Supplemental Information

Internalization of clay-siRNA nanohybrids by intact plant leaf cells efficiently silences a target gene

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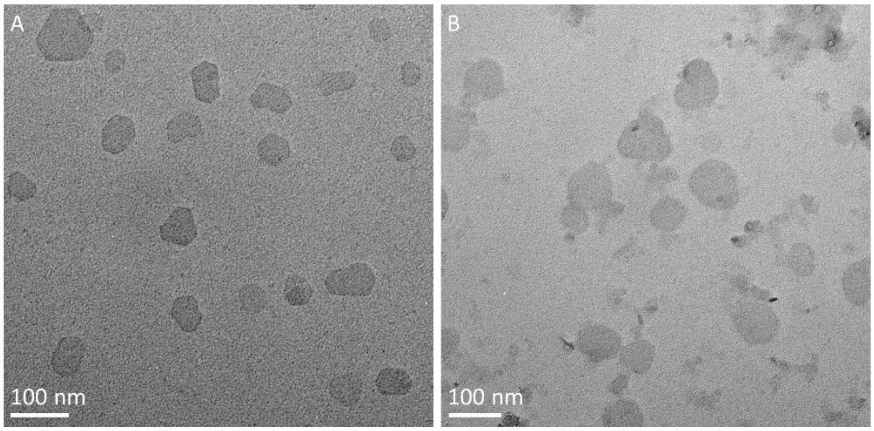
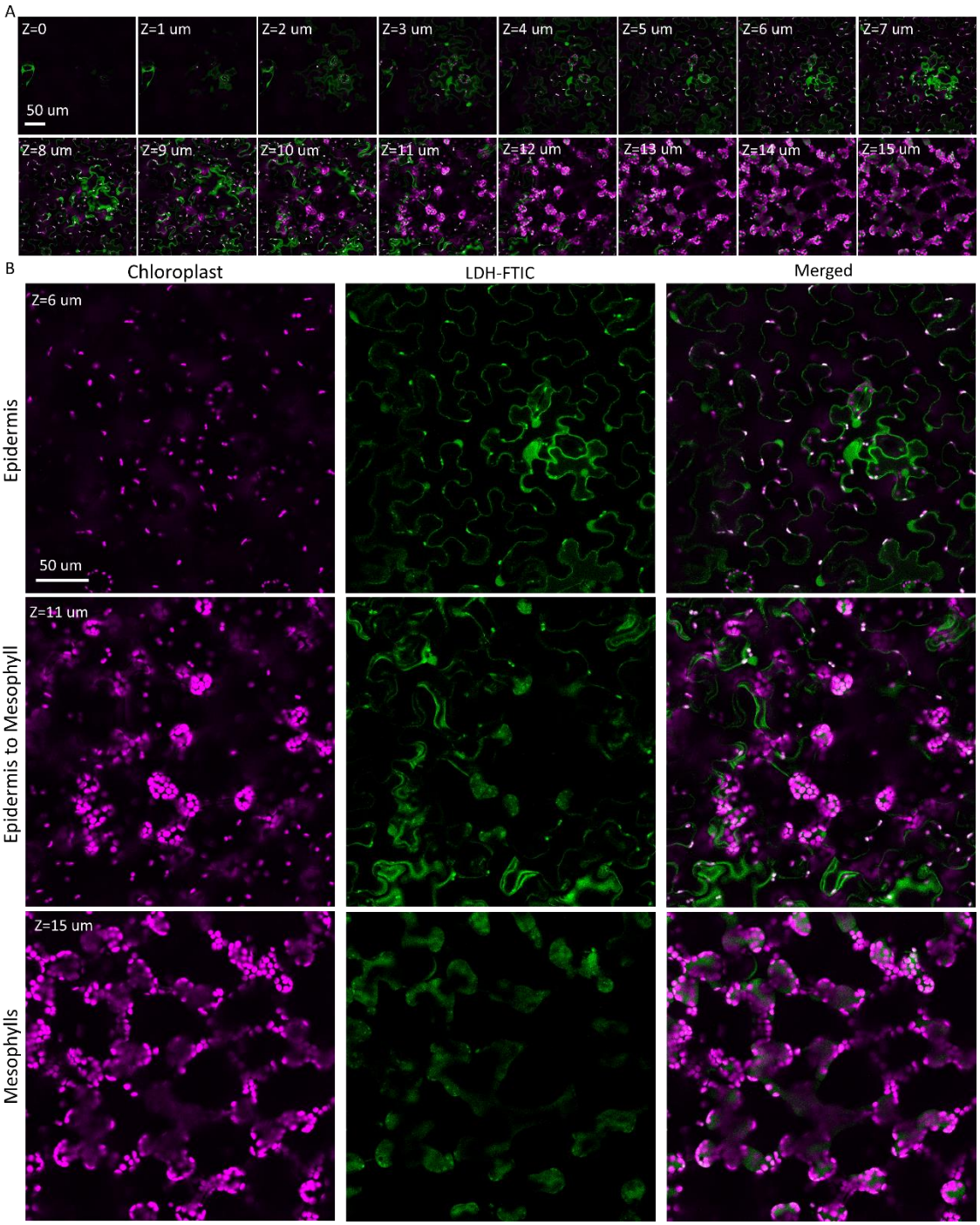
Supplemental figures S1-S20

Supplemental tables S1-S6

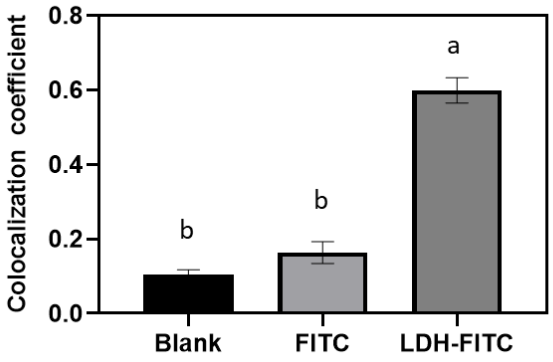
Supplemental datasets 1-3



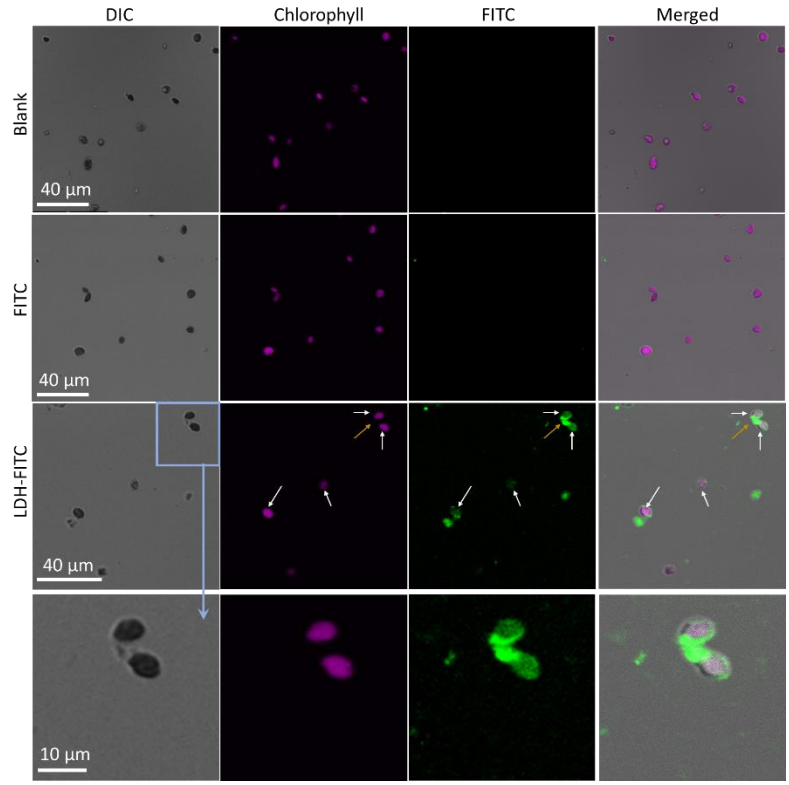
SupplementalFigure S1. Representative TEM images. Representative TEM image of A, LDH-FITC and B, LDH-siRNA.



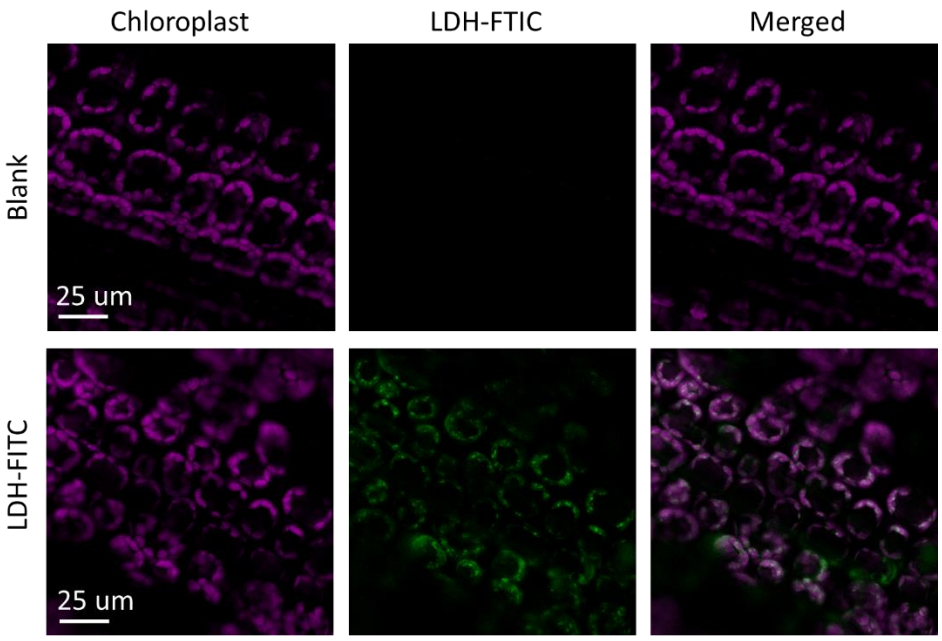
Supplemental Figure S2. Confocal images of *Nicotiana benthamiana* leaves at 90 min after infiltration with 200 mg/L LDH-FITC (100 µl). A, 3D z-stack images. B, Enlarged view of each channel and merged channel at the specific depth, indicating LDH-FITC nanoparticles were internalized by epidermal and chlorophyll cells, and colocalized with chloroplasts in chlorophyll cells.



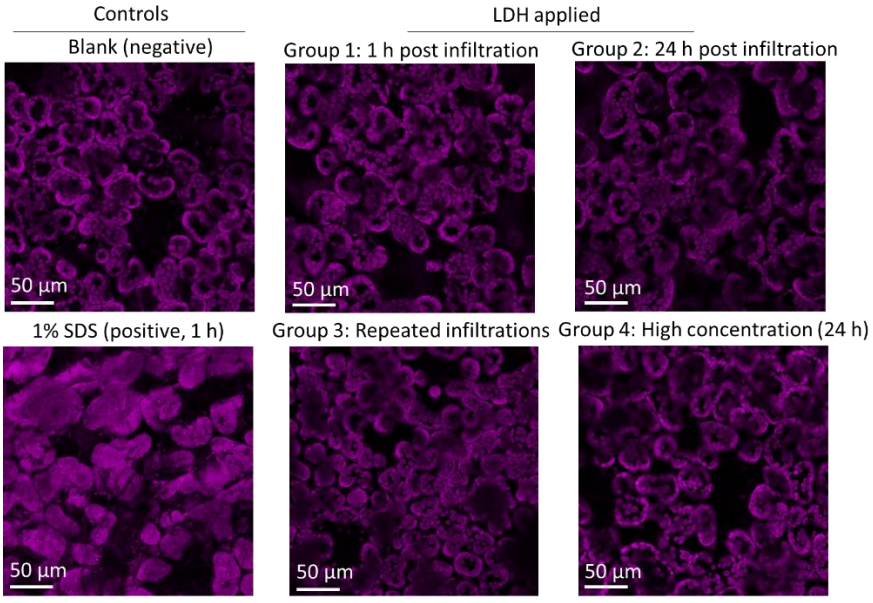
Supplemental Figure S3. Statistical colocalization coefficient of FITC with chloroplasts estimated with Pearson's correlation coefficient, n=3 (different lower case letters above the bars indicate statistical significance with $p<0.05$, by one-way ANOVA with post-hoc Tukey's analysis, data represented as Mean ± SEM).



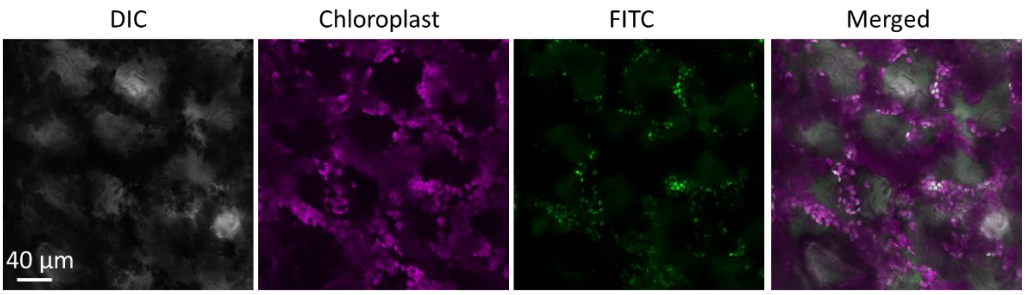
Supplemental Figure S4. Representative confocal images of isolated chloroplasts, showing internalization of LDH-FITC after co-incubation with 200 mg/L LDH-FITC for 90 min. White arrows indicates LDH-FITC were colocalized with the chloroplast, orange arrows indicate LDH-FITC were attached to outside of the chloroplast.



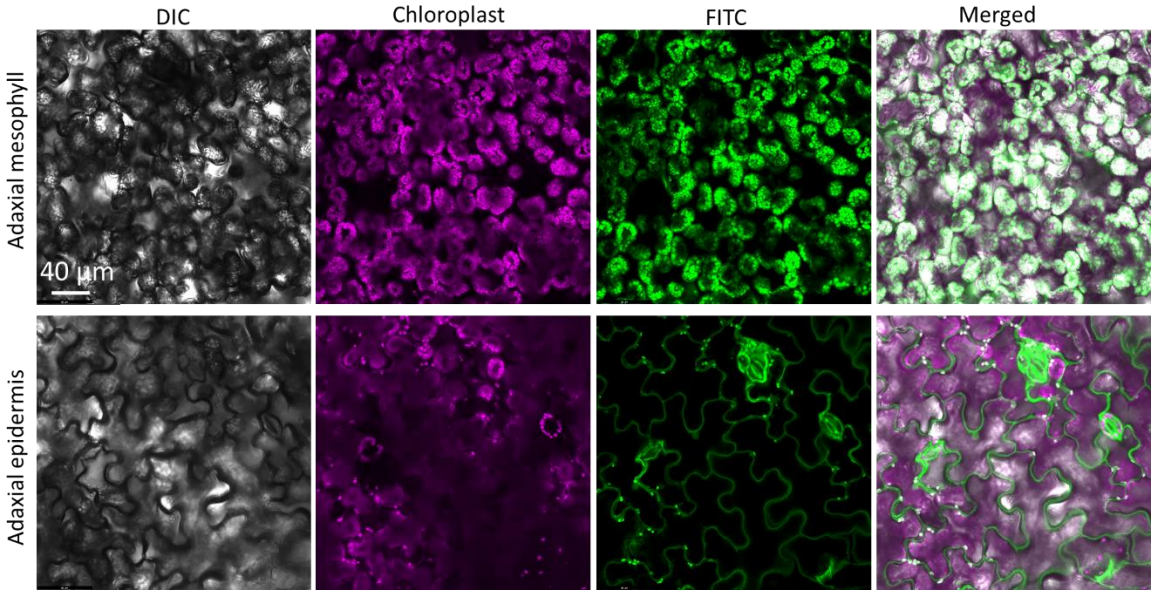
Supplemental Figure S5. Representative confocal microscope images of 4-weeks-old wheat leaf cells internalization of LDH-FITC after infiltration of 25 µl 200mg/L LDH-FITC for 90 min.



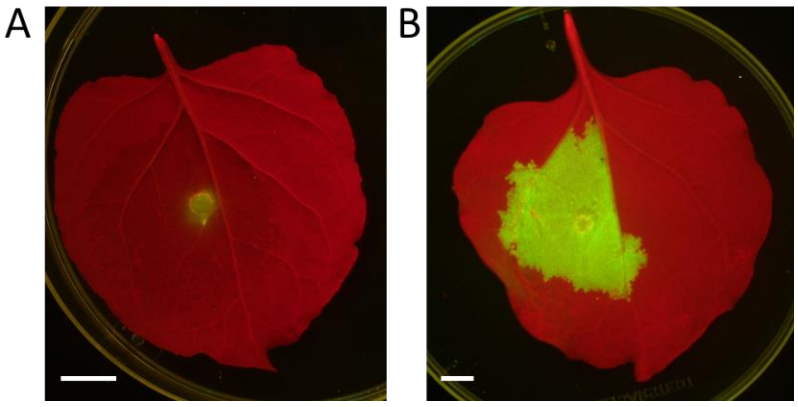
Supplemental Figure S6. Biocompatibility of LDH nanoparticles. Representative confocal microscope images of non-treated Nb leaves and leaves infiltrated with LDHs and 1% SDS (as positive control) after 1 h. LDHs treated groups includes group 1: infiltration of 100 µl of LDH (200 mg/L) after 1 and group 2: 24 h; group 3: repeated infiltration of 100 µl LDH (200 mg/L) at 0 and 48 h and observed at 72 h; and group 4: 100 µl of LDH (5000 mg/L) after 24 h.



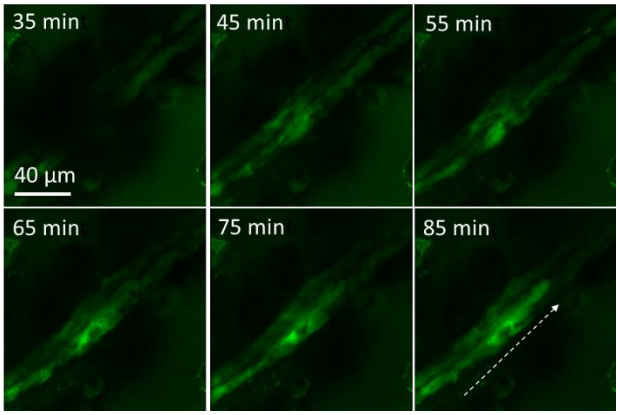
Supplemental Figure S7. Representative confocal images of LDH-FITC entering abaxial mesophyll cells from the extracellular region at ~30 min after infiltrated with 100 μl of LDH-FITC (200 mg/L).



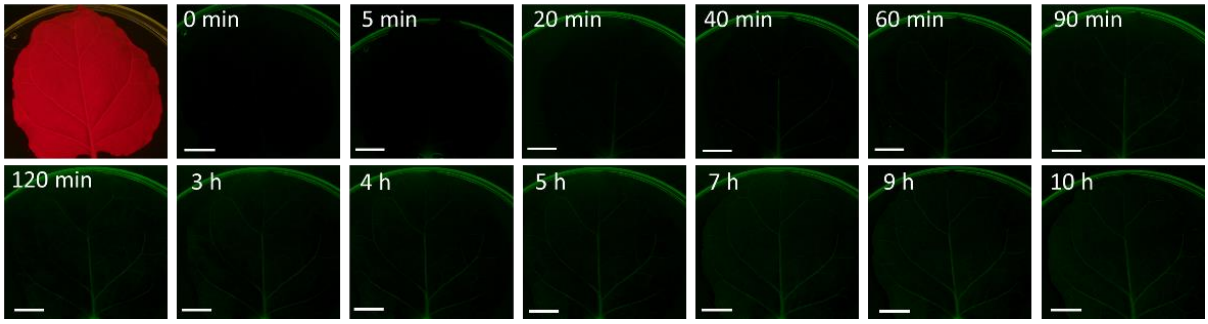
Supplemental Figure S8. Representative confocal images of adaxial side mesophyll and epidermal cells, showing internalization of LDH-FITC at 90 min after infiltrated with 100 μl of LDH-FITC (200 mg/L).



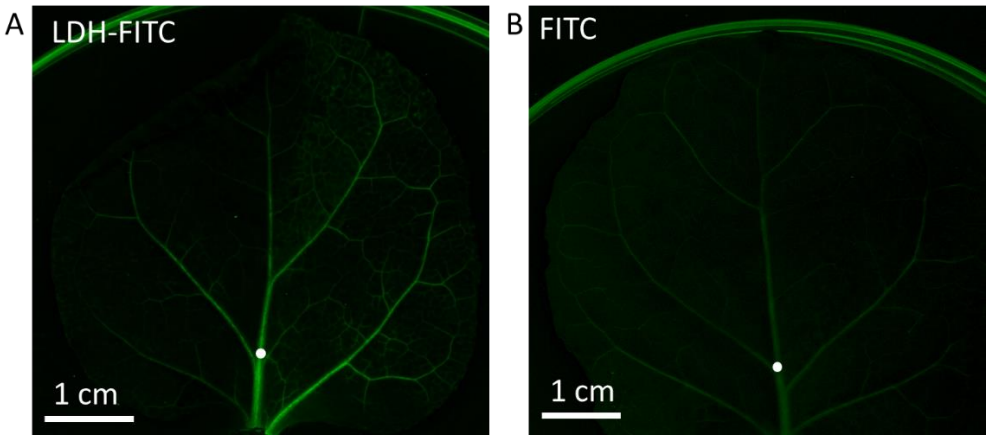
Supplemental Figure S9. Blue light excited images of leaves infiltrated with different nanoparticles. Representative images of leaves infiltrated with A, Large LDH nanoparticles (>100 nm, 200 mg/L, 100 µl) and B, 500 mg/L Quantum dots (3-5 nm) immediately after infiltration (bar, 1 cm).



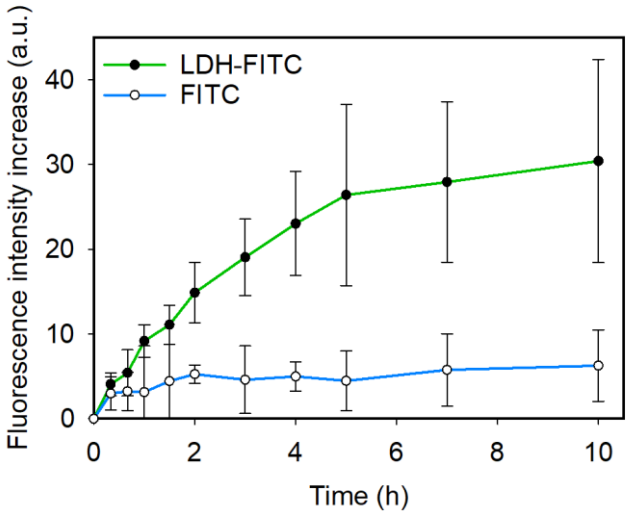
Supplemental Figure S10. Time-course confocal microscope images of LDH-FITC showing translocation and accumulation in vasculatures after infiltration of LDH-FITC (200 mg/L, 100 µl) and possible translocation direction (white arrow).



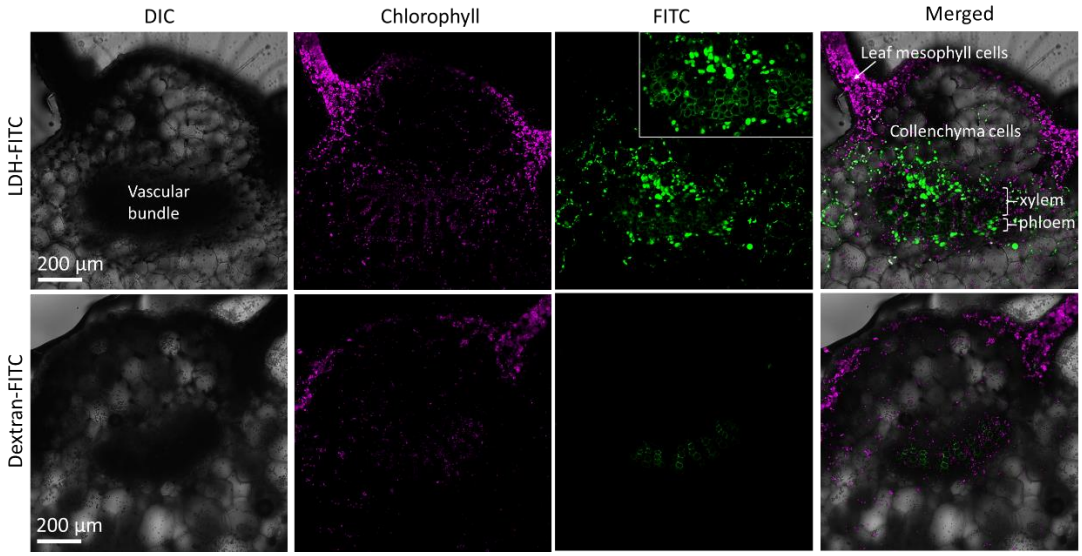
Supplemental Figure S11. Representative images of detached leaves treated with 10 mg/L FITC solution in pH 6.0 MES buffer (bar, 1 cm).



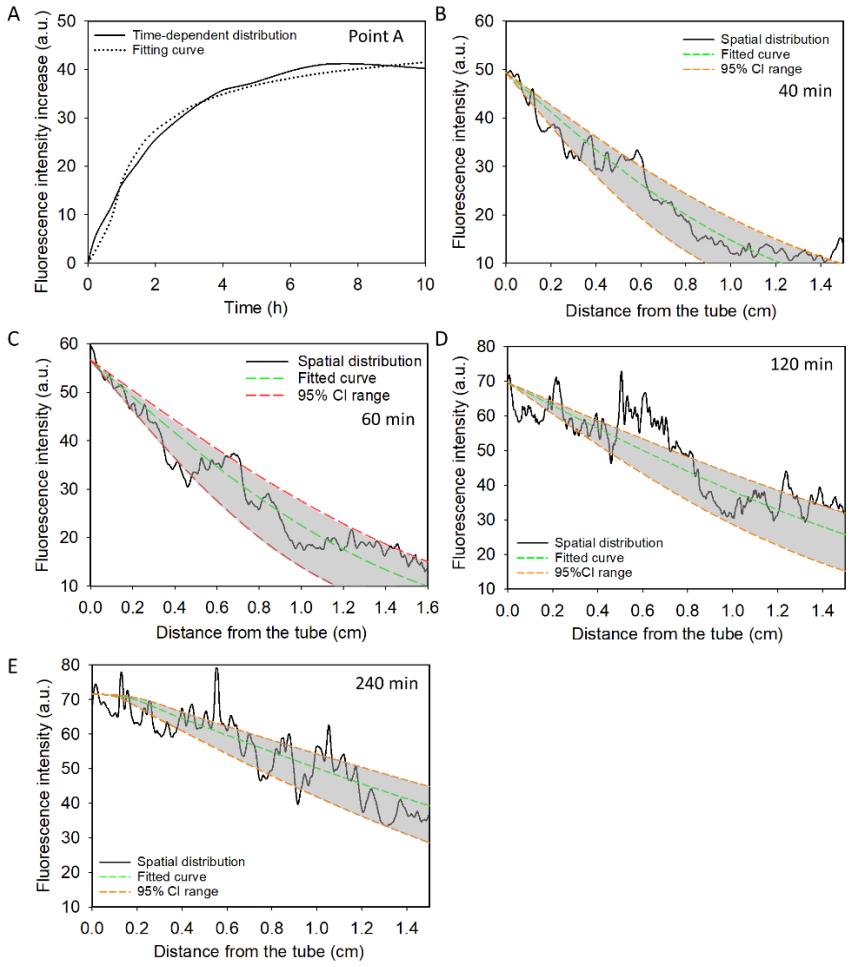
Supplemental Figure S12. Excised leaves petiole treated with nanoparticles. Representative images of excised leaves treated for 10 h with A, LDH-FITC (200 mg/L of LDH, 10 mg/L FITC and 100 µl); and B, FITC (10 mg/L, 100 µl). The panel A is the large version of the same picture in Figure 4A at 10 h, showing FITC signals in tertiary and quaternary veins and B in Figure S10 at 10 h for comparison.



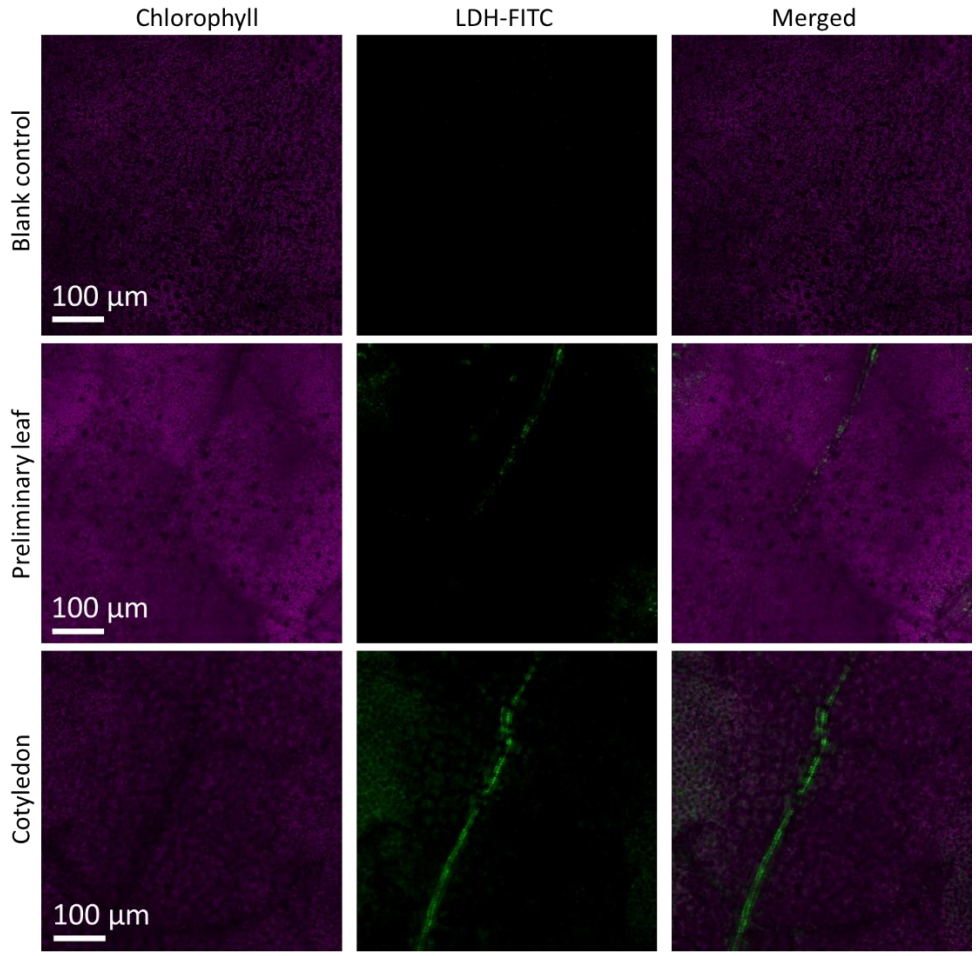
Supplemental Figure S13. Time-dependent fluorescence increase on the main veins at 1.0 cm away from the tube containing LDH-FITC (point B in Figure 4B and white dot in Figure S11A) and FITC (white dot in Figure S12B). Data presented as Mean ± SEM.



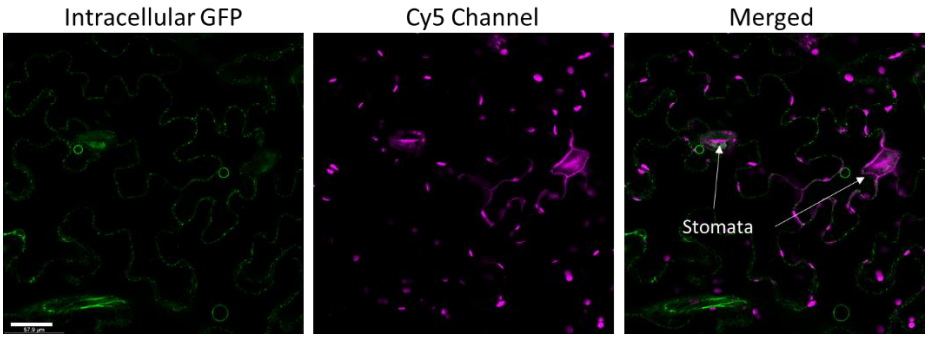
Supplemental Figure S14. Cross section images of FITC signals in midrib of N. benthamiana 1 cm from petiole, 4 h after petiole application of 200 mg/L LDH-FITC and 10 mg/L Dextran-FITC. Inset picture is higher magnification image showing LDH-FITC in xylem and phloem. Green fluorescence intensity in Dextran-FITC group were enhanced (2x) for better visualization.



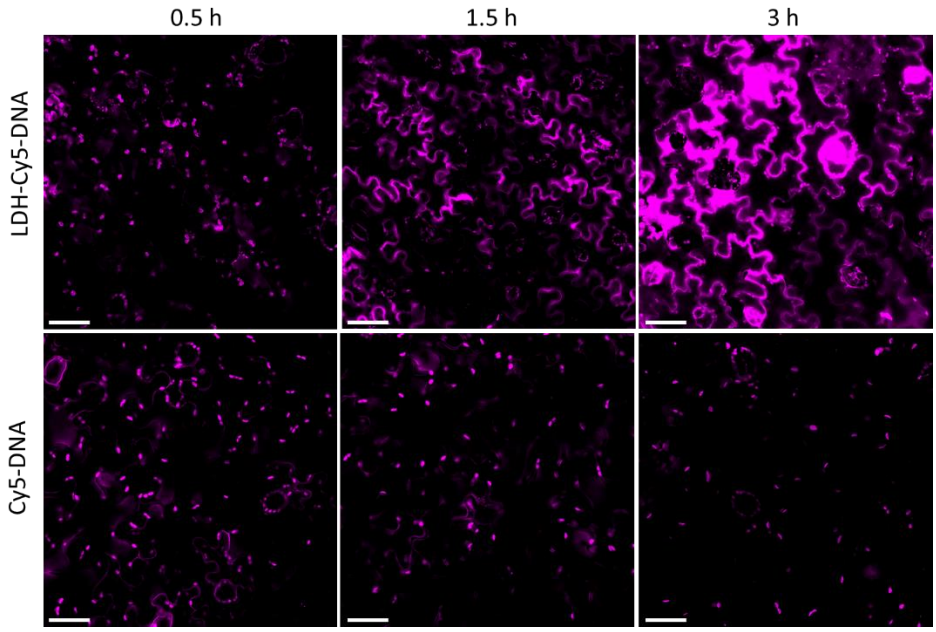
Supplemental Figure S15. Time- and spatial-dependent translocation and accumulation of LDH-FITC in veins. A, Fitting curve of Figure 4C (point A, R^2=0.9734) based on Fick's Law. And representative fitting curve of spatial-dependent accumulation based on Fick's Law with the average fitted diffusion coefficient from the time-dependent accumulation at 15 points at B, 40 min, C, 60 min, D, 120 min, E, 240 min. Green line stands for fitted curve with average diffusion coefficient and grey shadowed area indicating fitted curve with diffusion coefficient within 95% confidence interval (CI) of fitted diffusion coefficient from 15 points (0.1-1.5 cm, 0.1 cm interval). The fitted curves were plotted based approximating of one-dimensional solution of Fick's Law of Diffusion, $C(x,t) = C(0) * erfc\left(\frac{x}{2\sqrt{Dt}}\right)$, where C(x, t) stands for concentration at time t and point x, C(0) stands for concentration on the boundary, x is the distance from the LDH-FITC incubation solution, D the diffusion coefficient and t the time, *erfc* stands for complementary error function ($erfc(z) = 1 - \frac{2}{\sqrt{\pi}}\int_0^z e^{-t^2}$).



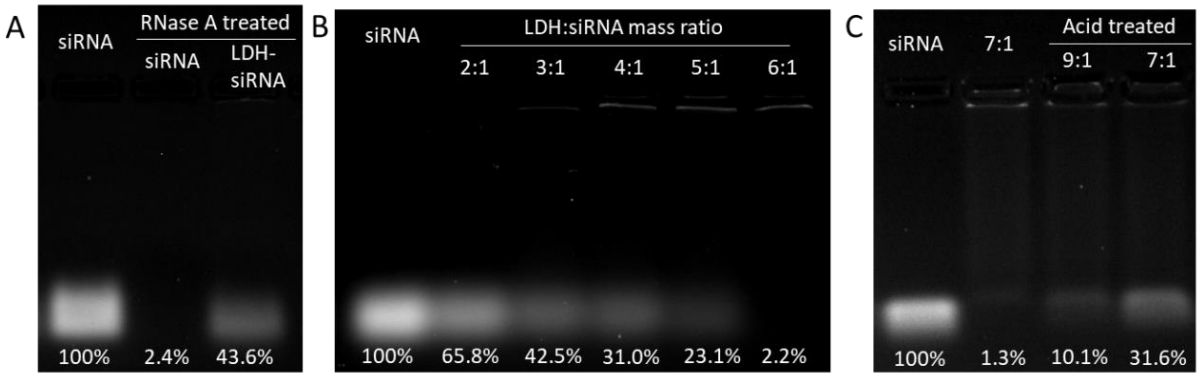
Supplemental Figure S16. Representative confocal images of LDH-FITC translocated to 2-week-old *A. thaliana* seedling cotyledon and young leaf, 10 h after application of 200 mg/L LDH-FITC on stem of excised shoot.



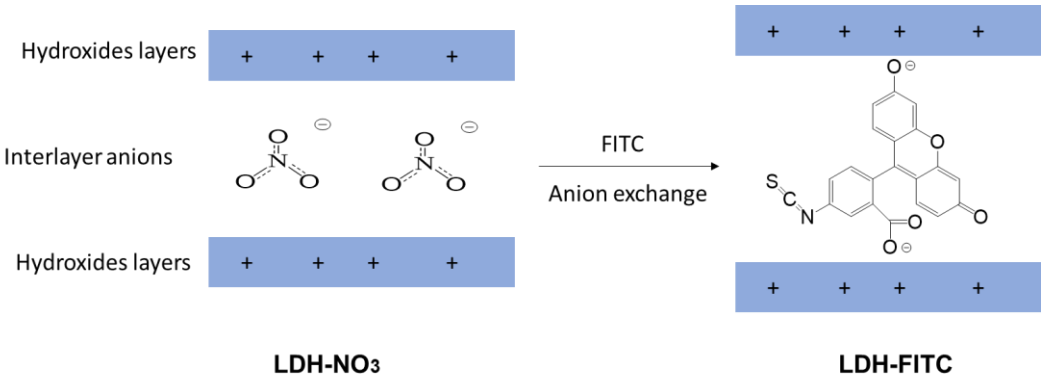
Supplemental Figure S17. Representative confocal images of infiltrated Cy5-DNA (100 µL, 10 mg/L) localized around abaxial side stomata and in stomata chock at 1.5 h post-infiltration. (Bar, 40 µm)



Supplemental Figure S18. Representative confocal images of time-dependent internalization by leaf abaxial epidermal cells after infiltrated with LDH-Cy5-DNA and Cy5-DNA (100 µL, 10 mg/L of Cy5-DNA). (Bar, 40 µm).



Supplemental Figure S19. Gel images on LDH loading, protection and release of siRNA. A, LDH protection of siRNA over RNase A. B, siRNA loading onto LDH nanoparticles at the different LDH:siRNA mass ratios. The complete conjugation of siRNA with LDHs occurred at 6:1, as a minimal amount of free siRNA migrated to the bottom. C, siRNA release from LDH-siRNA at different loading ratio after treatment at pH 6.0 for one day (24 h). A-C, white numbers below the bands indicate the normalized average intensity of each row compared to siRNA.



Supplemental Figure S20. Schematic illustration of FITC intercalated into LDH nanoparticles.

Supplemental Table S1. Summary of characteristic properties of LDH, LDH-FITC and LDH-siRNA.

Sample	Measurement method	Average size (nm)	Average thickness (nm)	ζ-potential (mV)	PDI	Mass loading ratio
LDH	AFM	37.8 ± 11.5 *	5.8 ± 1.0 #	-	-	-
LDH	TEM	40.8 ± 12.0 *	-	-	-	-
LDH	DLS	34.3 ± 2.2	-	43.0	0.155	-
LDH-FITC	DLS	37.7 ± 4.3	-	27.9	0.179	4.2 %
LDH-siRNA	DLS	45.2 ± 10.6	-	24.6	0.406	12.5 %

*The average size from AFM was the mean of 100 particles, the average size from TEM was the mean of more than 200 particles.

#The average thickness was the mean of the measured thickness of 20 measured particles.

Data shown as Mean ± SD.

Supplemental Table S2. Simulated diffusion coefficient (D) and regression coefficient (R^2) of time-dependent curve at each point with 0.1 cm interval.

Position (cm)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
D value (cm^2/h)	0.010	0.043	0.104	0.119	0.253	0.394	0.463	0.332
R^2	0.934	0.973	0.869	0.864	0.945	0.860	0.946	0.880
Position (cm)	0.9	1.0	1.1	1.2	1.3	1.4	1.5	
D value (cm^2/h)	0.444	0.975	0.923	1.219	1.331	1.829	1.985	
R^2	0.878	0.859	0.974	0.915	0.872	0.844	0.883	

Average D = 0.695 cm^2/h; standard deviation = 0.647

Supplemental Table S3. Detail data of GFP gene silencing induced by LDH-delivered siRNA in Figure 6B.

	Blank	800 nM LDH-ns-siRNA	800 nM Naked siRNA	LDH-siRNA (nM)		
				200	400	800
Relative GFP fluorescence intensity (%)	102.8	99.9	87.3	65.1	70.6	51.6
	107.1	97.5	97.1	74.7	90.6	72.1
	90.0	94.1	93.2	91.9	61.4	45.9
Average (%)	100 ± 5.1	97.2 ± 1.7	92.5 ± 2.8	77.2 ± 7.8	74.2 ± 8.6	56.5 ± 7.9

Supplemental Table S4. Detail data of GFP gene silencing induced by LDH-delivered siRNA in Figure 6C.

	Blank	Days post infiltration				
		1	2	3	5	7
Relative GFP fluorescence intensity (%)	83.8	68.3	59.1	48.1	69.0	105.7
	110.7	56.8	53.2	48.5	69.9	96.1
	91.7	64.7	48.2	44.8	75.4	82.5
	113.8	61.5	66.9	68.4	67.1	83.3
Average (%)	100 ± 7.3	62.8 ± 2.4	56.9 ± 4.0	52.5 ± 5.3	70.3 ± 1.8	91.9 ± 5.5

Supplemental Table S5. Detail data of GFP gene silencing induced by LDH-delivered siRNA in Figure 6D.

	Blank	LDH:siRNA mass ratio				
		1:1	3:1	5:1	7:1	9:1
Relative GFP fluorescence intensity (%)	83.8	80.1	85.6	95.4	48.1	66.5
	110.7	97.6	67.4	73.4	48.6	58.9
	91.6	86.0	76.0	57.4	44.8	63.4
	113.8	83.2	71.7	50.7	68.4	55.8
Average (%)	100 ± 7.3	86.7 ± 3.8	75.2 ± 3.9	69.2 ± 9.9	52.5 ± 5.3	61.2 ± 2.4

Supplemental Table S6. Detail data of GFP gene silencing induced by LDH-delivered siRNA in Figure 6E.

	Blank	800 nM Naked siRNA	800 nM LDH-siRNA
Relative *GFP* mRNA level in entire leaf (%)	100	79.8	23.5
	100	88.9	22.2
	100	71.9	22.1
Average (%)	100	80.2 ± 4.9	22.6 ± 4.5

Supplemental dataset.

Supplemental Dataset 1: Cy5-DNA sequence (5'→3'):

TTCTCCGAACGTGTCACGTTT

Supplemental Dataset 2: siRNA sequence (5'→3'):

siRNA antisense: UUC CGU AUG UUG CAU CAC CTT
siRNA sense: GGU GAU GCA ACA UAC GGA ATT

ns-siRNA sense: GGU UGC CAU CAA GAA AGU
ns-siRNA antisense: UUA ACU UUC UUG AUG GCA

Supplemental Dataset 3: RT-qPCR primers (5'→3'):

EF1 forward: TGG TGT CCT CAA GCC TGG TAT GGT TG
EF1 reverse: ACG CTT GAG ATC CTT AAC CGC AAC ATT CTT
mGFP5 forward: AGT GGA GAG GGT GAA GGT GAT G
mGFP5 reverse: GCA TTG AAC ACC ATA AGA GAA AGT AGT G