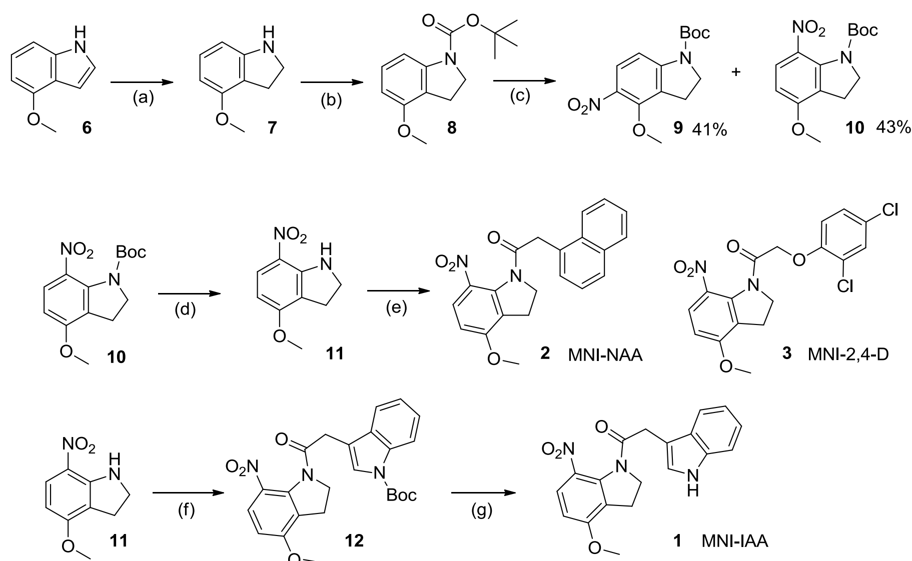
Supplemental information

Development of 4-methoxy-7-nitroindolinyl (MNI)-caged auxins which are extremely stable in planta

Ken-ichiro Hayashi [a*], Naoyuki Kusaka [a], Soma Yamasaki [a], Yunde Zhao[b], Hiroshi Nozaki [a]

a Department of Biochemistry, Okayama University of Science, Okayama, 700-0005 Japan
b Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA 92093-0116, United States



Experimental Section

General: UV spectra were recorded on a V-630 spectrophotometer (JASCO, Japan). ^{1}H and ^{13}C NMR were run on JEOL ECS400 spectrometer (JEOL, Japan), with chemical shifts shown as δ-values from TMS as the internal reference. Peaks multiplicities are quoted in Hz. Mass spectra were measured on a JMS-700 spectrometer (JEOL, Japan).

4-methoxy-2,3-dihydro-indole-1-carboxylic acid *tert*-butyl ester (8)

4-methoxyindoline (**7**) was synthesized according to the reported procedures. To 4-methoxyindole (**6**) (500 mg, 3.4 mmol) in acetic acid (5.0 mL), sodium cyanoborohydride (236 mg, 5.5 mmol) was slowly added over 10 min, the mixture was stirred at room temperature for 3h. This reaction mixture was adjusted to pH10 by the addition of 0.5N sodium hydroxide and then extracted with EtOAc (20 mL × 3). The organic layer was washed with brine (10 mL). After dried over Na_2SO_4, the layer was concentrated *in vacuo*. The residue was purified by a silica gel column chromatography [c.c.], hexane–EtOAc (8:2) to give 4-methoxyindoline **(7)** (497 mg, Yield 98 %) as a white solid. To the solution of 4-methoxyindoline **(7)** (493 mg, 3.30

mmol) and triethylamine (0.7 mL, 5.0 mmol) in dry THF (10 mL), di-*tert*-butyl dicarbonate 1.9mL (8.3 mmol) was added and then stirred for 3 h at room temperature. The reaction mixture was poured into the saturated NH_4Cl solution (10 mL) and then extracted with EtOAc (3 × 20ml). The EtOAc layer was washed with brine (10 mL) and dried over Na_2SO_4 and then concentrated in vacuo. This residue was purified by a silica gel c.c. (hexane–EtOAc=9:1) to give 4-methoxy-2,3-dihydro-indole-1-carboxylic acid tert-butyl ester (**8**) (765 mg, Yield 93%) as a white powder: mp 108-111°C; ^{1}H-NMR (400MHz, $CDCl_3$): δ_H=7.13 (1H, t, J=8.2), 6.49 (1H, d, J=8.7), 3.98 (2H, t, J=8.2), 3.82 (3H, s), 2.99 (2H, t, J=8.3), 1.55 (9H, s); ^{13}C-NMR(100 MHz, $CDCl_3$): δ_C=155.82, 152.5, 128.68, 107.77, 104.52, 55.23, 47.98, 28.41, 24.34. Positive FAB MS *m/z* 250 $[M+H]^+$, HR-FAB MS: calcd. for $C_{14}H_{20}NO_3$: 250.1443 $[M+H]^+$, found for 250.1469.

7-Methoxy-4-nitro-2,3-dihydro-1H-indole-3-carboxylic acid *tert*-butyl ester (9)

To the solution of **8** (760 mg, 3.1 mmol) and silver nitrate $AgNO_3$ (1000 mg, 5.9 mmol) in CH_3CN (12 mL), acetyl chloride (480 mg, 6.5 mmol) in CH_3CN (3mL) was slowly added and then stirred 2h at room temperature. The resulting solution was poured into saturated sodium carbonate solution, and extracted with EtOAc. The organic layer was washed with brine, and then dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by a silica gel C.C. (hexane–EtOAc=8:2) to give **9** as pale yellow oil (387 mg, 43% yield) ^{1}H-NMR (400MHz, $CDCl_3$): δ_H= 7.77 (1H, d, J=9.1), 6.59 (1H, d, J=9.1), 4.20 (2H, t, J=8.3), 3.91 (3H, s), 2.99 (2H, t, J=8.3), 1.47 (9H, s); ^{13}C-NMR(100 MHz, $CDCl_3$): δ_C= 158.93, 152.4, 137.91, 134.12, 125.79, 122.76, 105.37, 82.63, 55.88, 50.47, 28.03, 25.52; Positive FAB MS *m/z* 295 $[M+H]^+$, HR-FAB MS: calcd. for $C_{14}H_{18}N_2O_5$: 295.1294 $[M+H]^+$, found for 295.1306. and regiosiomer **10** (371 mg, 41% yield) as yellowish powder.

4-Methoxy-7-nitroindoline (11)

Compound **9** (150 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (2 mL) and trifluoroacetic acid (0.2mL) was added at 0ºC then stirred for 30 min at 0ºC. The reaction solution was further stirred for 3h at room temperature. The resulting solution was poured into saturated sodium carbonate solution, and extracted with EtOAc. The organic layer was washed with brine, and then dried over Na_2SO_4, evaporated under reduce pressure. The residue was purified by a silica gel C.C. (n-hexane:EtOAc=8:2) to give **11** as pale brownish powder (92mg, 93% yield); mp 118-121°C; ^{1}H-NMR (400MHz, $CDCl_3$): δ_H= 7.85 (1H, d, J=9.5), 6.26 (1H, d, J=9.5), 3.89 (3H, s), 3.86 (2H, t, J=8.8), 3.09 (2H, t, J=8.8); ^{13}C-NMR(100 MHz, $CDCl_3$): δ_C= 159.86, 150.84, 125.48, 125.01, 116.17, 102.18, 55.74, 47.14, 25.64. Positive FAB MS *m/z* 195 $[M+H]^+$, HR-FAB MS: calcd. for $C_9H_{11}N2O_3$: 195.077 $[M+H]^+$, found for 195.0796.

1-(4-Methoxy-7-nitro-2,3-dihydro-indol-1-yl)-2-naphthalen-1-yl-ethanone, MNI-NAA (2)

To the solution of 4-methoxy-7-nitroindoline (**11**) 15 mg (0.077 mmol) and 1-napthalene acetic acid 30mg (2eq, 0.154 mmol) in toluene (1 mL), thionyl chloride (50 μL) was added and then stirred 8h at 75ºC. The resulting solution was added dropwise to aqueous sodium carbonate solution, and extracted with EtOAc. The organic layer was washed with brine, and then dried over Na_2SO_4, evaporated in vacuo. The residue was purified by a silica gel c.c. ($CHCl_3$:EtOAc=95:5) to give 2 as pale yellow powder (16 mg, 56% yield): ^{1}H-NMR (400MHz, $CDCl_3$): δ_H=8.01 (1H, d, J=8.5), 7.87 (1H, d, J=8.3), 7.80 (1H, d, J=7.6), 7.75 (1H, d, J=9.0), 7.59 (1H, t, J=8.0, 8.3), 7.52 (1H, t, J=8.0, 8.5), 7.46-7.41 (2H, m), 6.63 (1H, d, J=9.0), 4.28 (2H, s), 4.10 (2H, t, J=8.1), 3.88 (3H, s), 2.99 (2H, t, J=8.1); ^{13}C-NMR(100 MHz, $CDCl_3$): δ_C= 169.63, 158.69, 136.73, 135.4, 133.84, 131.94, 130.3, 128.72, 128.08, 127.03, 126.58, 126.02, 125.49, 125.49, 123.69, 122.66, 106.32, 55.94, 50.38, 40.83, 26.40; Positive FAB MS *m/z* 363 $[M+H]^+$, HR-FAB MS: calcd. for $C_{21}H_{19}N_2O_4$: 363.1345 $[M+H]^+$, found for 363.1321; UV λmax MeOH: 244 nm (ε= 20,580), 249 nm (ε= 26,360), 255 nm (ε= 27,840), 261 nm (ε= 20,150).

2-(2,4-dichlorophenoxy)-1-(4-methoxy-7-nitroindolin-1-yl)ethanone, MNI-2,4-D (3)

To the solution of 4-methoxy-7-nitroindoline (**11**) 15 mg (0.077 mmol) and 2,4-dichlorophenoxyacetic acid 34 mg (0.154 mmol) in toluene (1 mL), thionyl chloride (50 μL) was added and then stirred 8h at 75ºC. The resulting solution was added dropwise to aqueous sodium carbonate solution, and extracted with EtOAc. The organic layer was washed with brine, and then dried over Na_2SO_4, evaporated in vacuo. The residue was purified by a silica gel c.c. ($CHCl_3$:EtOAc=95:5) to give **3** as pale yellow powder (16 mg, 56% yield): ^{1}H-NMR (400MHz, $CDCl_3$): δ_H=7.78 (1H, d, J=9.1), 7.36 (1H, s), 7.28 (1H, d, J=9.0), 7.00 (1H, d, J=9.0), 6.66 (1H, d, J=9.1), 4.88 (2H, s), 4.36 (2H, t, J=7.9), 3.91 (3H, s), 3.09 (2H, t, J=7.9); ^{13}C-NMR(100 MHz, $CDCl_3$): δ_C= 167.33, 159.04, 151.50, 136.33, 134.97, 130.04, 127.99, 127.08, 125.66, 123.06, 122.93, 114.44, 106.76, 68.98, 56.01, 50.24, 26.65; Positive FAB MS *m/z* 397 $[M+H]^+$, HR-FAB MS: calcd. for $C_{17}H_{15}N_2O_5$: 397.0358 $[M+H]^+$, found for 397.0361; UV λmax MeOH: 244 nm (ε= 18,100), 249 nm (ε= 19,900), 254 nm (ε= 17,340), 260 nm (ε= 12,680).

3-[2-(4-Methoxy-7-nitro-2,3-dihydro-indol-1-yl)-2-oxo-ethyl]-indole-1-carboxylic acid tert-butyl ester (12)

To the solution of 4-methoxy-7-nitroindline **(11)** 20mg (0.1 mmol) and 2-(1-(*tert*-butoxycarbonyl)-1H-indol-3-yl)acetic acid 57mg (2eq, 0.2 mmol), thionyl chloride (50 μL) was added and then stirred 6h at 70ºC. The resulting solution was added dropwise to

aqueous sodium carbonate solution, and extracted with EtOAc. The organic layer was washed with brine, and then dried over Na_2SO_4, evaporated in vacuo. The residue was purified by a silica gel c.c. ($CHCl_3$:EtOAc=95:5) to give **12** as pale yellow powder (38 mg, 81% yield): ^{1}H-NMR (400MHz, $CDCl_3$): δ_H=8.14 (1H, d, J=8.4), 7.73-7.66 (3H, m), 7.32 (1H, t, J=7.6), 7.24 (1H, t, J=7.6), 6.87 (1H, d, J=8.8), 4.42 (2H, t, J=8.4), 4.02 (2H, s), 3.95 (3H, s), 3.11 (2H, t, J=7.6), 1.67 (9H, s); ^{13}C-NMR(100 MHz, $CDCl_3$): δ_C=169.38, 159.71, 150.24, 137.65, 136.33, 136.22, 131.38, 125.72, 125.25, 125.14, 123.95, 123.23, 120.54, 115.70, 114.95, 107.38, 84.21, 56.53, 50.97, 33.06, 28.22, 26.95; Positive FAB MS *m/z* 452 $[M+H]^+$, HR-FAB MS: calcd. for $C_{24}H_{26}N_3O_3$: 452.1822 $[M+H]^+$, found for 452.1826.

2-(1H-indol-3-yl)-1-(4-methoxy-7-nitroindolin-1-yl)ethanone, MNI-IAA (1)

Compound **12** (24 mg, 0.052 mmol) was dissolved in CH_2Cl_2 (1 mL) and trifluoroacetic acid (0.1mL) was added at 0ºC then stirred for 30 min at 0ºC. The reaction solution was further stirred for 3h at room temperature. The resulting solution was poured into saturated sodium carbonate solution, and extracted with EtOAc. The organic layer was washed with brine, and then dried over Na_2SO_4, evaporated under reduce pressure. The residue was purified by a silica gel C.C. ($CHCl_3$:acetone=95:5) to give **1** as pale yellow powder (17mg, 94% yield); ^{1}H-NMR (400MHz, $CDCl_3$): δ_H= 8.27 (1H, s), 7.74 (1H, d, J=9.0), 7.78 (1H, d, J=8.1), 7.34 (1H, d, J=8.1), 7.12-7.20 (2H, m), 6.60 (1H, d, J=9.0), 4.13 (2H, m), 3.95 (2H, s), 3.87 (3H, s), 2.95 (2H, t, J=8.0); ^{13}C-NMR(100 MHz, $CDCl_3$): δ_C= 170.12, 158.71, 136.89, 136.05, 135.31, 127.02, 125.39, 123.12, 122.74, 122.21, 119.67, 118.43, 111.33, 107.91, 106.19, 55.93, 50.28, 33.29, 26.34. Positive FAB MS *m/z* 352 $[M+H]^+$, HR-FAB MS: calcd. for $C_{19}H_{18}N_3O_4$: 352.1219 $[M+H]^+$, found for 352.1321. UV λmax MeOH: 249 nm (ε= 21,900), 255 nm (ε= 22,790), 261 nm (ε= 18,030).

Plant materials

The *Arabidopsis thaliana* ecotype Columbia and transgenic Arabidopsis reporter line *DR5::GUS* were used for all experiments. Plants were grown vertically in GM agar medium (0.5 x Murashige and Skoog salts [Gibco BRL], 1% sucrose, 1x B5 vitamins, 0.5 g/L 2-(4-morpholino)-ethanesulfonic acid (MES), and 14 g/L agar pH 5.8) for 5-6 days under continuous light at 24°C.

In vitro uncaging rate of MNI-caged auxins

Caged auxins solution (100 µM in 85% aqueous EtOH) in a 1 cm quartz cell was irradiated with UV light (365 nm) with a fluorophotometer (Shimadzu RF-1500, Japan). Aliquots of the irradiated solution were analyzed by reversed-phase HPLC at regular intervals during photolysis.

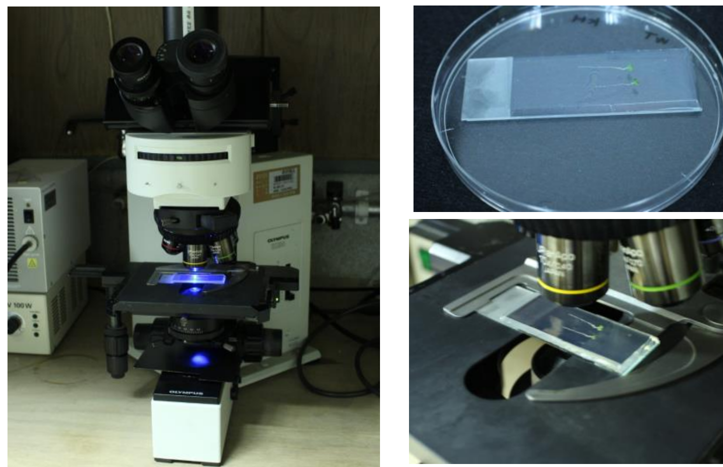
The analysis condition are indicated as follow; For analysis of MNI-IAA: YMC-Pack ODS-A column (4.6 mm ID × 250 mm, YMC Co., Ltd., Japan); Flow rate = 0.5 mL/min; Mobile phase: MeOH:H_2O=3:2 + 0.5% AcOH; Detection: UV 254nm;. The retention times of MNI-IAA and IAA were 22.8 min and 9.8 min, respectively. For analysis of MNI-NAA, YMC-Pack ODS-A column (4.6 mm ID × 250 mm, YMC Co., Ltd., Japan); Flow rate = 0.5 mL/min; Mobile phase: MeOH:H_2O=2:1 + 0.5% AcOH; Detection: UV 254nm. The retention times of MNI-NAA and NAA were 28.2 min and 14.0 min, respectively For analysis of MNI-2,4-D, YMC-Pack ODS-A column (4.6 mm ID × 250 mm, YMC Co., Ltd., Japan); Flow rate = 0.5 mL/min; Mobile phase: MeOH:H_2O=4:1 + 0.5% AcOH; Detection: UV 254nm. The retention times of MNI-2,4-D and 2,4-D were 15.0 min and 9.6 min, respectively

Uncaging in culture medium and in vivo stability of caged auxins

Caged auxins were dissolved in GM liquid medium. The GM media containing the caged auxins were then irradiated for 15 min using a 16 W UV hand lamp that was equipped with a band-pass filter (350-400 nm) at a distance of 5 cm (1820 $\mu W/cm^2$) from the solution. The *DR5::GUS* seedlings (n=10) were incubated in the photolyzed caged auxin media or non-irradiated media at 24°C for 5 h in the dark. After *DR5::GUS* induction, the seedlings were washed with GUS staining buffer and then histochemically stained by X-Gluc. For quantitative measurement of the GUS enzyme activity, the seedlings (n=15) were immediately frozen until the assay was performed.

In vivo Photolysis of intercellular caged auxins

To load the caged auxins into cell, the seedlings were incubated in a liquid GM medium containing caged auxins for 30 min. After washing with a fresh GM medium, the seedlings were placed on the surface of GM agar medium solidified over slide glass. The light (360-370 nm) was irradiated to root for 4-5 s by a fluorescent microscopy (Olympus BX50) with a high-pressure marcury lamp, and then incubated for additional 5 h in a culture dish under dark. For control treatment, the seedlings were treated with same procedures without light-irradiation. After incubation, the induced *DR5::GUS* reporter enzyme was histochemically stained and microscopic pictures were recorded (Olympus SZX16).



Spatiotemporal manipulation of cellular auxin level in planta

5-days-old *DII-VENUS* root was incubated in liquid GM medium containing auxin biosynthesis inhibitors, L-kynurenine (5 μM) and yucasin (50 μM) for 5 h under continuous light at 24°C. The seedlings were immersed in GM medium containing 10 μM MNI-IAA and biosynthesis inhibitor for 20 min. The roots were immediately irradiated for 4-5 s by a fluorescent microscopy (Olympus BX50) with a high-pressure marcury lamp (360 nm light) as a spot illumination or to whole root. For the control, the roots were immersed in 2μM IAA within a slide glass covered with cover slip. The degradation of nuclear-localized DII-VENUS protein was monitored by fluorescent microscopy with YFP filter set at 2, 20, and 40 min after exposure and IAA treatment.

Histochemical and quantitative measurements of GUS reporter activity

For histochemical GUS enzyme staining, the transgenic *DR5::GUS* seedlings after hormonal induction were washed with a staining buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 0.1 % Triton X-100) and transferred to a staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), the

substrate for histochemical staining. The seedlings were then incubated at 37 °C until sufficient staining developed (3-4 h). For quantitative measurements, after induction of GUS reporter gene, the seedling (n = 15) were homogenized in an extraction buffer. GUS activity of the supernatant after centrifugation was measured by a fluorophotometer (*Ex* 365nm, *Em* 455nm) using 1 mM 4-methyl umbelliferyl *β*-D-glucuronide as a fluorogenic substrate at 37 °C. The protein concentration was determined by Bradford protein assay (Bio-Rad, Japan).

Light control of auxin-response in root by using MNI-IAA

For primary root growth assay, 5-days old wild type Col mutant seedlings were incubated in GM liquid media containing 10 μM MNI-IAA for 20 min. After washing with a fresh medium, seedlings were placed vertically on GM agar plate. The seedlings were then irradiated for 5 min by a 16 W UV hand lump (350-400 nm, 1820 $\mu W/cm^2$)) at distance 5 cm from plate. After photolysis, the seedlings were vertically cultured at 24°C under dark. The picture was taken at indicated time. For restoration assys using *yuc 3 5 7 8 9*mutant, 6-days old wild type Col and *yuc* Q mutant seedlings were incubated in GM liquid media containing 50 μM MNI-IAA for 20 min. After inbucation, the seedlings were placed on GM agar medium solidified over slide glass. The light (360-370 nm) was irradiated to root tip for 3-5 s by a fluorescent microscopy (Olympus BX50) and then incubated for additional 15 h in a culture dish under dark.