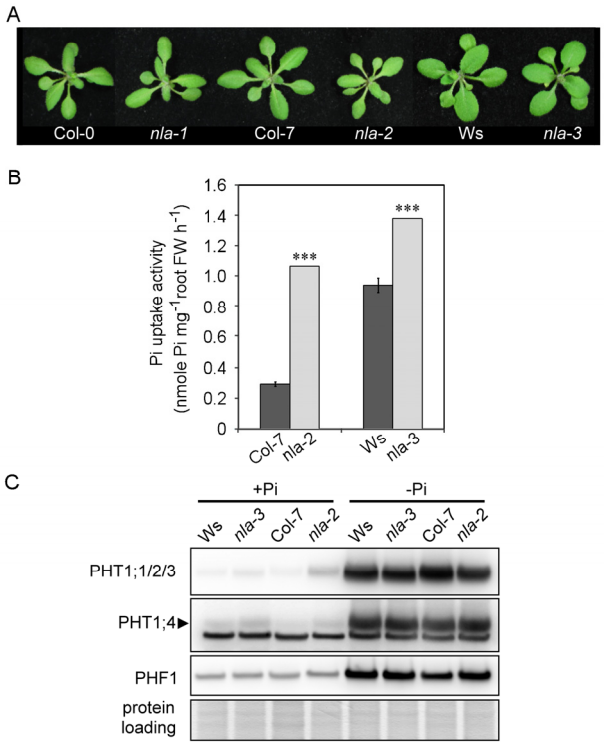
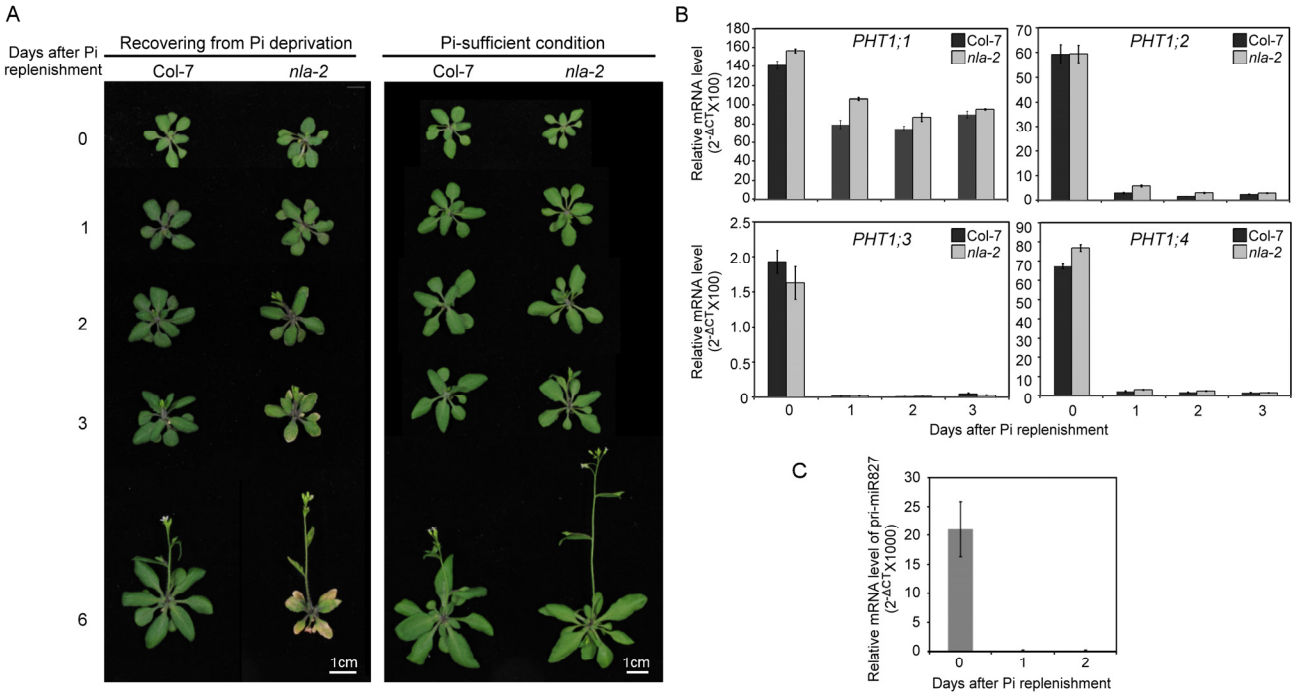


Supplemental Figure 1. Mutation in *NLA* Causes Increased Pi Uptake Activity and PHT1 Protein Amounts**.**

(A) Shoot morphology of 19-day-old *nla* mutants under Pi-sufficient conditions.

(B) [^{33}P]Pi uptake activity of 19-day-old WT and *nla* mutants. N = 8; error bar = SEM; Student's *t* test, mutants versus WT, ***, P < 0.005. FW, fresh weight; h, hour.

(C) Protein expression of PHT1;1/2/3, PHT1;4 and PHF1 in the roots of WT and *nla* mutants under Pi-sufficient (+Pi) and -deficient conditions (-Pi).

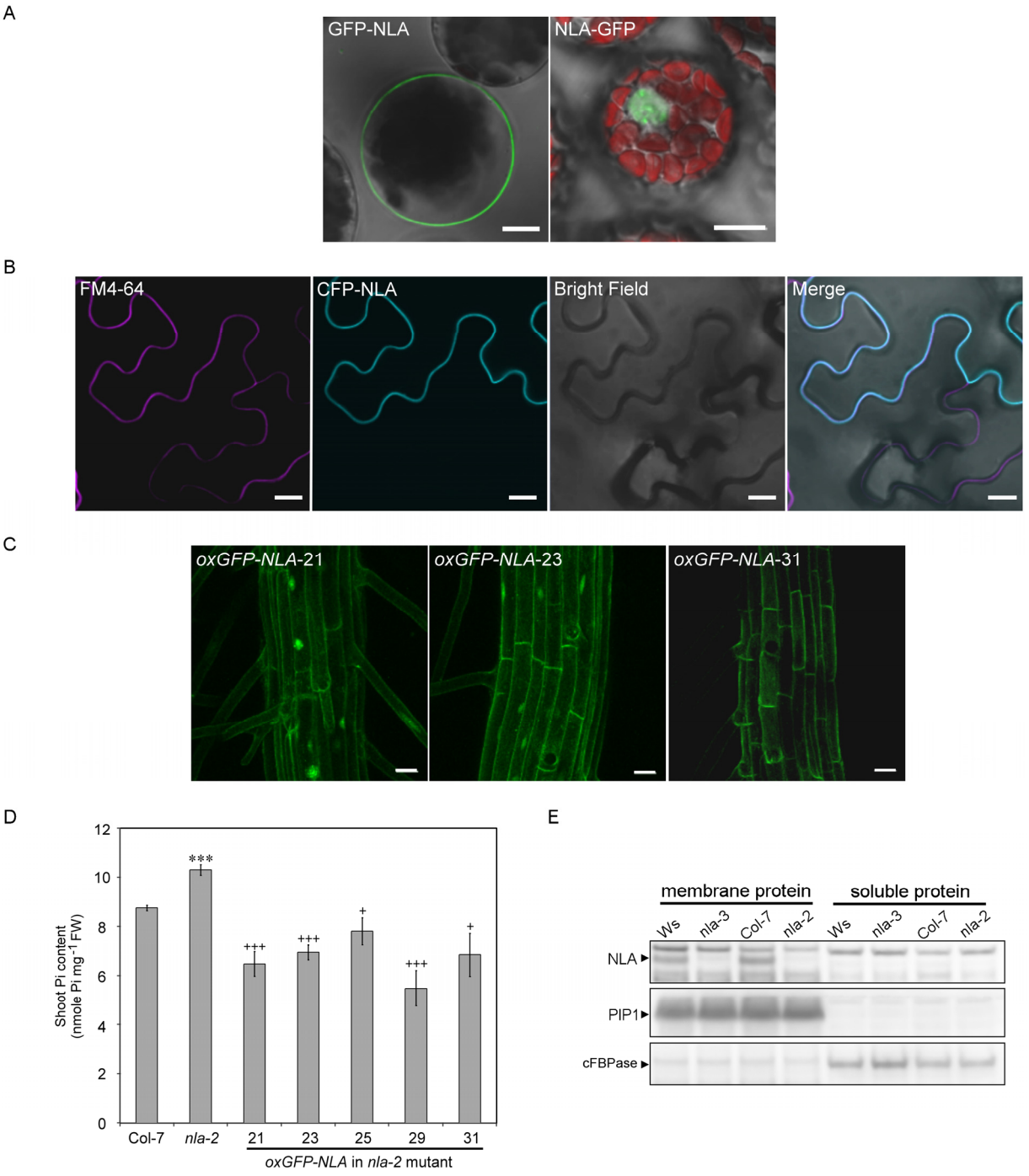


Supplemental Figure 2. The Responses of WT and *nla-2* Mutants after Recovery from Pi Deprivation.

(A) Shoot morphology of the WT and *nla-2* mutants under Pi-sufficient conditions (right) or after recovery from 4-day Pi deprivation (left). Severe necrosis at leaf edges appeared in the mutants after recovery for 3 days.

(B) Expression of *PHT1;1*, *PHT1;*2, *PHT1;*3 and *PHT1;4* in the roots of WT and *nla-2* mutants after Pi replenishment. There was no significant difference in mRNA level between the WT and *nla* mutants. N = 3; error bar = SEM.

(C) Expression of primary miR827 (pri-miR827) in the WT after Pi replenishment. N = 3; error bar = SEM.



Supplemental Figure 3. Subcellular Localization of NLA.

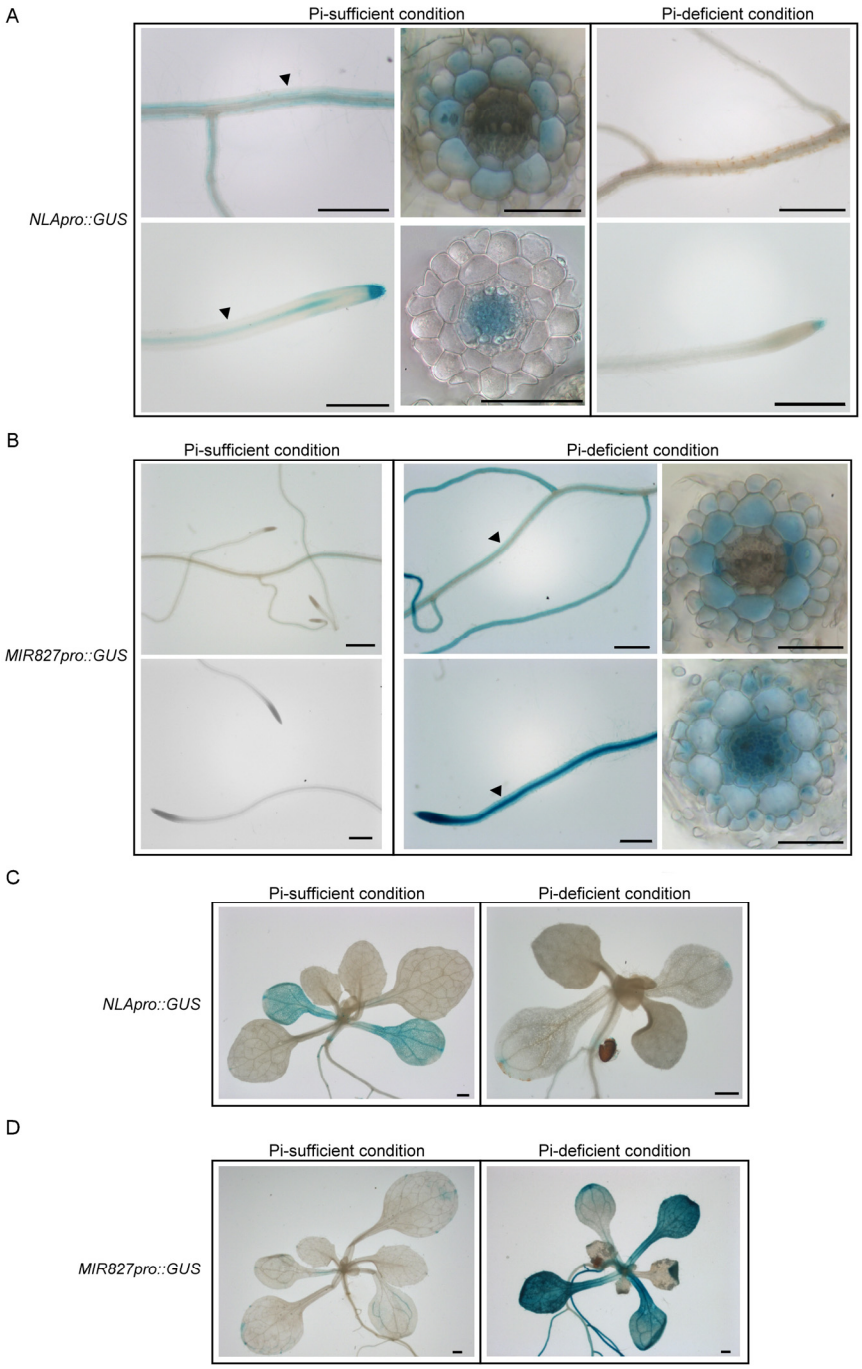
(A) Subcellular localization of GFP-NLA or NLA-GFP in Arabidopsis mesophyll protoplasts. Bar = 20 µm.

(B) Colocalization of CFP-NLA with FM4-64 at the PM after staining for 15 minutes. Bar = 10 µm.

(C) Subcellular localization of GFP-NLA in 3 independent *nla-2* lines (12-day-old) overexpressing GFP-NLA. Bar = 20 µm.

(D) Pi content of overexpressing GFP-NLA in 12-day-old *nla-2* mutants grown in high Pi medium. N = 3; error bar = SEM; Student's *t* test, *nla-2* mutants versus WT, ***, P < 0.005; overexpressing lines versus *nla-2*, $^{+}$, P < 0.05 ; $^{+++}$, P < 0.005. FW, fresh weight.

(E) NLA proteins were detected only in the membrane protein fraction and not in the soluble protein fraction. PIP1, cFBPase and PHF1 were used as markers for PM proteins, cytosolic proteins and ER proteins, respectively.

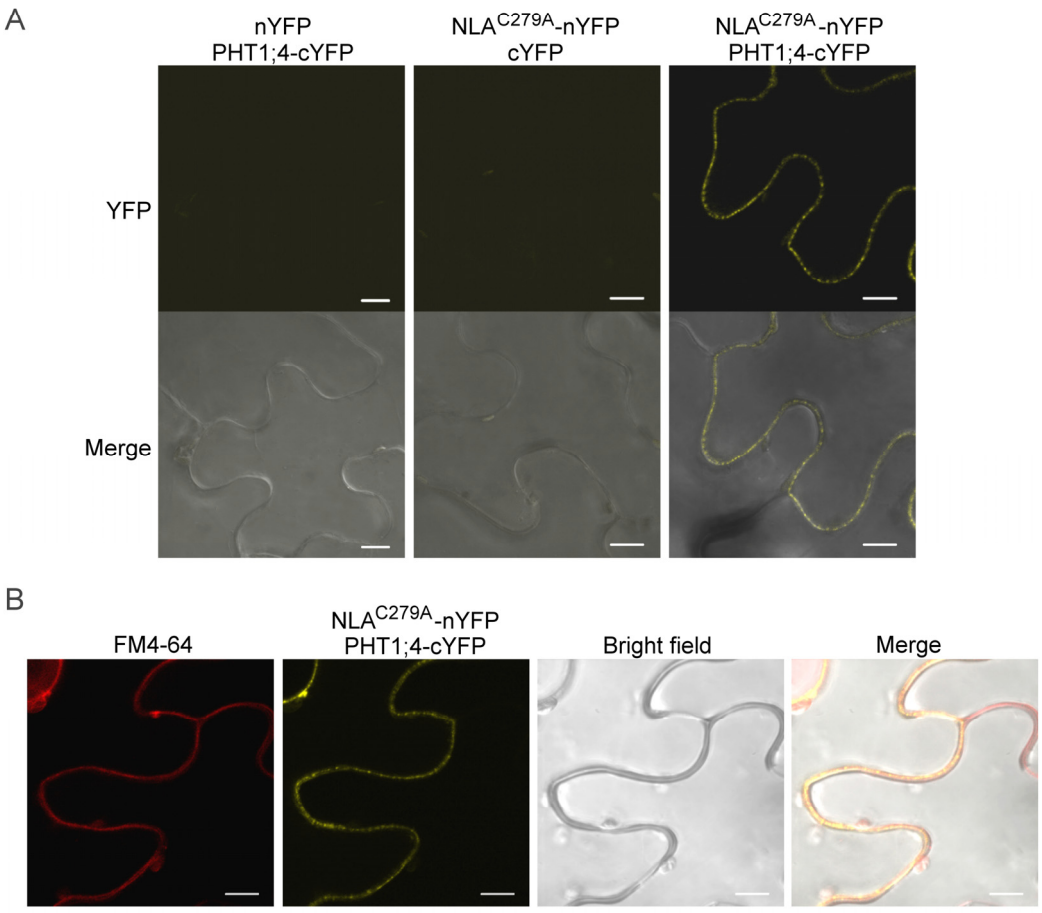


Supplemental Figure 4. Expression Pattern of *NLA* and *MIR827*.

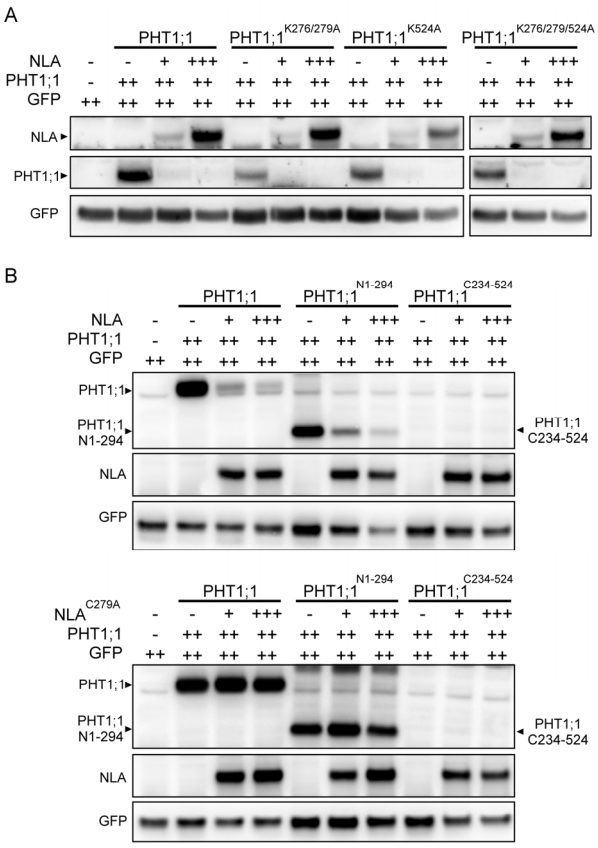
GUS staining of roots of 12-day-old seedlings for examining the expression pattern of *NLA* **(A)** and *MIR827* **(B)**. The cross sections of roots were from the areas marked with arrowheads in the left panels.

Expression pattern of *NLA* and *MIR827* in shoots are shown in **(C)** and **(D)**, respectively. Bar = 50 μm.

Six independent transgenic lines of each construct were examined. Representative lines are shown.



Supplemental Figure 5. Analysis of Protein-Protein Interaction between NLA and PHT1;4**.** Coexpression of PHT1;4-cYFP with NLA^{C279A}-nYFP in the tobacco leaves shown BiFC fluorescence in the PM **(A)** which was colocalized with FM4-64 after staining for 15 minutes **(B)**. The “merge” panel is an overlaid image of YFP and bright field **(A)** or YFP, FM4-64 and bright field **(B)**.

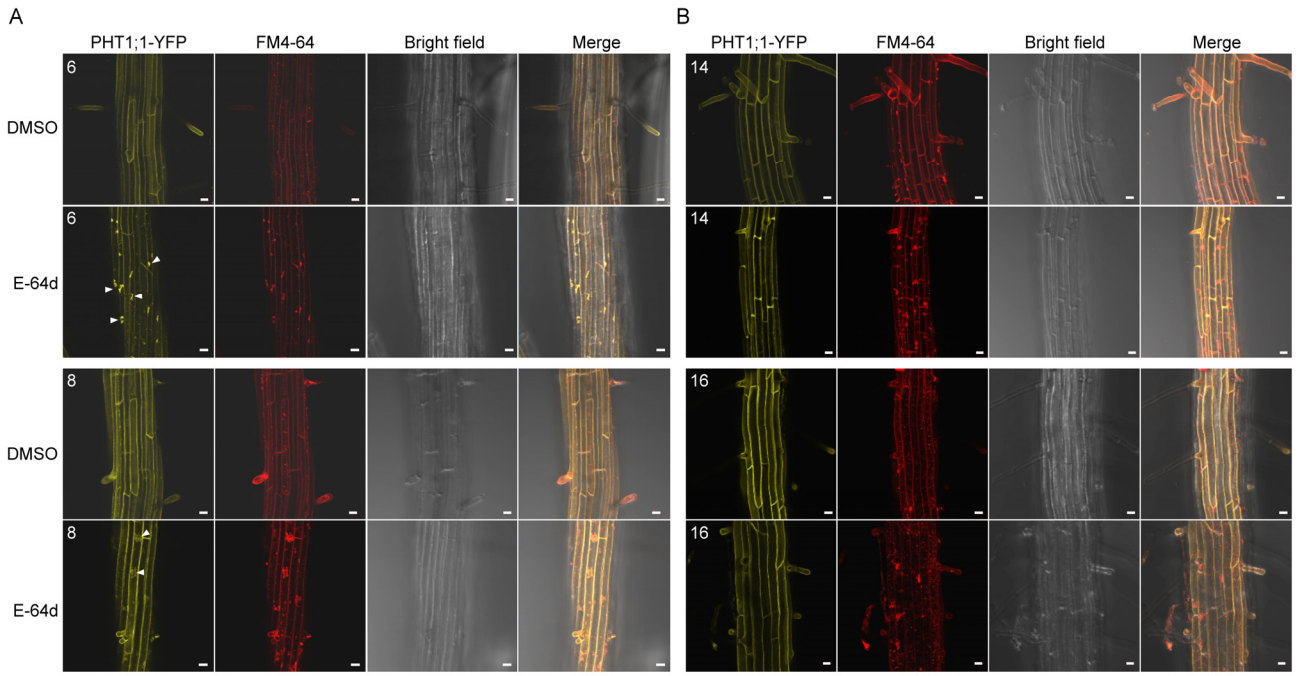


Supplemental Figure 6. The N-terminal Portion of PHT1;1 Is Sufficient for NLA-Mediated Degradation.

(A) None of the mutated PHT1;1 proteins (PHT1;1^{K524A}, PHT1;1$^{K276/279A}$, PHT1;1$^{K276/279/524A}$) were resistant to NLA-mediated degradation when coexpressed with NLA in tobacco leaves.

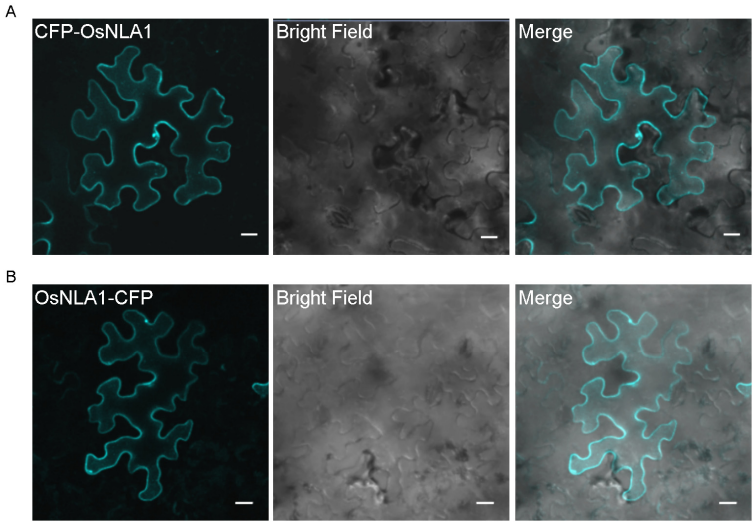
(B) Coexpression of N- (PHT1;1^{N1-294}) or C- (PHT1;1$^{C234-524}$) terminal portion of PHT1;1 with NLA or NLA^{C279A} in tobacco leaves. PHT1;1^{N1-294} was degraded by NLA but not by NLA^{C279A}. The signal of PHT1;1$^{C234-524}$ was too weak to be conclusive.

Microsomal proteins were used for this experiment. PHT1;1 and its mutated and truncated forms were detected by anti-PHT1;1/2/3 antibodies. Co-infiltration of 35S::GFP construct was used as an internal control. Note that "+++" and "++" denotes 5- and 2.5-fold increases in volume of infiltration relative to "+", respectively.

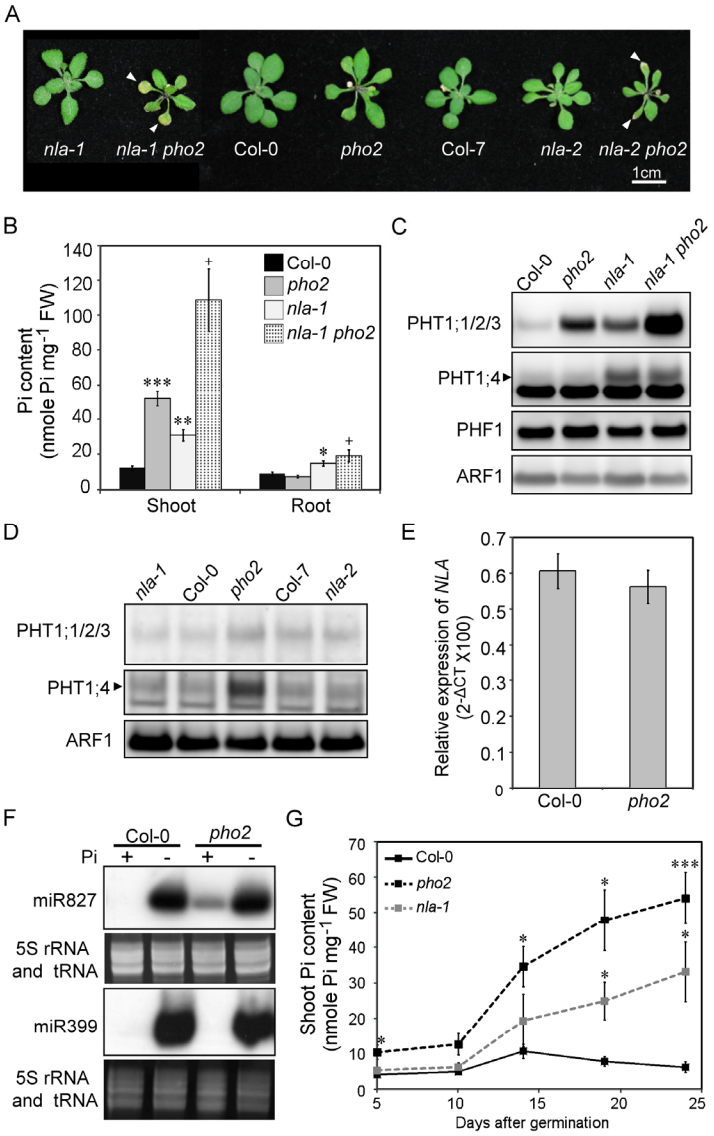


Supplemental Figure 7. NLA-Mediated Ubiquitination Triggers PHT1;1 Sorting into the Vacuole through the Endosomal Trafficking Pathway.

The effect of E-64d treatment on PHT1;1-YFP trafficking in two additional independent transgenic lines, #6 and #8 in the WT background **(A)** and #14 and #16 in *nla-2* mutants **(B)**. The "merge" panel is an overlaid image of YFP, FM4-64 and bright field. The aggregation of PHT1;1-YFP in the WT **(A)** is indicated by arrowheads. Bar = 20 μm.



Supplemental Figure 8. Subcellular localization of Os-NLA1.
Subcellular localization of CFP-OsNLA1 **(A)** and OsNLA1-CFP **(B)** in tobacco leaves. The “merge” panel is an overlaid image of CFP and bright field. Bar = 20 μm.



Supplemental Figure 9. The Difference between NLA- and PHO2-Dependent Regulation of PHT1s.

(A) The leaf phenotype of 19-day-old *nla pho2* double and respective single mutants. The necrosis (arrowhead) was clearly observed at the leaf edge of double mutants.

The Pi content **(B)** and protein expression of PHT1;1/2/3, PHT1;4 and PHF1 **(C)** in *nla-1 pho2* double and the respective single mutants. N = 3; error bar = SEM. Student's *t* test, single mutants versus WT, *, P < 0.05 ; **, P < 0.01 ; ***, P < 0.005; *nla-1 pho2* versus *pho2*, $^{+}$,P < 0.05. FW, fresh weight. The detection of ARF1 was used as a loading control.

(D) Immunoblot analysis of PHT1;1/2/3 and PHT1;4 in 10-day-old mutants and the corresponding WT. The detection of ARF1 was used as a loading control.

(E) The mRNA expression of *NLA* in 19-day-old WT and *pho2* plants. There was no significant difference of *NLA* expression between WT and *pho2* mutants. N = 3; error bar = SEM.

(F) Expression of mature miR827 and miR399 in the roots of 19-day-old WT and *pho2* plants.

(G) The Pi content of *nla-1* and *pho2* mutants during plant growth. N = 3, error bar = SEM. Student's *t* test, single mutants versus WT, *, P < 0.05; ***, P < 0.005. FW, fresh weight.

Supplemental Table 1. Sequence of Primers Used in This Study

Gene	Primer	Sequence (5'→3')
AT1G02860	Forward	CTTTTTTTTTGTTTGAGGGCTGAAT
NLA	Reverse	TCATTTATTTTTTCTCTGAATTTCACAAC
AT2G33770	Forward	AGGTTTGAAGCTCCACCCTCA
PHO2	Reverse	CCCAAGATGTGATTGGAGTTCC
AT2G38940	Forward	GGTCCCAATAGTTTAGGTGAT
PHT1;4	Reverse	AGTTGCTAGAGACAAGGAGAA
AT3G59884	Forward	CCACGAAAGAGTTTGTTGATGGT
MIR827	Reverse	TCCTTGTGTTGATCGATTGGTT
AT5G43350	Forward	GCCATGACGAGAAATAATTATGT
PHT1;1	Reverse	TAACTTAAGGTCAACGAGCCAAT
AT5G43360	Forward	CGAGGCTGAGGTTGATAAATGAT
PHT1;3	Reverse	CACACATCGCAAAACCAATGAC
AT5G43370	Forward	AGCCATCATTGGAGCCTTC
PHT1;2	Reverse	ACCTTAGCCTTGTCTTGATT
AT4G05320	Forward	GGCCTTGTATAATCCCTGATGAATAAG
UBQ10	Reverse	AAAGAGATAACAGGAACGGAAACATAGT

Construct	Primer	Sequence (5'→3')
35S::NLA *35S::GFP-NLA* *35S::CFP-NLA*	Forward	AGCTCCATGAAGTTTTGTAAGAAGT
	Reverse	ACCTCGAACTATATCATATTCCAGT
35S::NLA-GFP	Forward	AGCTCCATGAAGTTTTGTAAGAAGT
35S::NLA-CFP	Reverse	TATTCCAGTGAAGCTTCGGCA
35S::NLA^{C279A}	Forward	GCCCGTGAGGATGGGGTTTATAAAGGTGC
35S::CFP-NLA^{C279A}	Reverse	AAGCGGGCATTTTTCAGTTGCTTCTGCG
35S::CFP-NLA^{SPX}	Forward	AGCTCCATGAAGTTTTGTAAGAAGT
	Reverse	TCAAGAGAGCTCGCAGGAAAG
35S::NLA^{SPX}-CFP	Forward	AGCTCCATGAAGTTTTGTAAGAAGT
UBQ$_{pro}$::NLA^{SPX}-nYFP	Reverse	AGAGAGCTCGCAGGAAAG
35S::CFP-NLA^{RING}	Forward	ATGAAAATGCGAATAGAAATC
	Reverse	ACCTCGAACTATATCATATTCCAGT
35S:: NLA^{RING}-CFP	Forward	ATGAAAATGCGAATAGAAATC
	Reverse	TATTCCAGTGAAGCTTCGGCA
35S::PHT1;1	Forward	ATGGCCGAACAACAACTAGGAGT
	Reverse	TTATTTCTCGTCATGGCTAACCTC
UBQ$_{pro}$::PHT1;1-cYFP	Forward	ATGGCCGAACAACAACTAGGAGT
	Reverse	TTTCTCGTCATGGCTAACCTCA
PHT1;1$_{pro}$::PHT1;1-YFP	Pro-for	CCCGGGTCTAGGGACGTCCAAGTTCACTGAAT
	P2	CACAGCTCCACCTCCACCTCCAGGCCGGCCAGAGAGTTCTTCAAGGGACTTGCCT
	FP-for	GGCCGGCCTGGAGGTGGAGGTGGAGCTGTGAGCA
	FP-rev	GGCCCCAGCGGCCGCAGCAGCACCAGCAGGATC
	P3	TGCTGGTGCTGCTGCGGCCGCTGGGGCCGGTGAGGCTGAGGTTAGCCATG
	P4	CGAAATATTAGACAAGATTTAAATGATATC

Supplemental Table 1. Sequence of Primers used in This Study (Continued)

Construct	Primer	Sequence (5'→3')
35S::PHT1;1-YFP	P1	ATGGCCGAACAACAACTAGGAGT
	P2	CACAGCTCCACCTCCACCTCCAGGCCGGCCAGAGAGTTCTTCAAGGGACTTGCCT
	FP-for	GGCCGGCCTGGAGGTGGAGGTGGAGCTGTGAGCA
	FP-rev	GGCCCCAGCGGCCGCAGCAGCACCAGCAGGATC
	P3	TGCTGGTGCTGCTGCGGCCGCTGGGGCCGGTGAGGCTGAGGTTAGCCATG
	P4	CGAAATATTAGACAAGATTTAAATGATATC
35S::PHT1;4	Forward	ATGGCAAGGGAACAATTACAAGTG
	Reverse	CTAAACTATTGGGACCGTTCTACTATCA
UBQ_{pro}::PHT1;4-cYFP	Forward	ATGGCAAGGGAACAATTACAAGTG
	Reverse	AACTATTGGGACCGTTCTACTATCATT
35S::PHT1;4-YFP	P1	ATGGCAAGGGAACAATTACAAGTG
	P2	CACAGCTCCACCTCCACCTCCAGGCCGGCCGCTATTCTCATTGTCTTCATTTTCACC
	FP-for	GGCCGGCCTGGAGGTGGAGGTGGAGCTGTGAGCA
	FP-rev	GGCCCCAGCGGCCGCAGCAGCACCAGCAGGATC
	P3	TGCTGGTGCTGCTGCGGCCGCTGGGGCCAACAATGATAGTAGAACGGTCCC
	P4	GGTCAATTACGAAGGAGAGCCTTCTACTT
35S::PHT1;1-HA	Forward	ATGGCCGAACAACAACTAGGAGT
	Reverse	TTAAGCGTAATCTGGAACATCGTATGGGTATTTCTCGTCATGGCTAAC
35S::PHT1;1 ^{K524A}-HA	Forward	GCATACCCATACGATGTTCCAGATTACGCT
	Reverse	CTCGTCATGGCTAACCTCAGCCTCAC
35S::PHT1;1 $^{K276/279A}$-HA	Forward	GACCCCGCACAAAACTATGGCTTGTTCTCC
35S::PHT1;1$^{K276/279/524A}$-HA	Reverse	TGCGACGTCATCCTCCACCCTTTCCTCAAG
35S::OsNLA1	Forward	ATGAAGTTTGCCAAGAAGTAC
35S::CFP-OsNLA1	Reverse	TCACATGCCCAAGAATGCC
35S::OSNLA1-CFP	Forward	ATGAAGTTTGCCAAGAAGTAC
	Reverse	CATGCCCAAGAATGCCCTG
UBQ_{pro}::IRT1-cYFP	Forward	ATGGCTTCAAATTCAGCAC
	Reverse	GCGGCCGCTTAAGCCCATTTGGCGATAA
$MIR827_{pro}$::GUS	Forward	CACTATGTTAAACTCAGCTT
	Reverse	ACCTATAACGTTTCATGGAAGT
NLA_{pro}::GUS	Forward	AGCCAAATAGGTATTAGTCT
	Reverse	GAACATATCTTCTTCTTCGT
pDL-Nub-NLA^{SPX}	Forward	CGCTGGATCCATGAAGTTTTGTAAGAAGT
	Reverse	TCAAGAGAGCTCGCAGGAAAG
pTMBV4-PHT1;4-Cub	Forward	ACATCTAGAATGGCAAGGGAACAATTAC
	Reverse	TATGTCTAGAAACTATTGGGACCGTTCTAC

Supplemental Table 2. Constructs

Purpose	Construct	Plasmid backbone	Insert or PCR product	Template used for cloning	Cloning method
promoter::reporter assay	NLA_{pro}::GUS	pMDC164[1]	2.2 kb upstream genomic sequence of NLA from start codon	Arabidopsis genomic DNA	Gateway LR
	$MIR827_{pro}$::GUS	pMDC164[1]	1.3 kb upstream genomic sequence of MIR827 from stem loop	Arabidopsis genomic DNA	Gateway LR
overexpression	35S::PHT1;1	pMDC32[1]	PHT1;1 CDS	TOPO-PHT1;1 CDS	Gateway LR
	35S::PHT1;4	pMDC32[1]	PHT1;4 CDS	TOPO-PHT1;4 CDS	Gateway LR
	35S::NLA	pMDC32[1]	NLA CDS	Arabidopsis cDNA	Gateway LR
	35S::NLA^{C279A}	pMDC32[1]	NLA^{C279A} CDS	TOPO-NLA CDS	site-directed mutagenesis/ Gateway LR
	35S::OsNLA1	pMDC32[1]	OS-NLA1 CDS	rice cDNA	Gateway LR
HA-tagged PHT1;1 and point-mutated PHT1;1	35S::PHT1;1-HA	pMDC32[1]	PHT1;1-HA	TOPO-PHT1;1 CDS	Gateway LR
	35S::PHT1;1 ^{K524A}-HA	pMDC32[1]	*PHT1;1* ^{K524A}*-HA*	TOPO-PHT1;1-HA	site-directed mutagenesis/ Gateway LR
	35S::PHT1;1 $^{K276/279A}$-HA	pMDC32[1]	*PHT1;1*$^{K276/279A}$*-HA*	TOPO-PHT1;1-HA	site-directed mutagenesis/ Gateway LR
	35S::PHT1;1 $^{K276/279/524A}$-HA	pMDC32[1]	*PHT1;1* $^{K276/279/524A}$*-HA*	TOPO-PHT1;1$^{K276/279A}$_HA	site-directed mutagenesis/ Gateway LR
fluorescent protein-tagged fusion protein	$PHT1;1_{pro}$::PHT1;1-YFP	pMDC99[1]	PHT1;1 genomic DNA &YFP	Arabidopsis genomic DNA and pK7WGY2	three piece PCR[4] /Gateway LR
	35S::PHT1;1-YFP	pMDC32[1]	PHT1;1 CDS &YFP	TOPO-PHT1;1 CDS and pK7WGY2	three piece PCR[4] /Gateway LR
	35S::PHT1;4-YFP	pMDC32[1]	PHT1;4 CDS &YFP	TOPO-PHT1;4 CDS and pK7WGY2	three piece PCR[4] /Gateway LR
	35S::GFP-NLA	pK7WGF2[2]	NLA CDS	TOPO-NLA CDS	Gateway LR
	35S::NLA-GFP	pMDC83[1]	NLA CDS	TOPO-NLA CDS	Gateway LR
	35S::CFP-NLA	pK7WGC2[2]	NLA CDS	TOPO-NLA CDS	Gateway LR
	35S::CFP-NLA^{C279A}	pK7WGC2[2]	NLA^{C279A} CDS	TOPO-NLA CDS	site-directed mutagenesis/ Gateway LR
	35S::NLA-CFP	pK7CWG2[2]	NLA CDS	TOPO-NLA CDS	Gateway LR
	35S::CFP-NLA^{SPX}	pK7WGC2[2]	NLA^{1-220} CDS	TOPO-NLA^{SPX}	Gateway LR
	35S::CFP-NLA^{RING}	pK7WGC2[2]	$NLA^{158-335}$ CDS	TOPO-NLA^{RING}	Gateway LR
	35S::CFP-OsNLA1	pK7WGC2[2]	Os-NLA1 CDS	TOPO-OsNLA1 CDS	Gateway LR
	35S::OsNLA1-CFP	pK7CWG2[2]	Os-NLA1 CDS	TOPO-OsNLA1 CDS	Gateway LR
bimolecular fluorescence complementation (BiFC)	UBQ_{pro}::NLA-nYFP	pUBC-nYFP[3]	NLA CDS	TOPO-NLA CDS	Gateway LR
	UBQ_{pro}::NLA^{SPX}-nYFP	pUBC-nYFP[3]	NLA^{1-220} CDS	TOPO-NLA^{SPX}	Gateway LR
	UBQ_{pro}::PHT1;1-cYFP	pUBC-cYFP[3]	PHT1;1 CDS	TOPO-PHT1;1 CDS	Gateway LR
	UBQ_{pro}::PHT1;4-cYFP	pUBC-cYFP[3]	PHT1;4 CDS	TOPO-PHT1;4 CDS	Gateway LR
	UBQ_{pro}::CHL1-cYFP	pUBC-cYFP[3]	CHL1 CDS	pENTRTM/D-TOPO-CHL1	Gateway LR
	UBQ_{pro}::IRT1-cYFP	pUBC-cYFP[3]	IRT1 CDS	pDL-NubG-IRT1 (Shin et al., 2013)	Gateway LR
split-ubiquitin yeast-two-hybrid	pTMBV4-PHT1;4	pTMBV4	PHT1;4 CDS	TOPO-PHT1;4 CDS	*Xba*I
	pDL-NubG-NLA^{SPX}	pDL-NX2	NLA^{1-220} CDS	TOPO-NLA^{SPX}	*Bam*HI/*Sma*I

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