New Phytologist

***New Phytologist* Supporting Information**

Article title: NIN is essential for development of symbiosomes, suppression of defence and premature senescence in *Medicago truncatula* nodules

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The following Supporting Information is available for this article:

Fig. S1 Expression profile of Medicago NIN/Medtr5g099060 (probe id. Mtr.28094.1.S1.st) based on *M. truncatula* Gene Atlas in various tissues.

Fig. S2 Live/dead staining shows prematurely death of rhizobia in Medicago *nin-16* nodules.

Fig. S3 Medicago *nin-13* mutant nodules show the same phenotype as *nin-16.*

Fig. S4 F1 plants obtained by crossing Medicago *nin-13* and *nin-16* showed the same nodule phenotype as the parental plants.

Fig. S5 *NF-YA1* expression pattern in Medicago *nin-16* nodule.

Fig. S6 *Tnt1* was transcribed in Medicago *nin-13* and *nin-16* mutant nodules.

Fig. S7 *NIN* RNA transcribed from the Medicago *nin-16* allele was altered by *Tnt1* insertion.

Fig. S8 Complementation of Medicago *nin-16* nodule phenotype with $ProNIN_{3C\text{-}5kb}:NIN_{\Delta PB1}$ and $ProNIN_{3C\text{-}5kb}:NIN$.

Table S1 Primers used in this study.

Table S2 Genes with transcripts detected in Medicago *nin-16* or R108 (wild type). (separate Excel file)

Table S3 Genes differentially expressed in Medicago *nin-16*. (separate Excel file)

Table S4 Genes specifically expressed in different Medicago wild type nodule developmental

zones differentially expressed in *nin-16*. (separate Excel file)

Table S5 Differentially expressed gene families/metabolism pathways in Medicago *nin-16* (separate Excel file)

Fig. S1 Expression profile of Medicago NIN/Medtr5g099060 (probe id. Mtr.28094.1.S1.st) based on *M. truncatula* Gene Atlas in various tissues. Mt *NIN* is specifically expressed in nodules.

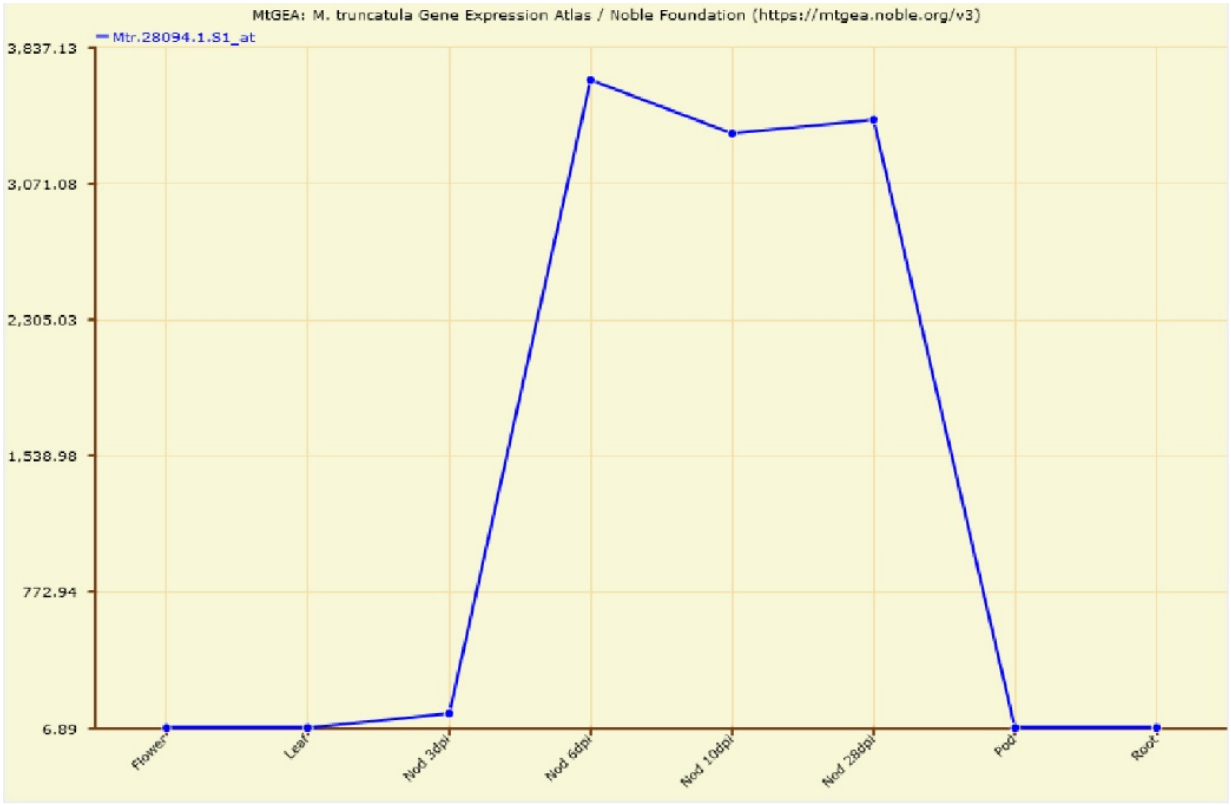


Fig. S2 Live/dead staining shows prematurely death of rhizobia in Medicago *nin-16* nodules. Sections of two weeks post inoculation (wpi) nodules, imaged by a confocal microscope after live/dead staining assay. Green (SYTO 9) and red (propidium iodide) stain alive and dead bacteria respectively. Compare with wildtype (a, b), the early death of the bacteria in the *nin-16* nodule was detected (c, d). Bars: (a, c) 250 µm and (b, d) 50 µm.

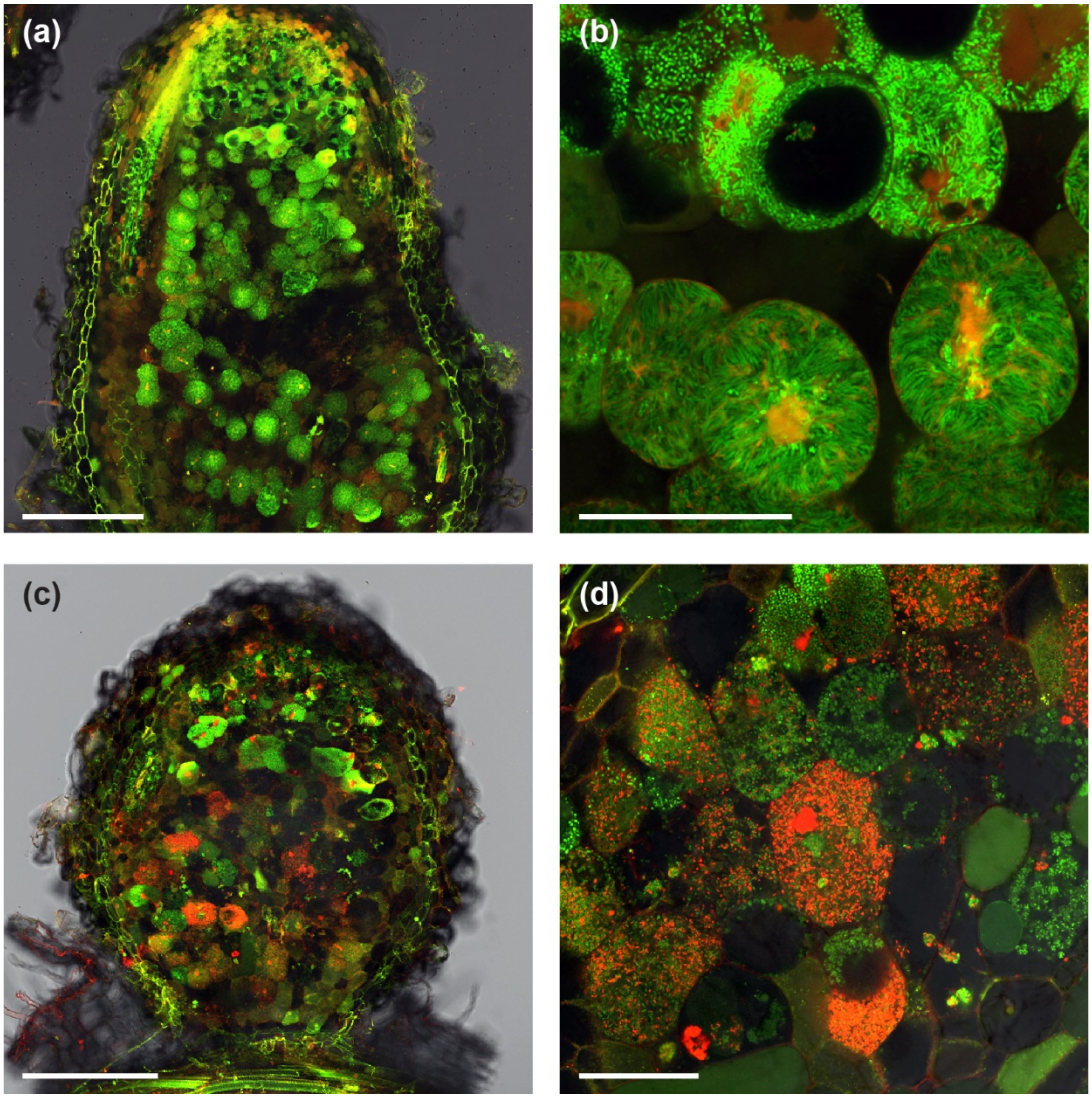


Fig. S3 Medicago *nin-13* mutant nodules show the same phenotype as *nin-16*. Like *nin-16*, *nin-13* formed white nodules at two wpi (a). Inoculation with rhizobial carrying *nifH:GFP* showed that *nifH* was not induced in *nin-13* nodules (e). Sections of these nodules showed that meristem was formed (b), rhizobia were released and divided (c), but bacteria differentiation were arrested, and premature senescence was induced (d). The bacteria death was confirmed by live/dead staining (f, g). Green (SYTO 9) and red (propidium iodide) stain alive and dead bacteria respectively. Potassium permanganate/methylene blue staining shows accumulation of phenolic compound in *nin-13* nodules (h). Bars: (a) 2 mm; (b) 300 µm; (c, d, g) 30 µm; (e, f) 250 µm and (h) 500 µm.

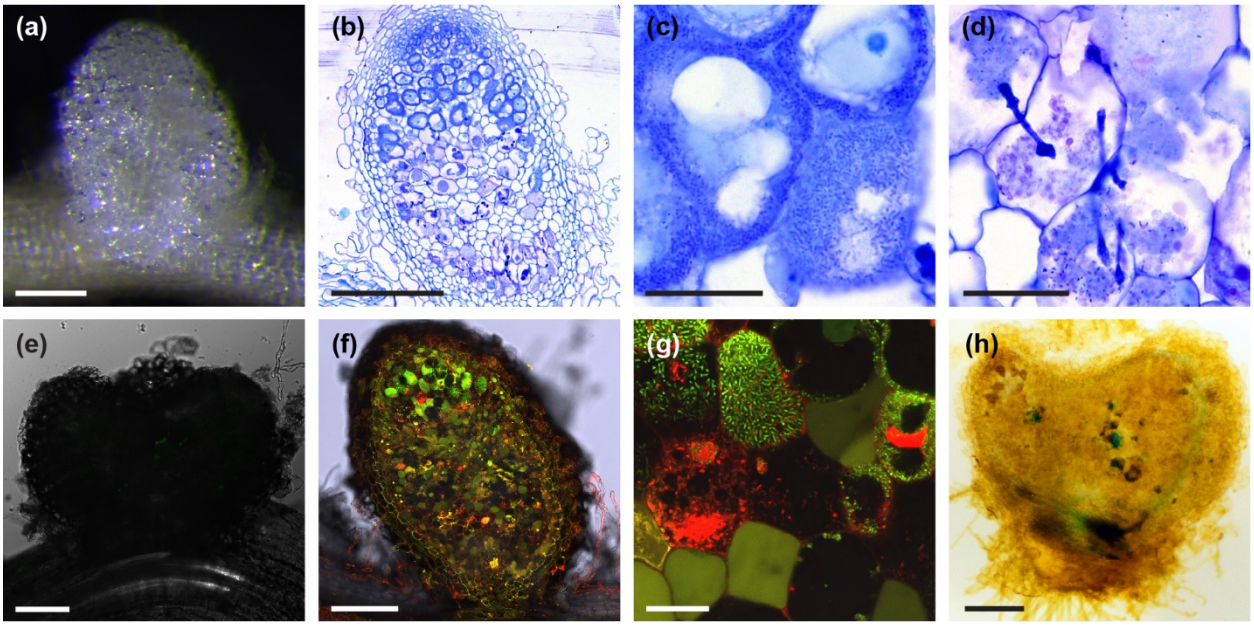


Fig. S4 F1 plants obtained by crossing Medicago *nin-13* and *nin-16* showed the same nodule phenotype as the parental plants. Transmitted light macroscopy images of root nodules formed F1 plants obtained by crossing *nin-13* and *nin-16* (a). Semi-thin sections of these nodules stained with toluidine blue display the same nodule phenotype as the *nin-13* and *nin-16* (b). Bars: (a) 2 mm and (b) 300 µm.

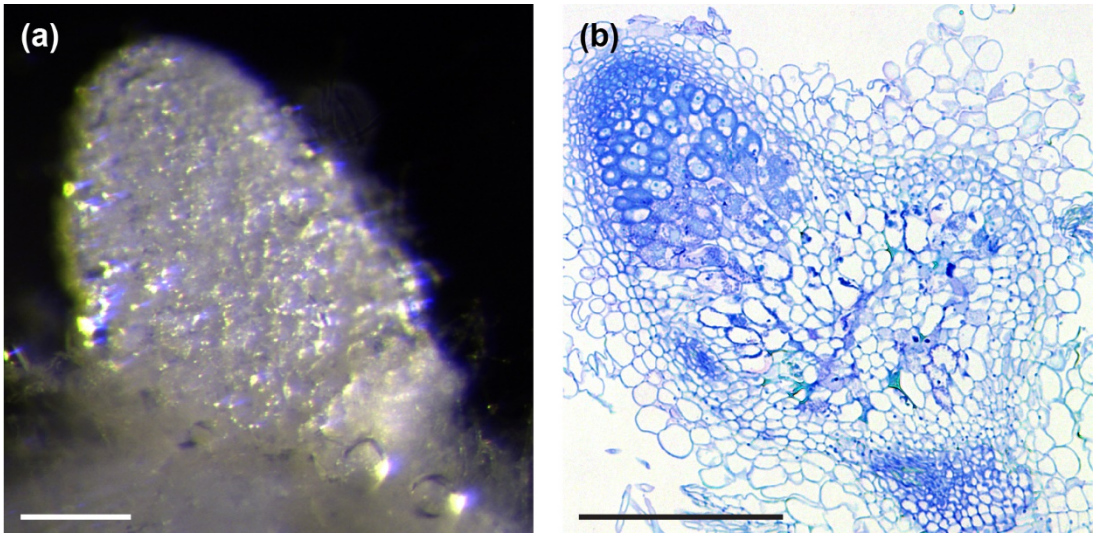


Fig. S5 *NF-YA1* expression pattern in Medicago *nin-16* nodule. RNA *in situ* localization of *NF-YA1* in *nin-16* nodule at two wpi. Hybridization signals are visible as red dots. Scale bar: 200 µm.

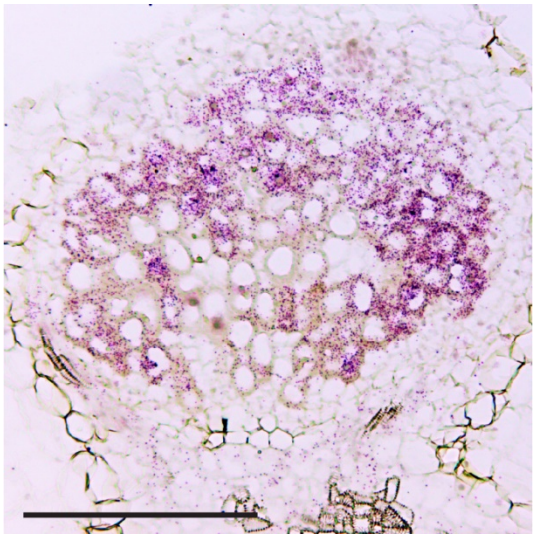


Fig. S6 *Tnt1* was transcribed in Medicago *nin-13* and *nin-16* mutant nodules. Quantitative real-time (qRT-PCR) using different primer sets targeting *NIN*, upstream junction of *NIN* and *Tnt1* (*NIN-Tnt1*), within *Tnt1* insertion (*Tnt1*) and downstream junction of *Tnt1* and *NIN* (*Tnt1-NIN*) show that *Tnt1* was transcribed in *nin-13* (a) and *nin-16* (b) nodules. Data are means ± SD of three biological replicates.

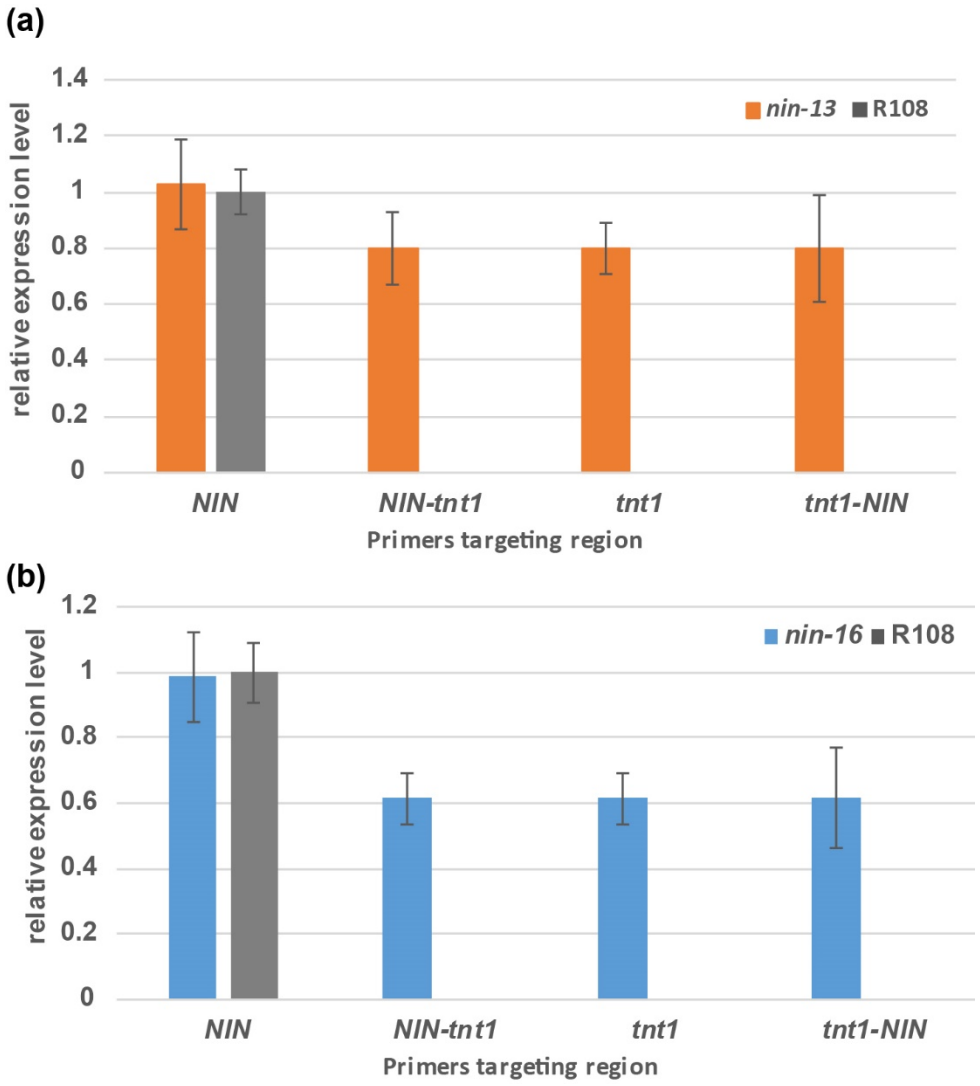
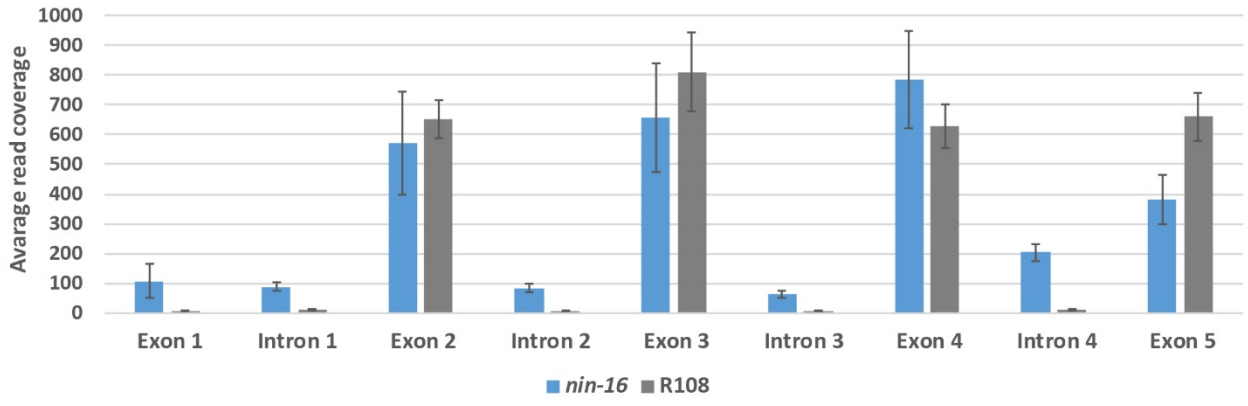


Fig. S7 *NIN* RNA transcribed from the Medicago *nin-16* allele was altered by *Tnt1* insertion. (a) Mapping of the reads at the *NIN* locus in R108 (above) and *nin-16* (below). Arrow heads indicate the reads that mapped in *NIN* intron regions and arrow indicates the much longer 5' UTR in *nin-16.* (b) Quantification of average read coverage for each exon/intron region of *NIN* in *nin-16* and R108. The read coverage was determined with mosdepht (version 0.3.0) using default settings (Pedersen & Quinlan, 2018). Data are means ± SD. (c) Non-spliced intron rate of *NIN* RNA in *nin-16* and R108. Non-spliced intron rate was calculated by intron reads coverage divided by the average exon reads coverage. Calculation of average read coverage in exon regions were based on the exons 2, 3 and 4. This is because *nin-16* contains a *Tnt1* insertion in exon 5 which leads to low read coverage, and exon 1 is in the non-coding region and there the read coverage is much lower than in the coding region. The average read coverage in exons of R108 and *nin-16* were similar, in R108 it is 697.23 and in *nin-16* it is 671.39. Theoretically it is possible that only one or all introns are aberrantly spliced. Therefore, we can only indicate a range within which the transcripts are wrongly spliced. The frequency of aberrantly spliced *NIN* transcripts in *nin-16* is between 30.24% (all introns are aberrantly spliced) and 64.80% (a single intron is aberrantly spliced). This is markedly higher than the frequency in R108 which is between 1.39% and 4.37%.

(a)

(b)



(c)

Non-spliced intron rate of *NIN* in R108 and *nin-16*

	Intron 1	Intron 2	Intron 3	Intron 4	Sum intron 1-4
R108	1.39%	0.66%	0.95%	1.38%	4.37%
nin-16	12.98%	12.26%	9.32%	30.24%	64.80%

Fig. S8 Complementation of Medicago *nin-16* nodule phenotype with *ProNIN*$_{3C\text{-}5kb}$*:NIN*$_{\Delta PB1}$ and *ProNIN*$_{3C\text{-}5kb}$*:NIN*. Overview (a, c, e) and close-up (b, d, f) images of semi-thin longitudinal sections of nodules formed on *nin-16* roots transformed with *ProNIN*$_{3C\text{-}5kb}$*:NIN*$_{\Delta PB1}$ (a, b), *ProNIN*$_{3C\text{-}5kb}$*:NIN* (c, d) and empty vector (e, f). Fully elongated symbiosomes were observed in five out of 30 sectioned nodules formed on *nin-16* roots transformed with *ProNIN*$_{3C\text{-}5kb}$*:NIN*$_{\Delta PB1}$ and five out of 60 nodules formed on *nin-16* roots transformed with *ProNIN*$_{3C\text{-}5kb}$*:NIN,* but not in nodules formed on the roots transformed with empty vector (n=55). In addition, the defence-related phenotype was markedly reduced in the complemented nodules. Bars: (a, c, e) 300 µm and (b, d, f) 30 µm.

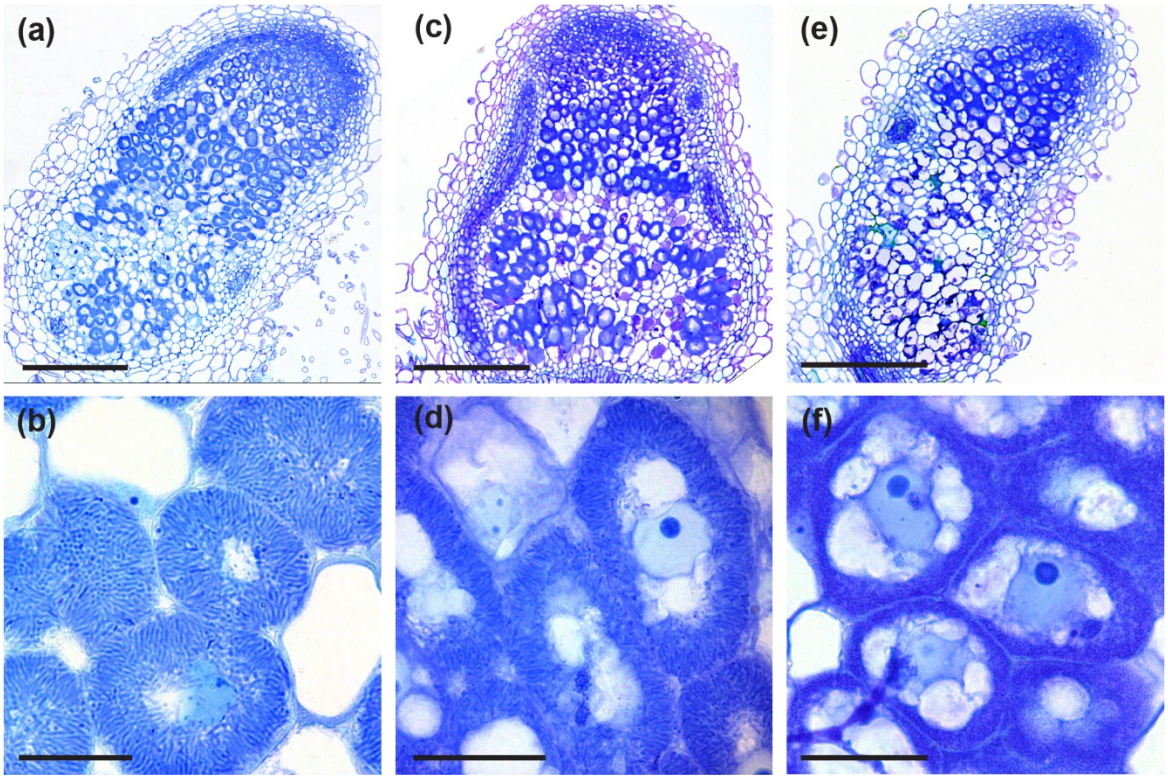


Table S1 Primers used in this study.

Name	Sequence (5′→3′)
qPCR-NIN-F	TACTTTGCCGGAAGCCTAAA
qPCR-NIN-R	ATCTGTATGGCACCCTCTGC
qPCR-NIN-Tnt1-F	GAGTTGATCATGCCTTCATGC
qPCR-NIN-Tnt1-R	GGTTGGCTACCAAACCAAAG
qPCR-Tnt1-F	GCGTTTGAAATCCCAGAGAG
qPCR-Tnt1-R	AACCGAACACCTTCAGATGC
qPCR-Tnt1-NIN-F	TCAGAAGGGTTTTCCACGTAA
qPCR-Tnt1-NIN-R	CCACAGTTGGTCTTGGAGGT
qPCR-ACTIN2-F	TGGCATCACTCAGTACCTTTCAACAG
qPCR-ACTIN2-R	ACCCAAAGCATCAAATAATAAGTCAACC
Genotyping-NIN-F	TGCTAATGGTGGTGATGGTAAT
Genotyping-NIN-R	GGTTAAATCGCCTTGCAATCTC
Genotyping-Tnt1-R	TGTAGCACCGAGATACGGTAATTAACAAGA
$NIN_{\Delta PB1}$-F	**CACC**ATGGAATATGGTGGTGGGTT
$NIN_{\Delta PB1}$-R	GTAGTCCTGGATATTAATATTAGATGCA
$NIN_{\Delta PB1}$-35Ster-BP-F	**GGGGACAGCTTTCTTGTACAAAGTGGAA**ATGGAATATGGTGGTGGGTTAGTG
$NIN_{\Delta PB1}$-35Ster-BP-R	**GGGGACAACTTTGTATAATAAAGTTGC**TCACTGGATTTTGGTTTTAGGAATTA
NIN-CDs-F	**CACC**ATGGAATATGGTGGTGGGTTAGTGG
NIN-CDs-R	GCTAGGAGGATGGACTGCTGCTGCT
NINGFP-35Ster-BP-F	**GGGGACAGCTTTCTTGTACAAAGTGGAA**ATGGAATATGGTGGTGGGTTAGTG
NINGFP-35Ster-BP-R	**GGGGACAACTTTGTATAATAAAGTTGC**TCACTGGATTTTGGTTTTAGGAATTA
GFPNIN-35Ster-BP-F	**GGGGACAGCTTTCTTGTACAAAGTGGAA**ATGGTGAGCAAGGGCGAGGA
GFPNIN-35Ster-BP-R	**GGGGACAACTTTGTATAATAAAGTTGC**TCACTGGATTTTGGTTTTAGGAATTA

Sequences designated in boldface are added to primers for TOPO cloning or BP recombination

References:

Pedersen BS, Quinlan AR. **2018**. Mosdepth: Quick coverage calculation for genomes and exomes. *Bioinformatics* **34**: 867–868.