Evolution by gene duplication of *Medicago truncatula PISTILLATA*-like transcription factors. Roque E. *et al.*

SUPPLEMENTARY DATA

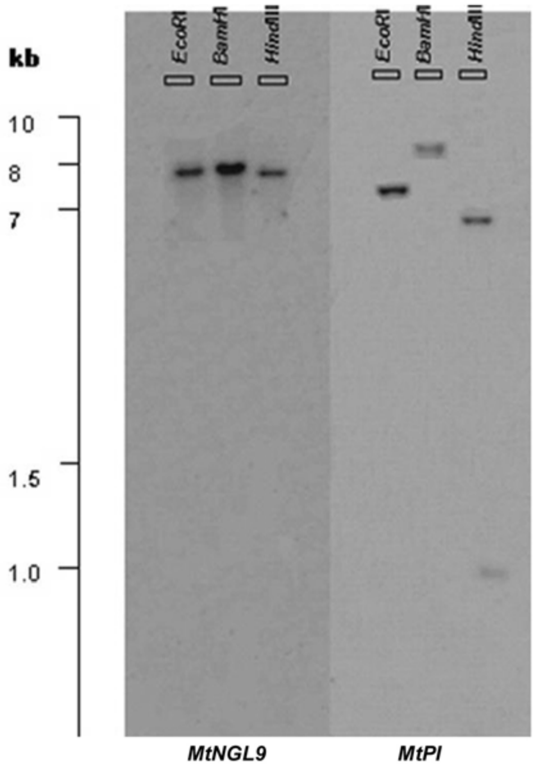

Fig. S1. Southern blot analysis of the *Medicago truncatula PI*-like genes. *MtPI* and *MtNGL9* are present in the *Medicago* genome as single copies.

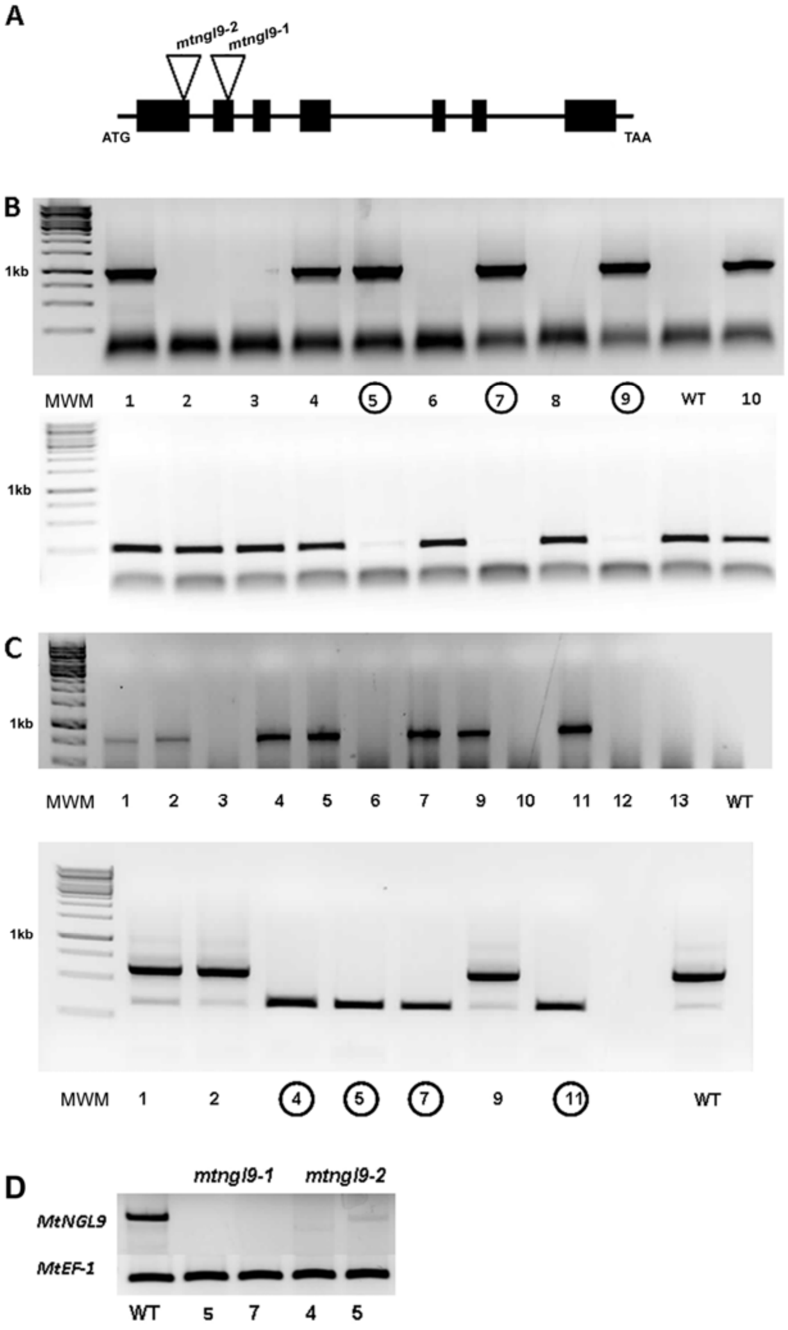

Fig. S2. Molecular characterization of the *Tnt1* insertions in the *MtNGL9* locus. A. Schematic diagram of the genomic structure of the *MtNGL9* gene. In NF14948 lines (*mtngl9-1*), *Tnt1* was inserted 360 bp downstream of the ATG codon, at the second exon. In NF12316 lines (*mtngl9-2*). *Tnt1* was inserted 183 bp downstream of the ATG codon, at the end of the first exon. **B.** Genotyping of *mtngl9-1* mutant using NGL9-F/Tnt1-R (upper) and NGL9-291/NGL9-612G (lower) primer pairs. Homozygous plants are indicated in circled numbers. **C.** Genotyping of *mtngl9-2* mutant using NGL9-F/Tnt1-R (upper) and NGL9-F/NGL9-590G (lower) primer pairs. Homozygous plants are indicated in circled numbers. **D.** RT-PCR results show no detectable *MtNGL9* expression in four homozygous mutant plants. MWM: molecular weight markers; WT: wild-type control.

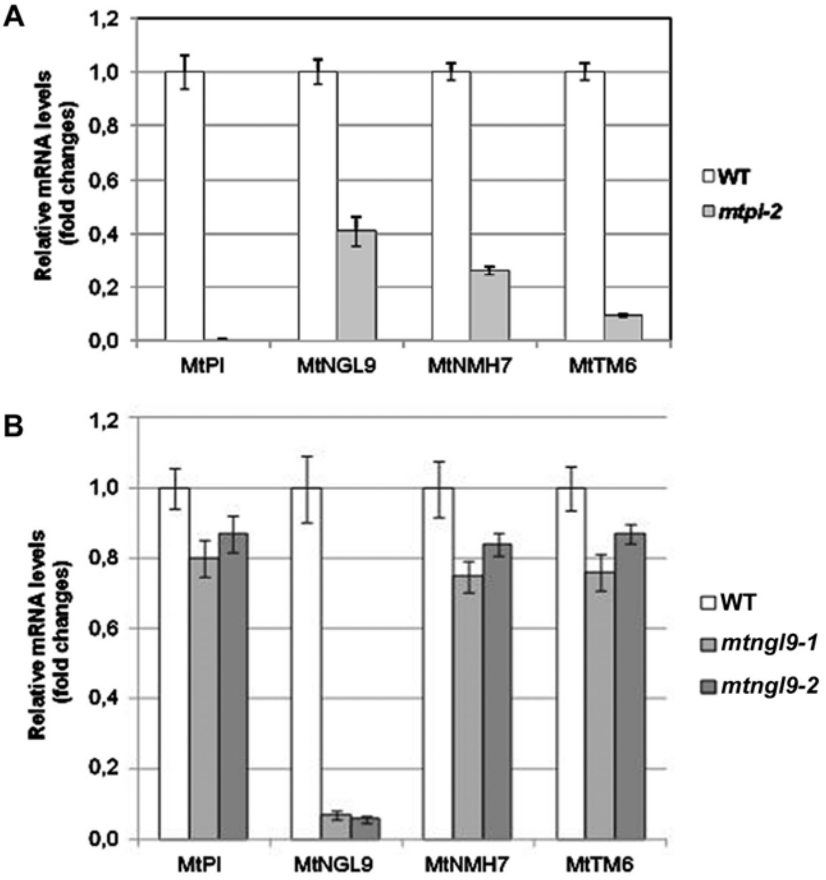

Fig. S3. qRT-PCR expression analyses of loss-of-function plants. A. mRNA levels of the B-function genes in floral buds of *mtpi-2.* **B.** mRNA levels of the B-function genes in floral buds of *mtngl9-1* and *mtngl9-2*. The height of the bars for a given gene indicates relative differences in expression levels in floral buds. The expression value of all B-function genes in WT was set to 1.00. Expression levels were plotted relative to this value.

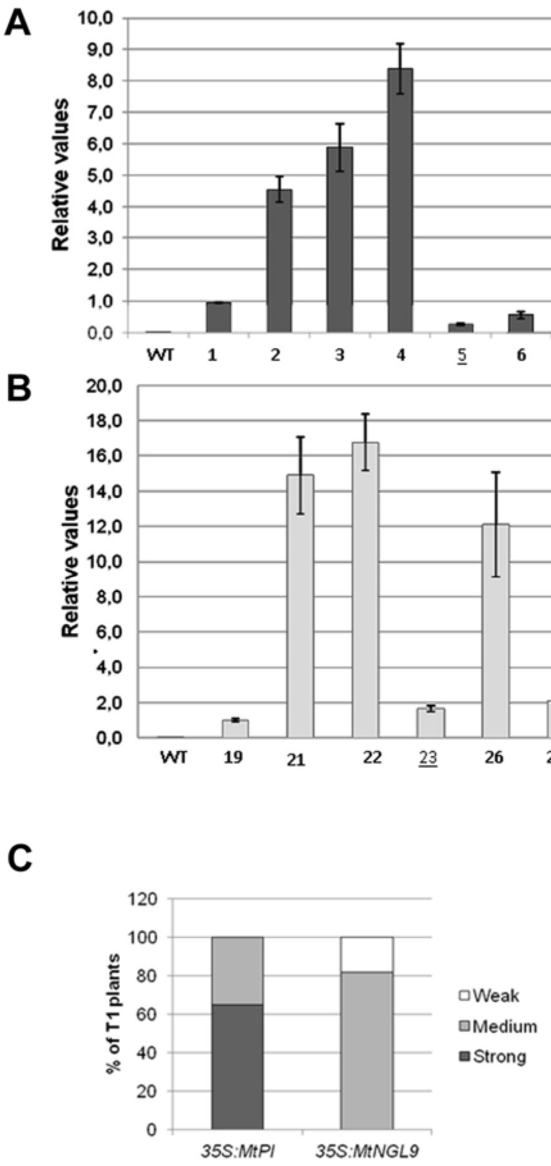

Fig. S4. Analyses of *35S:MtPI* and *35S:MtNGL9* plants. A. Transcript abundance of *MtPI* in 35S*::MtPI* plants. The expression value of *MtPI* in plant number 7 was set to 1.00. Expression levels were plotted relative to this value. Numbers indicate the transgenic line. Underlined number indicates plants classified as medium phenotype, and the remaining plants showed the strong phenotype. **B.** Transcript abundance of *MtNGL9* in 35S*::MtNGL9* plants. The expression value of *MtPI* in plant number 19 was set to 1.00. The number of the transgenic line is indicated. Underlined number indicates plants classified as weak phenotype, and the remaining plants showed the medium phenotype. **C.** Relative proportion of phenotypes observed in plants overexpressing *MtPI* (N^{o} of transgenic plants= 17) or *MtNGL9* (N^{o} of transgenic plants= 33).

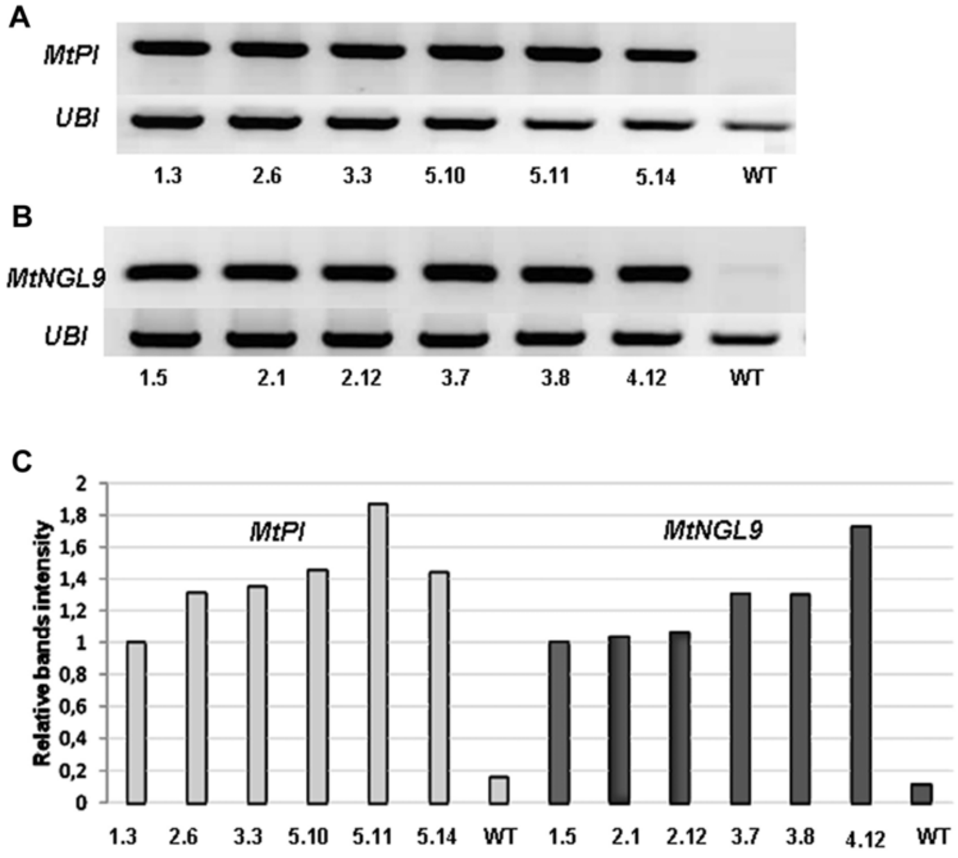

Fig. S5. RT-PCR expression analyses in *pi-1/pi-1*; *35S:MtPI* and *pi-1/pi-1*; *35S:MtNGL9* complementation lines. A. Expression of *MtPI* in *pi-1/pi-1*; *35S:MtPI* lines. **B.** Expression of *MtNGL9* in *pi-1/pi-1*; *35S:MtNGL9 lines.* **C.** Comparative expression level of *MtPI* and *MtNGL9* in their respective complementation lines. Levels were quantified by measuring the intensity of individual bands using ImageJ densitometry software, and expressed relative to UBIQUITIN signal, as a measure of transcript relative abundance in the different samples. The numbers of the transgenic lines are indicated.

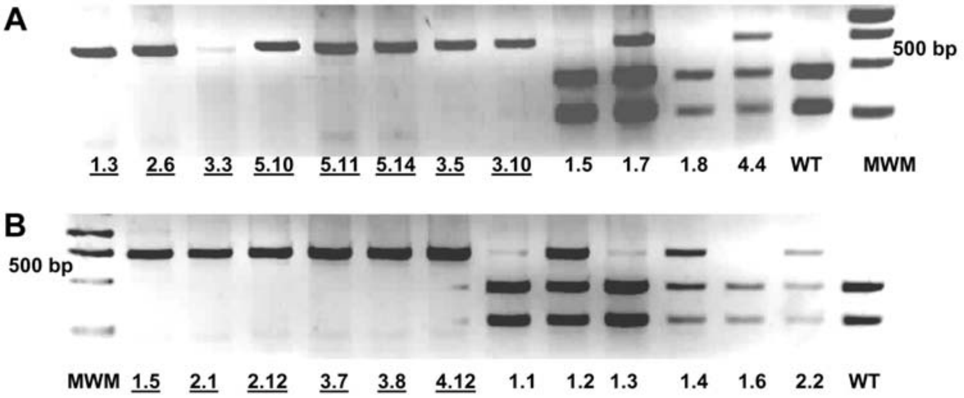

Fig. S6. Analyses of the *pi-1* complementation by *MtPI* and *MtNGL9.* A. Genotyping of some individuals of the complementation lines by *MtPI.* **B.** Genotyping of some individuals of the complementation lines by *MtNGL9***.** Genomic PCR product (729 bp) was digested by *Bse*GI. *pi-1*/*PI*; *35::MtPI* or *pi-1*/*PI*; *35S::MtNGL9* background resulting in three fragments: 729, 451 and 278 bp. Two fragments (278 and 451 bp) were obtained in the case of wild-type plants (*PI*/*PI*; *35S::MtPI* or *PI*/*PI*; *35S::MtNGL9*. *Bse*GI does not cut the 729 bp PCR fragment in *pi-1*/*pi-1; 35::MtPI* or *pi-1*/*pi-1; 35::MtNGL9* background (underlined numbers).

Classification	Taxa	PI-homologues	Gymnosperm B-class genes
GYMNOSPERMS			
Coniferales	*Picea abies*		*DAL13-1* (AF158539)
Pinaceae			*DAL13-2* (AF158541)
	Pinus radiata		PrDGL (AF120097)
Cupressaceae	*Cryptomeria japonica*		*CryMADS2* (AF097747)
			CryMADS1 (AF097746)
Gnetaceae	*Gnetum gnemon*		*GnegGGM2* (AJ132201)
			GnegGGM13 (AJ132219)
			GnegGGM15 (AJ251555)
ANGIOSPERMS			
Nymphaceae	*Nuphar japonica*	*Nj PI-1* (AB158359)	
		NjPI-2 (AB158360)	
	Nuphar advena	*Nu.ad.PI* (AY337736)	
	Euryale ferox	*EfPI* (AB158350)	
MAGNOLIIDS			
Magnoliaceae	*Liriodendrum tulipifera*	*LtPI* (AF052864)	
	Magnolia praecocissima		
		MpMADS8 (AB050650)	
Laurales			
Calycanthaceae	*Calycanthus floridus*	*CfPI-1* (AF230708)	
		CfPI-2 (AF230709)	
Lauraceae	*Persea americana*	*Pe.am.PI* (AY337738)	
Piperales			
Piperaceae	*Peperomia hirta*	*PhiPI* (AF052865)	
	Piper magnificum	*PmPI-1* (AF052866)	
		PmPI-2 (AF052867)	
MONOCOTS			
Poales			
Poaceae	*Oryza sativa*	*OsMADS2* (L37526)	
		OsMADS4 (L37527)	
		nmads1 (AF095645)	
	Zea mays	*ZMM16* (AJ292959)	
		ZMM18 (AJ292960)	
		ZMM29 (AJ292961)	
Liliales			
Liliaceae			
	Phalaenopsis equestris	*PeMADS6* (AY678299)	
Alismatales			
Alismataceae	*Sagittaria montevidensis*	*SmPI* (AF230712)	
EUDICOTS (early divergent eudicots)			
Ranunculales	*Papaver nadicaule*	*PnPI-1* (AF052855)	
		PnPI-2 (AF052856)	
Papaveraceae	*Papaver somniferum*	*PapsPI-1* (EF071994)	
		PapspI-2 (EF071995)	
	Sanguinaria canadiensis	*ScPI* (AF130871)	
	Akebia kinata	*AkqPI* (AY162837)	
	Ranunculus bulbosus	*RbPI-1* (AF052859)	
		RbPI-2 (AF052860)	
	Ranunculus ficaria		
Ranunculaceae		*RfPI-1* (AF052858)	
	Delphinum ajacis	*RfPI-2* (AF130872)	
	Trochodendron aralioides	DaPI (AF052862)	
Trochodendraceae		*Tr.ar. PI-1* (EF436259)	
		Tr.ar. PI-2 (EF436260)	
CORE EUDICOTS			
Caryophyllales			
Caryophyllaceae	*Silene latifolia*	*SLM3* (X89108)	
Saxifragales			
Saxifragaceae	*Ribes sanguineum*	*Ri.sa. PI* (AY337742)	
ROSIDS			
Euforbiáceas			
Euphorbiaceae	*Ricinus communis*	*Ri.co. PI* (XM_002520262)	
Vitales		*VvPI* (DQ059750.1)	
Vitaceae	*Vitis Vinifera*		

Myrtales Myrtaceae	*Eucalyptus grandis*	*EGM2* (AF029976)	
Eurosid I			
Cucurbitales Cucurbitaceae	*Cucumis sativus*	*CUM26* (AF043255)	
Fabales Fabaceae	*Medicago sativa*	*MsNGL9* (AF335473) ***MsPI*** (KJ470631)	
	Medicago trunctula	*MtPI* (FJ403468) *MtNGL9* (FJ403469)	
	Pisum sativum	*PsPI* (AY842791) ***PsNGL9*** (KJ470632)	
	Lotus japonicus	*LjPIa* (AY770398) *LjPIb* (AY770399)	
	Glycine max	*GmPI1* (BT089901) *GmPI2* (BT090476)	
Malvales Malvaceae	*Gossypium hirsutum*	*GhMADS12* (FJ409869)	
Rosales Rosaceae	*Malus domestica*	*MdPI* (AJ291490)	
	Rosa rugosa	MASAKO BP (AB038462)	
EurosidsII			
Brassicales Brassicaceae	*Arabidopsis Thaliana*	AtPI (D30807)	
ASTERIDS			
Cornales Hydrangeaceae	*Hydrangea macrophylla*	*HmPI* (AF230711)	
EuasteridsI			
Lamiales Plantaginaceae	*Antirrhinum majus*	*AmGLO* (X68831)	
Solanales Solanaceae	*Nicotiana tabacum*	*NtGLO* (X67959)	
	Petunia hybrida	*PhGLO1* (M91190) *PhGLO2* (X69947)	
Euasterids II			
Asterales Asteraceae	*Gerbera hybrida*	*GGLO1*(AJ009726)	
	Chrysanthemun morifolium	*CDM86* (AY173061)	
	Helliantus annuus	*He.an.PI* (AY157725)	

Table S1. Sequences from different plant species used in the elaboration of the phylogenetic tree with their respective GenBank accession numbers.

Model	Log-likelihood (ℓ)	Parameter estimates	Amino acids
G&Y	-10937.758	$\omega = 0.147$	-
FRM	-10773.329	-	-
BSMN	-10837.050	$\omega_0 = 0.144$, $P_0 = 0.79$; $\omega_1 = 1$, $P_1 = 0.21$	-
BSM	-10836.367	$\omega_0 = 0.144$, $P_0 = 0.68$; $\omega_1 = 1$, $P_1 = 0.18$, $\omega_2 = 3.71$, $P_2 = 0.11$	Leu9, Ser47, Ser14, Arg40, Ala74.

Table S2. Maximum-likelihood test of adaptive evolution in the duplicated *M. truncatula PISTILATA*-like transcription factors. To identify signatures of adaptive evolution after gene duplication, we conducted four models, all implemented in the program PAML package version 4.7 (Yang, 2007): (a) G&Y model estimates the non-synonymous-to-synonymous rates ratio (ω) for the entire alignment and phylogenetic tree, (b) FRM model (Free-ratio model), estimates one ω per phylogenetic branch, (c) BSMN refers to the neutral branch-site model and allows inferring ω under a neutral evolution model ($0 < \omega \leq 1$) for a user-specified branch of the tree, which in this case was the branch leading to the MtNGL9 duplicate, and (d) BSM model is the branch-site model that allows ω to be greater than 1, hence making it possible the identification of positive selection. The proportion of sites under each ω category (Pi) was also estimated.

Genotype	**Weak rescue**	**Strong rescue**	**N^{o}.of F2 kanamycin resistant plants**	**N^{o}.of expected *pi-1/pi-1* plants**
pi-1l pi-1*; *35S::MtPI	5 (26,3 %)	14 (73,7 %)	65	16,24 (1/4)
pi-1l pi-1; 35S::MtNGL9	14 (100%)	0	64	16 (1/4)

Table S3. Summary of rescue phenotypes.

Primer name	**Sequence 5’-3’**	**Use**
MtNGL9-ATG	ATGGGAAGAGGAAAGATTGAG	For *PsNGL9* cDNA isolation
MtNGL9-638	GCTGAAGTAGTGAGTGAAGC	
MtPI-ATG	ATGGGAAGAGGTAAGATTGAG	For *MsPI* cDNA isolation
MtPI-548	GGTTAAATCCATTGTCCATGCA	
MsPI-363	GATGGCCCTTGAGGAATCCCTT	
MtNGL9-511	GGCAGTGGAAGGTGGGAGATAA	For southern blot hybridization and RNA *in situ* hybridization
MtNGL9-753	GAAAGTGTAAGCCCAGGGATC	
MtPI-504	GGCACTTGAAGGTGTGGGAAA	
MtPI-801	GAAACCAAATTCAATCACTTCATA	
MtPI-SalI	GGTCGACCCATGGGAAGAGGTAAGATTGAG	For overexpression constructs
MtPI-BamHI	GCGGATCCTTAAATCCATTGTCCATGCATG	
MtNGL9-SalI	GGTCGACCCATGGGAAGAGGAAAGATTGAG	
MtNGL9-BamHI	CGGGATCCTTATAATCCATTTATCTCCCA	
BAD-MtPI-FOR	GCTGGATCCGCATGGGAAGAGGTAAGATTGAG	For yeast two-hybrid constructs
BD-MtPI-REV	GTCGTCGACTTAAATCCATTGTCCATGCATG	
BAD-MtNGL9-FOR	GCTGGATCCGCATGGGAAGAGGAAAGATTGAG	
BD-MtNGL9-REV	GTCGTCGACAAAGCTGAAGTAGTGAGTGAAG	
IKC-MtNMH7-FOR	GCTGGATCCGCGCCTCAACAAAGCAGTTTTTCGAT	
AD-MtNMH7-REV	GTCGAATTCCTAAAAAAGTAAAGGGTAAGTGGTG	
IKC-MtTM6-FOR	GCTGGATCCGCCTCTCTACAAAGAAGATTATTGATC	
AD-MtTM6-REV	GTCCTCGAGTCAACCCAGGCGCAAATCGTCA	
Tnt1-R	CAGTGAACGAGCAGAACCTGTG	For *mtngl9* mutants genotyping
NGL9-F	GGGAAGAGGAAAGATTGAGATC	
NGL9-590G	GCTACCTGAGCTGAATTTGCATGTC	
NGL9-612G	CCAACCATATATAGCTAATCTAGCTACCTG	
CAPS-FOR	CCTAAAATTGAGAAAGAGATAGAGAG	For *pi-1* mutant genotyping
CAPS-REV	CGCATAAGATTTGGTCTTGATGGTTTC	
MtPI qRT-FOR	GCTGATGGCCCTTGAGGAAT	For qRT-PCR
MtPI qRT-REV	TCACGGACGCCAGTAAGACC	
MtNGL9 qRT-FOR	AACTCAAGAAAGAGAATGAGGA	
MtNGL9 qRT-REV	TAAGACATCCTCTAAGCTCATC	
MtNMH7 qRT-FOR	TTAGGTCCACGCATGTTTGC	
MtNMH7 qRT-REV	AGGATTAGGATGAGTAGGCTGTAAGC	
MtTM6 qRT-FOR	CGAAGGAGACGAGGAATCAG	
MtTM6 qRT-REV	AGAGTGGAAGCACCATTGGC	
Sec.Ag-qRT-FOR	TGGCTACTAGGGTTGCTGGC	
Sec.Ag-qRT-REV	CCTCACCCAGTCCAGTGGAA	
MsNGL9 qRT-FOR	ATAAAGAAGCTGAATCTGGGTTTTTG	
MsNGL9 qRT-REV	ACACGCATATATAATGTAATGTAACCTCAA	
MsPI qRT-FOR	GTGTGTTTCCTAAATAGCCATTGATC	
MsPI qRT-REV	GCCATATTAAAGCAACATACATTTTTTC	
PsPI qRT-FOR	TGGACAATGGATTTAACCAGATCA	
PsPI qRT-REV	GCATAATATTGAGCAAACTAATTCCCT	
PsNGL9 qRT-FOR	TGATGAATTTGATGAGAGTACTGGAAG	
PsNGL9 qRT-REV	GAAAATGGCATCTGGGAATTGA	
PsACTIN-11-FOR	AGGTGCTGTACCAACCATCCA	
PsACTIN-11-REV	CGTGAATTCCTGCTGCTTCC	
MsNGL9 qRT-FOR	ATAAAGAAGCTGAATCTGGGTTTTTG	
MsNGL9 qRT-REV	ACACGCATATATAATGTAATGTAACCTCAA	
MsPI qRT-FOR	GTGTGTTTCCTAAATAGCCATTGATC	
MsPI qRT-REV	GCCATATTAAAGCAACATACATTTTTTC	
MsEF-1-FOR	CAGGCTGATTGTGCAGTCCTT	
MsEF-1-REV	CCAGCTTCAAAACCACCAGTG	
AtUBQ FOR	GGCCTTGTATAATCCCTGATGAATAAG	
AtUBQ REV	AAAGAGATAACAGGAACGGAAACATA	
MtNGL9 qRT-FOR	GCTGATGGCCCTTGAGGAAT	For semi-quantitative RT-PCR
MtNGL9-507 REV	CCCTTGTTGCTGCTGGAGGATA	
MtPI-363	GATGGCCCTTGAGGAATCCCTT	
MtPI-548	GGTTAAATCCATTGTCCATGCA	
MtEF-1-FOR	AGAAGGAAGCTGCTGAGATGAAC	
MtEF-1-REV	CTGCTTCACTCCAAGAGTAAATGC	
AtUBQ FOR-S	GATCTTTGCCGAAAACAATTGGAGGATGGT	
AtUBQ REV-S	CGACTTGTCATTAGAAAGAAAGAGATAACAGG	

Table S4. Primers used in this work.