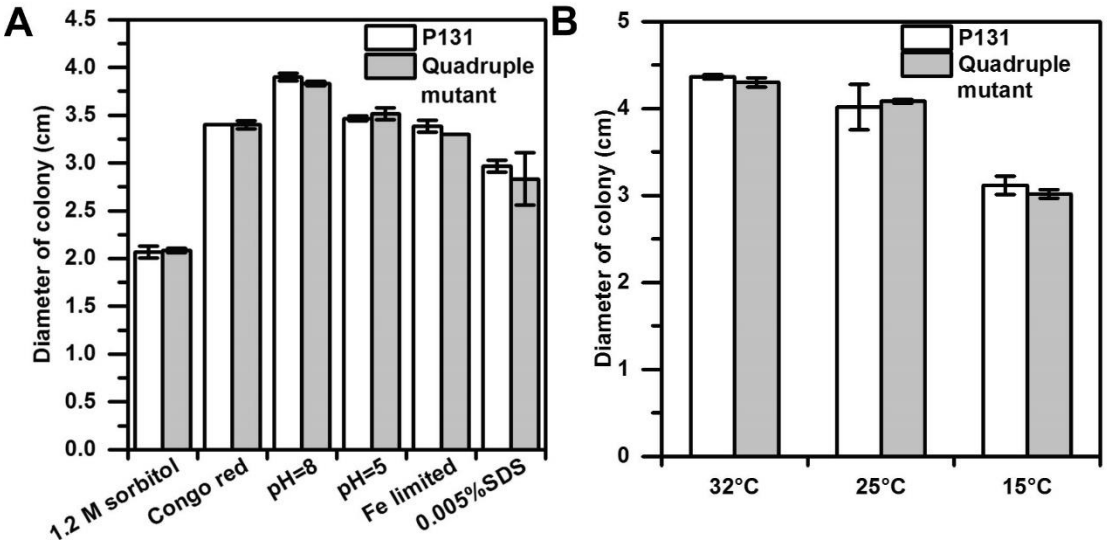

Fig. S6. Deletion of the *MoNLP* family does not affect the radial growth of *M. oryzae* under various adverse conditions. (A) The assays were performed by placing 5 μl conidial suspension (1×10^4 conidian ml^{-1}) on the center of CM plates supplemented with 1.2 M sorbitol, SDS (0.005%) or congo red (200 μg/ml), 1/10 CM supplemented with DTPA (8 μM), and rice medium with pH 8.0 and 5.0. The inoculated plates were incubated at 28°C for 6 days before measuring the colony diameter. (B) The comparison of radial growth of wild type and mutant strains on PDA plates. The colony diameter was measured 7 days except for 12 days at 15°C after inoculation. Data shown in A and B are means±sd (n=3). No significant difference is detected between P131 and mutant strain (Student's t test, $P<0.05$).

Table S1 The candidate elicitor genes were identified by screening the genomic library of *M. oryzae*

Candidate clones	Genes	Proteins	No. of family member	Signal peptide [a]	Phenotype
G7	MGG_10532	NEP1-like	4	Yes	Cell death
AD8	MGG_04098	Hypothetical protein1	1	No	Cell death
G4L12	MGG_14010	Hypothetical protein 2	1	No	Chlorosis
F6-H8	MGG_09499	Ras-2	2[b]	No	Chlorosis

[a]Signal peptide was predicted by SignalP 4.0.

[b]The identity of amino acid sequence is over 50%.

Table S2 Number of NLP genes from diverse microbial species.

Species	Number of *NLP* genes
Mycosphaerella graminicola[1]	1
Erwinia carotovora subsp. carotovora,[2]	1
Moniliophthora perniciosa[3]	2
Botrytis elliptica[4]	2
B. cinerea[5]	2
Sclerotinia sclerotiorum[6]	2
Magnaporthe oryzae[7]	4
Verticillium dahliae[8]	8-9
Hyaloperonospora arabidopsidis[9]	12
Phytophthora sojae[10]	33
P. capsici[11]	60*

⋆ Potential NLPs based on genome sequencing

Table S3 Primers used for deletion of *MoNLP* family, construction of plasmid, and qRT-PCR assays

Primer name	Primer Sequences (5'-3')	Remark
Mo10532 up For	**AAAAAGCAGGCT**CGAACATGATTAACAATTCCGGAG	MoNLP4 knock out vector construction
Mo10532 up Rev	ACATAACCACCCACTACATTGTGAATTTTGTGATGGGT	
Mo10532 down For	CACAAAATTCACAATGTAGTGGGTGGTTATGTGGTT	
Mo10532 down Rev	**AGAAAGCTGGGT**TACCTAGTTATTTCCCCTCC	
Mo10532 up left For	CAGGGTCTCCCATGCCAAGATT	Validation of MGG_10532 deletion
Mo10532 down right Rev	TCATCTTCCTCACTGCCGCCCT	
Mo08454 up For	**AAAAAGCAGGCT**CGATTGGCGATCTTTTGGATAC	MoNLP1 knock out vector construction
Mo08454 up Rev	AAGCCAACAAAGCTCACATCTTTTCTTTTTTCCTGAGGG	
Mo08454 down For	GAAAAAAGAAAAGATGTGAGCTTTGTTGGCTTTTTGG	
Mo08454 down Rev	**AGAAAGCTGGGT**AAAAGGACGGACTTGTTCATGCT	
Mo08454 up left For	CCTCCTTCTACATGCACCACTCCA	Validation of MGG_08454 deletion
Mo08454 down right Rev	CGCAGAGTGAATTCTGTGAGGACA	
Mo00401 up For-2	**AAAAAGCAGGCT**TGATCTTGTAACTGTGTGGT	MoNLP2 knock out vector construction
Mo00401 up Rev-2	AAAGACGGCGACGTGCCATGGTGTAGGATTAAAATG	
Mo00401 down For-2	TTAATCCTACACCATGGCACGTCGCCGTCTTTGTCG	
Mo00401 down Rev-2	**AGAAAGCTGGGT**CCCCTAAAACGACCCGAGATATTCG	
Mo00401 up left for-3	ACCCTTACAAATCGCATGTCTTGT	Validation of MGG_00401 deletion
Mo00401 down right Rev	GCCGTTTTATAGCCCGATATCC	
Mo02332 up For-2	AAAAAGCAGGCTAGAAATGGTTCTGACGGTGCATGT	MoNLP3 knock out vector construction
Mo02332 up Rev	CAGGACAAACCAATTACATGTTTCTCGGCTCGATCA	
Mo02332 down For	CGAGCCGAGAAACATGTAATTGGTTTGTCCTGTTGTT	
Mo02332 down Rev-2	AGAAAGCTGGGTCGTGAATGTGCTACGTCAATGAGA	
Mo02332 up left For-2	ATGGCATTGCCATGAGGAGTCTC	Validation of MGG_02332 deletion
Mo02332 down right Rev	TATACGCGTCAAACGGTCAATC	
attB1 adapter	GGGGACAAGTTTGTACAAAAAAGCAGGCT	For cloning genes by Gateway method
attB2 adapter	GGGGACCACTTTGTACAAGAAAGCTGGGT	
TrpC Rev	CTAGTGAATGCTCCGTAACACCC	For screening transformants
08454 rFor	CAGTCCGTGCCCAACAGCAA	MGG_08454 QRT forward
08454 rRev	ACGAGTGGCTGCCCGACGTTT	MGG_08454 QRT reverse
10532 rFor	CGGCAAGTACAAAAAGACGACCAGC	MGG_10532 QRT forward
10532 rRev	GTCCATGATGGGCTTGGTGTAGC	MGG_10532 QRT reverse
00401 rFor	GCTCAACAACATGTTCACGGGCA	MGG_00401 QRT forward
00401 rRev	GCAGCGCGACGTCCTTTTCA	MGG_00401 QRT reverse
02332 r2For	GGTCAACATCCGCCAGAAGC	MGG_02332 QRT forward
02332 r2Rev	TCGTCAAACGCACAGTCAAACT	MGG_02332 QRT reverse
MGActin F	GGTCTTGAGAGCGGTGGTATCCATG	*Actin* gene (MGG_03982) amplification for QRT
MGActin R	TGCCAGGGCAGTGATCTCCTTC	
MG40s ribosome For	ATGACCGAGATCATCCAGCGCG	*40S ribosomal protein S3aE* (MGG_06919) amplification for QRT
MG40s ribosome Rev	GCTTCAGCAGCTTAACCTTGCGG	

OPR11	**TAGAAGCTT**AGCTTGGGCTGCAGGTCGAG	PCR amplification for The modified inducible transcription unit
OPR12	**TAGAAGCTT**ATTCCTCGACTCGGTACCCC	
OPR13	TGCCTGCAGGATATCGTGGAT	PCR amplification for G10-90-XVE cassette.
OPR14	GCTTGTTTGGGATGTTTTACTCC	
OPR19	**AAAAAGCAGGCT**ATGCTTCCCAAGTTCTTCACTCTC	For MGG_08454 cloning
OPR20	**AGAAAGCTGGGT**AGCTCATTGGAAAACCTTGGCCAG	
OPR21	**AAAAAGCAGGCT**AACATGACTAGCAGCGTGTCGTCG	For MGG_02332 cloning
OPR22	**AGAAAGCTGGGT**CAATTAGCCTTGGCTCGTAGCAGG	
OPR17	**AAAAAGCAGGCT**ATTCACAATGAAGTCATTCG	For MGG_10532 cloning
OPR18	**AGAAAGCTGGGT**ATAAAACACATAACCACATAAC	
00401 F For	**AAAAAGCAGGCT**CCCGGATGCCTAAGCCT	For MGG_00401 cloning
OPR24	**AGAAAGCTGGGT**AAACCTACAACCTGCCACCGCTGC	
R Actin 1 For	TGGCATTGCTGACAGGATGAGC	Rice *Actin* gene (XM_015774830) for QRT
R Actin 1 Rev	CGTCGTACTCAGCCTTGGCAATC	
pTGD For	CATATGGGAGAGCTCCCAACGC	For construction of pMFKO DONR
pTGD Rev	ATATCAGTAGATGCCGACCGC	
phh-5 HmB For	ACAGAAGATGATATTGAAGGAGC	For construction of pMFKO DONR
HSV-tk Rev-2	AAGTTCCTTCCGGTATTGTCTC	

References

1 Motteram, J. *et al.* Molecular characterization and functional analysis of *MgNLP*, the sole NPP1 domain-containing protein, from the fungal wheat leaf pathogen *Mycosphaerella graminicola*. *Mol. Plant-Microbe Interact.* **22**, 790-799 (2009).

2 Mattinen, L., Tshuikina, M., Mäe, A. & Pirhonen, M. Identification and characterization of Nip, necrosis-inducing virulence protein of *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* **17**, 1366-1375 (2004).

3 Garcia, O. *et al.* Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*. *Mycol. Res.* **111**, 443-455 (2007).

4 Staats, M., van Baarlen, P., Schouten, A. & van Kan, J. A. Functional analysis of NLP genes from *Botrytis elliptica*. *Mol. Plant Pathol.* **8**, 209-214 (2007).

5 Arenas, Y. C. *et al.* Functional analysis and mode of action of phytotoxic Nep1-like proteins of *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* **74**, 376-386 (2010).

6 Dallal Bashi, Z., Hegedus, D. D., Buchwaldt, L., Rimmer, S. R. & Borhan, M. H. Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptides (NEPs). *Mol. Plant Pathol.* **11**, 43-53 (2010).

7 Mogga, V. *et al. Magnaporthe oryzae* effectors MoHEG13 and MoHEG16 interfere with host infection and MoHEG13 counteracts cell death caused by *Magnaporthe*-NLPs in tobacco. *Plant Cell Rep.*, 1-17 (2016).

8 Santhanam, P. *et al.* Evidence for functional diversification within a fungal NEP1-like protein family. *Mol. Plant-Microbe Interact.* **26**, 278-286 (2013).

9 Cabral, A. *et al.* Nontoxic Nep1-like proteins of the downy mildew pathogen *Hyaloperonospora arabidopsidis*: repression of necrosis-inducing activity by a surface-exposed region. *Mol. Plant-Microbe Interact.* **25**, 697-708 (2012).

10 Dong, S. *et al.* The NLP toxin family in *Phytophthora sojae* includes rapidly evolving groups that lack necrosis-inducing activity. *Mol. Plant-Microbe Interact.* **25**, 896-909 (2012).

11 Feng, B. *et al.* Characterization of necrosis-inducing NLP proteins in *Phytophthora capsici*. *BMC Plant Biol.* **14**, 126 (2014).