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Manipulation of Intracellular Auxin in a Single Cell by Light with Esterase-Resistant Caged Auxins

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Auxin, a plant hormone, is polar transported from its site of production. This auxin polar transport system establishes an auxin gradient in plant tissue that is necessary for proper plant development. Therefore, the spatial effect of the auxin gradient on plant development is highly important for the understanding of plant auxin responses. Herein we report the design, syntheses and biological properties of esterase-resistant caged auxins. The conventional caging group, 2-nitrobenzyl ester, was found to be enzymatically hydrolyzed in plant cells and released original auxin without photolysis. The esterase-resistant caging group, (2,5-dimethoxyphenyl)(2-nitrobenzyl) ester, (DMPNB) was designed to improve the stability of caged auxins. Three auxins, indole 3-acetic acid, naphthalene 1-acetic acid and 2,4-dichlorophenoxy acetic acid were caged with the DMPNB caging group. DMPNB-caged auxins were inactive within a plant cell until photolysis, but they release auxins with photoirradiation to activate auxin-responsive gene expression. We demonstrated spatial and temporal control of intracellular auxin levels with photoirradiation by using this caged auxin system and were able to photocontrol the physiological auxin response in Arabidopsis plants. Additionally, the photoirradiation of DMPNB-caged auxin within a single cell can manipulate the intracellular auxin level and triggers auxin response.

Introduction

Auxins regulate many aspects of plant development, including cell division, elongation and differentiation.[1,2] Indole 3-acetic acid (IAA), a natural auxin, is largely biosynthesized in shoot apical regions and then is polar transported to its responsive site.[3] This auxin polar transport system is necessary for proper plant development, especially in tropic growth, vascular development, lateral root formation, embryo and root development as well as apical dominance.[4] Molecular biology and genetic studies have demonstrated that auxin transport is modulated by the PIN family of auxin efflux proteins,[5] the AUX1 family of auxin influx proteins,[6] and the ABC-type multidrug resistance *p*-glycoproteins (ABCB/PGP).[7] These carrier and transporter proteins, especially PIN carrier and ABCB/PGP transporter proteins, drive the asymmetric distribution of auxin, called the auxin gradient, which causes asymmetric cell division and elongation leading to proper plant responses for survival (gravitropism, phototropism and apical hook formation). The PIN efflux carriers and ABCB/PGP transporters are polar localized at plasma membrane and determine the direction and rate of intracellular auxin movement.[5,8] Auxin itself has been revealed to inhibit PIN endocytosis and to promote PIN localization at the plasma membrane.[5,8] Many environmental stimuli and auxin itself regulate PIN localization, and this leads to asymmetric auxin accumulation. However, the regulatory mechanism of polar localization of PIN proteins is very complex and dynamic, and auxin exerts multiple effects on different tissues and cell types. Therefore, these complicated machineries prevent the precise and systematic analysis of plant auxin responses. Recently, new approaches with mathematical computational models have been applied to complement the indirect evidence for a physiological role of local auxin gradients in embryo patterning and root response.[9] However, the underlying mechanisms for the physiological role of local auxin gradients are still a matter of debate.

Here, we report that caged auxins are useful tools with which to investigate complex physiological auxin responses. Caged compounds are inactivated bioactive molecules with functional groups blocked by a photocleavable protecting group (caging group). The original bioactive molecules can be rapidly released upon photolysis with UV illumination in a fluorescence microscope, or with UV lasers and flash lamps (normally 350–360 nm ultraviolet light). Caged molecules have now become a standard tool for biological research. Diverse biologically active molecules, ATP (and many other nucleotides), calcium, IP_3, glutamate (and many other neurotransmitters), and peptides have been caged and are widely utilized as essential chemical tools for biologists.[10,11] Due to the nature of caged molecules, 1) uncaging can be intracellular and very fast, 2) the spatial and temporal resolution can be precisely controlled by optical devices, and 3) the intracellular concentration of released active compounds can be highly regulated by period and strength of light irradiation. Therefore, caged

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auxins would have many useful features and advantages in comparison with classical methods of auxin application, such as a solution, a waxy paste, or an agar-block-containing auxin. For instance, by controlling light irradiation, auxin can be directly and locally loaded into any position at any time during an experiment; this suggests that an artificial auxin gradient can be achieved at a desired position. Additionally, fine control of optical devices would enable the manipulation of auxin within a single cell (Figure 1 A).

The molecular design of the caged auxin is crucial for its successful application because chemical and biological properties, such as solubility, cell permeability and stability inside the cell, depend on the nature of the caged molecules. Napthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) are widely used as synthetic auxins together with IAA, a natural auxin (Figure 1 A). In past studies, 2-nitrophenylethyl (NPE)-caged NAA (**1 c**) has been synthesized, but the detailed biological properties have not been described thus far.[12,13] The transport and metabolism of these synthetic auxins are quite different from IAA. IAA is polar transported by both AUX1 influx and PIN efflux carrier proteins. PIN efflux proteins transport NAA, but AUX1 influx proteins do not. In contrast, 2,4-D is a substrate for AUX1, but not for PIN proteins.[3] Therefore, NAA cannot reproduce native behavior of the natural auxin, IAA, in plants. Thus, caged IAA may be the most desirable caged auxin.

In this study, we designed new types of caging groups for caged IAA, NAA and 2,4-D, because conventional caging groups used for mammalian biology were easily hydrolyzed by plant esterases to release auxins without photolysis. We designed esterase-resistant caged auxins and evaluated their uncaging efficiency and stability both in vitro and in vivo. We demonstrate the photocontrolled auxin response of roots in Arabidopsis plants and the subcellular manipulation of auxin in a single cell by using caged auxin systems.

Results and Discussion

Photolabile caged auxins

Caged compounds should be biologically inert until photolysis. To inactivate auxins by introducing a caging group, the carboxylic acid moiety in each auxin was esterified with a conventional 2-nitrobenzyl-type caging group that is widely used in many types of caged compounds in animal biology. Because the carboxylic acid in auxins is a common and essential group for auxin activity, as the initial step, we utilized a 1-(2-nitrophenyl) ethanol group to cage the auxins. *o*-Nitroacetophenone was reduced with $NaBH_4$ to yield 1-(2-nitrophenyl) ethanol (**8**). Auxins (IAA, NAA or 2,4-D) were condensed with alcohol **8** using *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*,*N*′-dimethylaminopyridine (DMAP) to give three NPE-caged auxins (**1 a**–**c**, Scheme 1).

To confirm the release of the original auxin from the NPE-caged auxin **1 a** by UV light (365 nm) in vitro, UV-irradiated solutions of **1 a** were analyzed by HPLC. This analysis indicated that the original IAA was released in proportion to photoexposure time (Figure 2). In contrast, no auxin was released from caged auxin under dark conditions. NPE-caged NAA and 2,4-D were also photolyzed to release NAA and 2,4-D, respectively (data not shown).

We next evaluated uncaging efficiency in plant culture medium and the in vivo stability of caged auxins using auxin responsive transgenic Arabidopsis *DR5::GUS* reporter lines.[14] This *DR5::GUS* line carries the β-glucuronidase (GUS) reporter gene, which is under the control of an auxin-responsive *DR5*

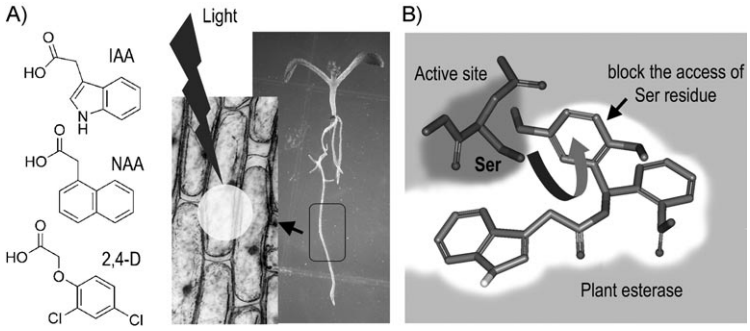
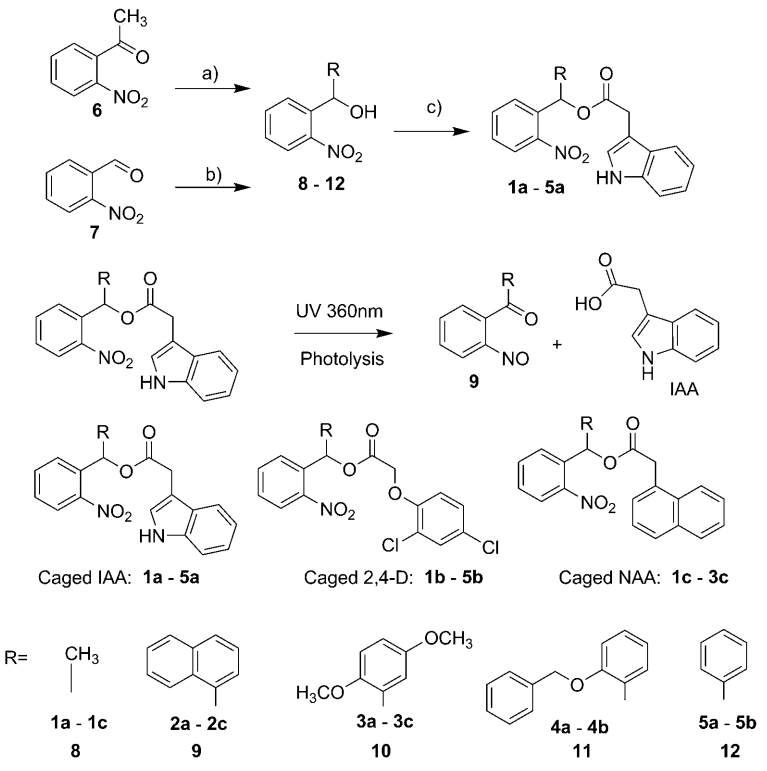


Figure 1. A) Photocontrolled auxin application and B) esterase-resistant caged auxin.



Scheme 1. Synthesis of caged auxins (**1 a**–**5 b**) : A) RMgBr, THF, −15 °C to RT; B) $NaBH_4$, MeOH, RT; C) DCC, DMAP, RT, THF.

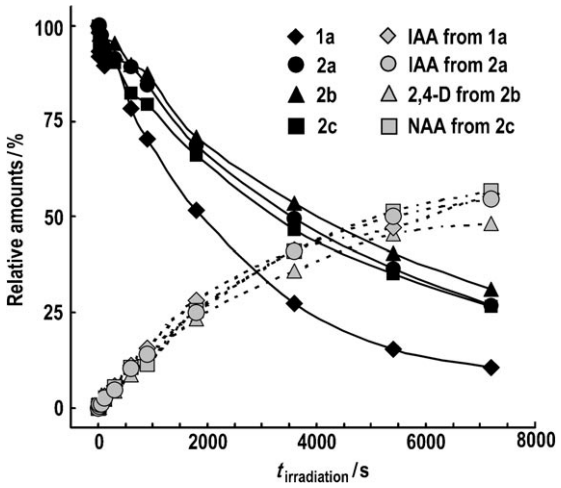


Figure 2. In vitro uncaging rates of NPE-caged IAA (**1a**) and DMPNB-caged auxins (**2a**–**c**). Caged auxin solution (50% aqueous EtOH) was photolyzed by a fluorophotometer (365 nm) and caged auxins and released auxins were measured by HPLC. Released auxin was plotted as uncaged yield [%] from caged auxins versus irradiation time at 365 nm.

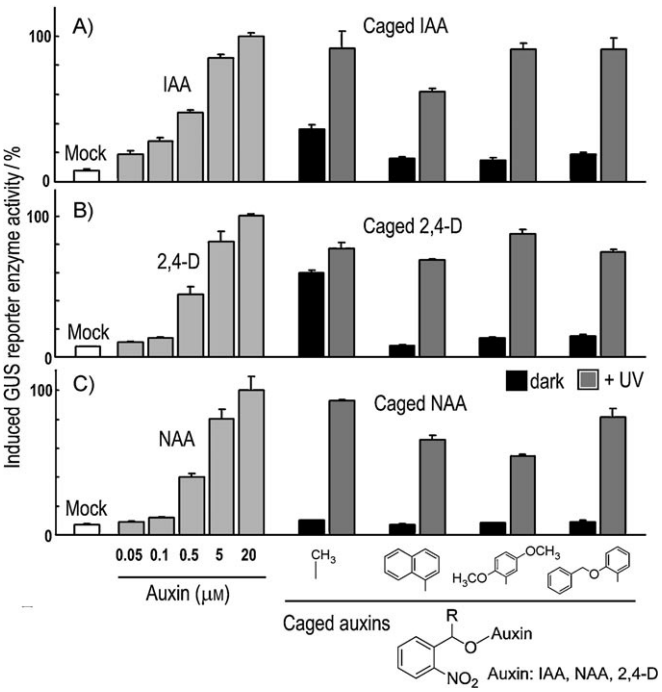


Figure 3. In vitro photolysis of caged auxins A) caged IAA, B) caged 2,4-D and C) caged NAA in culture medium. The caged auxin (20 μM) in GM medium was uncaged by using UV light. Arabidopsis *DR5::GUS* seedlings were cultured in the photolyzed caged auxin medium or nonirradiated caged auxin medium for 5 h in the dark. The induced GUS enzyme activity was measured by a fluorimeter and is adjusted to 100% value (20 μM auxin treatment)

promoter. This reporter line is highly sensitive and specific to biologically active auxins and the GUS reporter enzyme expression is tightly regulated by intracellular auxin in a dose-dependent manner.

To examine uncaging efficiency in culture medium, caged auxin in GM liquid medium was irradiated by using a UV lamp (365 nm), and the *DR5::GUS* line was incubated in photolyzed medium for 5 h in the dark. For the in vivo stability assay, the *DR5::GUS* seedlings were incubated in GM medium containing caged auxin for 5 h in the dark. In this in vivo stability assay, caged auxin was supplied constantly into the plant cells from the culture media containing caged auxin, since the seedling was incubated in caged auxin solution throughout the reporter gene induction. The auxin-induced GUS enzymatic activity was fluorometically determined using 4-methyl umbelliferyl-β-D-glucuronide as a fluorogenic substrate.

Figure 3 shows that the effects of photoirradiation on the NPE-caged auxins enhanced *DR5::GUS* expression. Although NPE-caged auxins **1a**–**c** were stable in the dark in vitro, *DR5::GUS* reporter expression was activated by NPE-caged IAA (**1a**) and NPE-caged 2,4-D (**1b**) without irradiation. In contrast, NPE-caged NAA (**1c**) was stable in vivo until UV irradiation. This suggests that the ester linkage between NPE-caged groups and IAA and 2,4-D is enzymatically hydrolyzed by esterases in plant cells to release auxins without photolysis.

We next examined the uncaging of intracellular caged auxins. Arabidopsis *DR5::GUS* seedlings were immersed for 30 min in a liquid GM medium containing 20 μM caged auxins to load caged auxins inside cells. After incorporation of caged auxin, the seedlings were washed three times with culture medium to remove caged auxin outside cells and then placed on GM agar medium solidified on slide glass. Immediately, the root was irradiated with UV light (360 nm) with a fluorescence microscope (see the Supporting Information). The irradiated seedling was incubated to induce GUS reporter expression for an additional 5 h in the dark. The auxin-induced GUS enzymatic activity was visualized by blue histochemical staining by using X-Gluc as a chromogenic substrate. Figure 4 shows that NPE-caged IAA, 2,4-D and NAA (**1a**–**c**) incorporated into plant cells were uncaged intracellularly to induce *DR5::GUS* reporter expression. Although extracellular caged auxin was washed out in this assay condition, NPE-caged IAA and 2,4-D were still enzymatically hydrolyzed to release original auxins during the incubation and, consequently, activated GUS expression without photoirradiation (Figure 4).

As the results demonstrate, caged auxins with a conventional NPE-caging group are found to be biologically unstable in plant cells. Therefore, the NPE-caging group would be insufficient for in vivo application for plants.

Esterase-resistant caged auxin is stable in plant cells

To enhance the stability of caged auxins in plant cells, we designed esterase-resistant caging groups. A serine nucleophile in typical serine esterases attacks the carbonyl carbon in an ester bond (Figure 1B). We speculated that bulky groups such as phenyl derivatives instead of the methyl group in the NPE caging moiety would block the access of serine nucleophile in esterase active site to the carbonyl carbon (Figure 1B).

Aromatic Grignard reagents were reacted with 2-nitrobenzaldehyde (**7**) in THF to afford corresponding 2-nitrobenzylalcohol (**9**–**12**) with an aromatic moiety. These alcohols were then con-

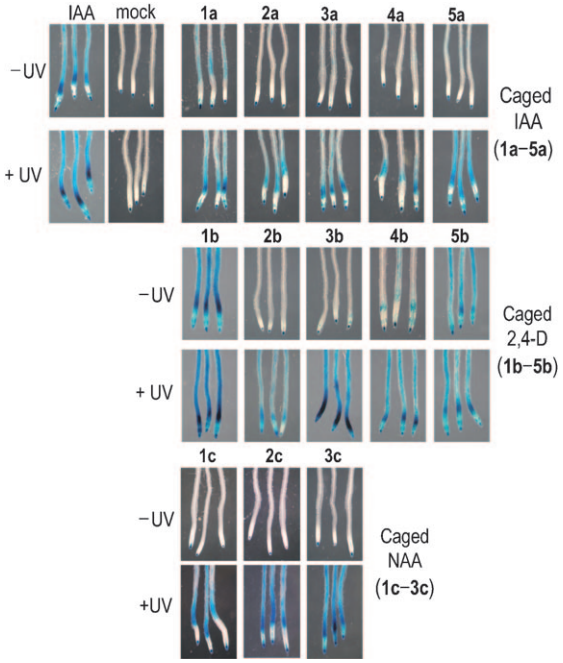


Figure 4. In vivo photolysis of intracellular caged auxins. The intracellular caged auxin was uncaged by using UV light after loading into the Arabidopsis *DR5::GUS* auxin responsive reporter line. The auxin responsive GUS reporter gene was induced in respond to uncaged auxin, and then the induced GUS enzyme was visualized by histochemical blue staining.

densed with IAA, NAA or 2,4-D with DCC and DMAP to yield corresponding caged auxins (**2a**–**5b**).

The introduction of a simple phenyl ring in place of the methyl group of the NPE moiety improved the stability of PNB-caged IAA (**5a**) within a cell, but PNB-caged 2,4-D (**5b**) was still hydrolyzed in vivo during the incubation (Figure 4). Therefore, the more bulky naphthyl (**9**), 2,4-dimethoxyphenyl (**10**) and 2-benzyloxyphenyl (**11**) groups were introduced to the 2-nitrobenzyl caging group to improve stability. These caging groups (**9**-**11**) were reacted with auxin to give naphthyl 2-nitrobenzyl (NNB)-caged auxins (**2a**–**c**), 2,4-dimethoxyphenyl 2-nitrobenzyl (DMPNB)-caged auxins (**3a**–**c**), 2-benzyloxyphenyl-2-nitrobenzyl (BOPNB)-caged auxins (**4a**–**b**). BOPNB-caged 2,4-D (**4b**) slightly activated *DR5::GUS* induction without photoirradiation (Figure 4); this indicates that BOPNB-caged 2,4-D is enzymatically hydrolyzed to release 2,4-D within a cell. Two types of caged auxins, NNB- and DMPNB-caged auxins, did not induce *DR5::GUS* expression without UV irradiation under our experimental conditions (Figure 3 and 4); this suggests that these caged auxins are highly stable in plant cells. This result was consistent with the concept of molecular design for esterase-resistant caging groups. NNPB-caged auxins were less soluble in culture medium than DMPNB-caged auxins. Therefore, we further evaluated DMPB-caged auxins as the most promising molecular probes to investigate auxin biology.

Light-control of intracellular auxin level

We next assessed light-controlled manipulation of the intracellular auxin concentration. The release of auxin from caged auxin depends on irradiation time or strength, and this suggests that we could regulate intracellular auxin concentration by controlling uncaging rate. In vitro, HPLC analysis revealed that uncaged auxin levels from DMNPE-auxins could be controlled by light irradiation time (Figure 2). The intracellular auxin level was indirectly estimated by the auxin-dependent GUS reporter gene expression in the *DR5::GUS* line. Figure 3 shows that *DR5::GUS* expression was proportional to exogenous auxin concentration. To regulate intracellular auxin levels by using light, DMNPE-caged auxin was loaded into cells by incubation of *DR5::GUS* seedlings in caged auxin solution. The seedlings were placed on GM agar plates after washing with medium three times, and then were irradiated for various lengths of time by using a UV hand lamp. After light irradiation, the seedlings were incubated for 5 h in the dark to induce *DR5::GUS* expression, and then immediately frozen. The induced GUS activities were fluorometically determined. Figure 5 indicates that *DR5::GUS* induction depends on photo-

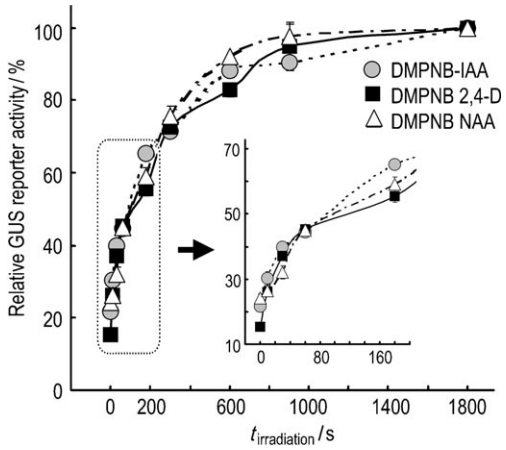


Figure 5. Photocontrol of intracellular auxin levels. The intracellular caged auxin was uncaged by using UV light after loading into the auxin responsive Arabidopsis *DR5::GUS* reporter line. The seedling was cultured in the dark for 5 h after indicated UV irradiation time. The induced GUS enzyme activity was measured by a fluorometer and is adjusted to 100% values (1800 s irradiation).

irradiation time. The GUS expression was elevated in proportion to irradiation time until 1 min and saturated after 10 min irradiation. Therefore, this data clearly demonstrates that intracellular auxin levels from caged auxins can be manipulated by light. Additionally, UV light was irradiated to the root tip after loading of DMPNB-caged auxins (**3a** and **3c**) and then incubated in the presence or absence of auxin transport inhibitor, *N*-1-naphthylphthalamic acid (NPA) for 4 h. Auxin released from DMPNB-caged auxin at the root tip basipetally moved from the root tip and then activated *DR5::GUS* expression. In the presence of NPA, basipetal auxin transport was blocked and *DR5::GUS* was expressed only at root tips (Supporting Information). These data demonstrate that the caged auxin system enables control of the auxin level at specific cells or tissue.

Light-regulated physiological auxin response

In the *DR5::GUS* assay, caged auxins were confirmed to release auxin within cells by photoirradiation. We next investigated the control of physiological plant auxin response by light. Auxin strongly inhibits primary root elongation and promotes lateral root development (Figure 6A). This inhibition of primary root elongation is a very rapid auxin response and is easy to observe. Therefore, we used this root response for a physiological auxin assay to demonstrate the light-regulation of auxin response by caged auxins. After loading of DMPNB-caged IAA (**3a**), the seedlings were placed vertically on agar medium and irradiated by UV light. The root elongation of seedlings treated with DMPNB-caged auxin was blocked by UV irradiation, but the elongation was not inhibited without irradiation (Figure 6B). UV light alone did not influence the root elongation (data not shown). Photolysis of DMPNB-caged auxins not only releases auxin, but also yields photolyzed byproducts (**13**) from caging groups. If this byproduct and the caged auxin itself are biologically toxic, it would perturb the analysis of auxin-elicited physiological responses. Therefore, it is crucial to confirm the biological inactivity of the photolyzed byproduct. To confirm the toxic effects of photolyzed byproducts, we used DMPNB-caged acetates that release acetic acid and the identical byproduct of DMPNB-caged auxin. The intracellular photolysis of DMPNB-caged acetate did not block the root elongation (Figure 6C). These results demonstrate that the plant physiological auxin response can be controlled with light by using a DMPNB-caged auxin.

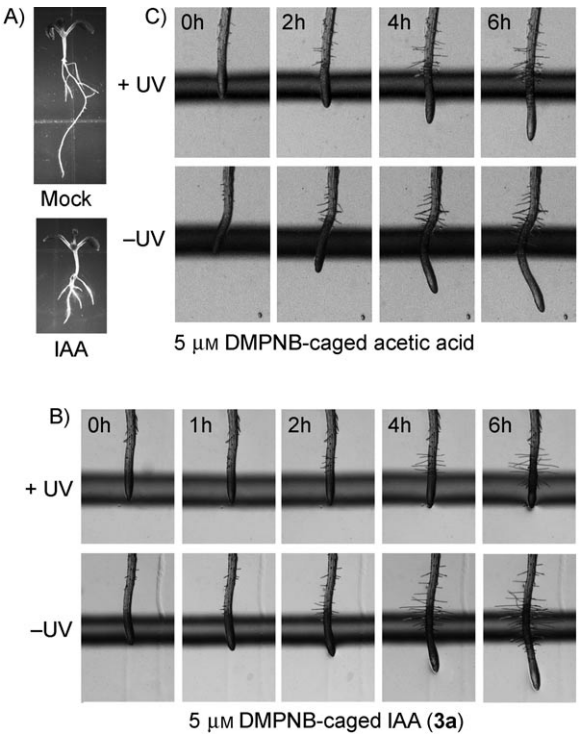


Figure 6. Photocontrolled root growth by modulating intracellular auxin levels. A) *Arabidopsis* seedlings treated with IAA. Four day old seedlings were immersed in GM liquid media containing 5 μM DMPNB-caged auxins B) or 5 μM DMPNB-caged acetic acid C) for 30 min. After washing with fresh medium, seedlings were irradiated for 20 min with UV light. After photolysis, the seedlings were vertically cultured at 24 °C in the dark.

Manipulation of intracellular auxin levels in a single cell

In the next step, photocontrol of intracellular auxin level in a single cell was examined. For this purpose, we used the transgenic tobacco suspension cell line BY-2 expressing a GFP-reporter driven by the auxin-responsive *DR5* promoter (BY-2 *DR5::GFP*).[15] Activation of the *DR5* promoter by auxin rapidly enhanced a cytoplasmic green fluorescence. This is consistent with studies demonstrating that auxin responses at the gene-expression level can be detected in the range of a few minutes.[16] A bicellular file of BY-2 cells was selected such that the fluorescence intensity was equal in both cells (Figure 7A). After releasing IAA by UV-irradiating the tip of the right cell, we followed the fluorescence intensity within this bicellular file over a time period of 50 min. The time series showed that the fluorescence intensity in the cytoplasm of the right cell increased continuously, whereas the cytoplasmic fluorescence in the adjacent cell remained largely unaltered.

To further investigate the manipulation of auxin response in a single cell, we used a transgenic BY-2 cell line as an imaging control stably expressing a fusion protein of the yellow fluorescent protein (YFP) and the actin-binding domain of mouse talin.[17] Due to talin overexpression, the microfilaments are constitutively bundled in this cell line; this results in artefactual cytoskeletal visualizations. However, the normal organization of actin (formation of fine actin microfilaments) can be restored when the culture medium is supplemented with IAA (Figure 7C), as demonstrated in a previous study.[18] DMPNB-caged IAA (**3a**) was loaded into these transgenic BY-2 cells, and the right cell of a bicellular file was irradiated with UV light (Figure 7B). Spotlight illumination induced localized auxin responses, namely the dissociation of actin bundles and the formation of fine microfilament strands in the irradiated cell, but not in the neighboring cell where no uncaged IAA was present (Figure 7B). Upon the localized uncaging of **3a** within the right cell, the massive actin bundles were changed to numerous fine actin filaments over a period of 20 min. Thus, it is possible to use caged IAA as a trigger to allow the release of a signal in a defined and delineated manner, both in space and in time.

Conclusions

To shed light on the crucially important mechanisms of polar auxin transport, it is necessary to manipulate the spatial distribution of auxin. Therefore, it is very important to use a trigger that allows one to release the auxin signal in a defined and delineated manner, both in space and in time. However, one has to keep in mind that this is not trivial, for example, in suspension cell culture, because chemical compounds that are added to the culture will act globally on all cells. As a methodological advance, caged auxin compounds comply with all of these re-

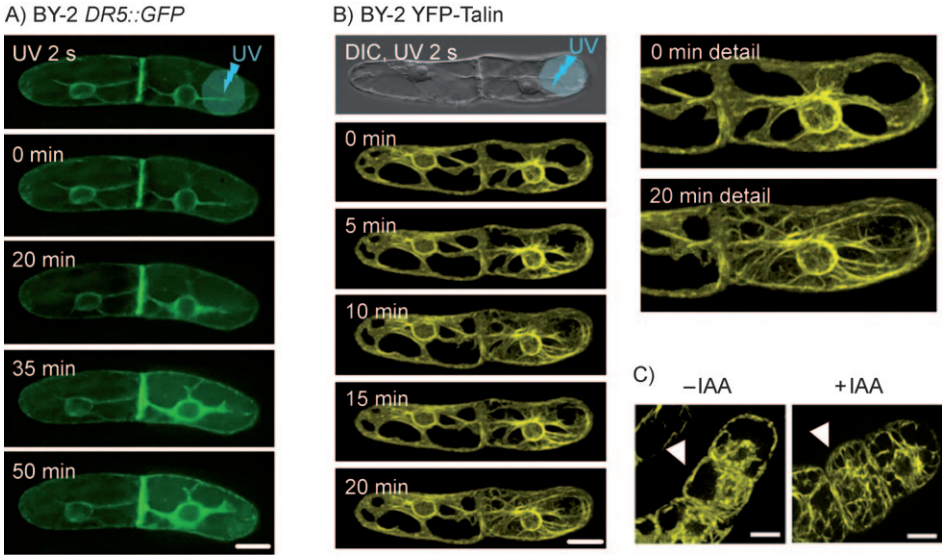


Figure 7. Detection and manipulation of intracellular auxin levels in single cells by UV-light activated release of DMPNB-caged IAA, **3a**. A) Time series of a bicellular BY-2 file expressing DR5::GFP, a marker for the activity of auxin-induced gene expression, after release of caged IAA in the right cell. B) Time series of a bicellular BY-2 file expressing YFP-Talin after release of caged IAA in the right cell. C) BY-2 cells overexpressing YFP-Talin in the absence or presence of 2 μM IAA in the culture medium. For each cell a focal section in the perinuclear region is shown. Bars = 20 μm.

quirements. For the first time in a physiological context, these modified caged auxins open the exciting possibility of studying polar auxin transport on the cellular level.

Experimental Section

General: UV spectra were recorded on a U-3210 spectrophotometer (Hitachi). ^{1}H and ^{13}C NMR were run on a JEOL lambda 500 spectrometer (JEOL, Tokyo, Japan), with chemical shifts shown as δ values with tetramethylsilane (TMS) as the internal reference. Peak multiplicities are quoted in Hz. Mass spectra were measured on a JMS-700 spectrometer (JEOL, Japan).

Synthesis of (2,5-dimethoxyphenyl)(2-nitrobenzyl) indole 3-acetate, DMPNB-caged IAA (3a): 1-Bromo-2,5-dimethoxy-benzene (1200 mg, 5.5 mmol) was added to a suspension of magnesium turnings (128 mg, 5.3 mmol) in dry THF (5.0 mL), and the mixture was stirred at room temperature until the magnesium was dissolved. The resulting Grignard reagent solution was slowly added to 2-nitrobenzaldehyde (256 mg, 1.7 mmol) **7** in dry THF at −15 °C over 10 min. The reaction mixture was stirred for 15 min and then warmed up to −15 °C over 15 min. This reaction was quenched by the addition of HCl (0.2 N) and then extracted with EtOAc (3× 100 mL). The organic layer was washed with saturated NH_4Cl solution and brine. After drying over Na_2SO_4, the layer was concentrated in vacuo. The residue was purified by silica gel column chromatography by using hexane-EtOAc (8:2) as the solvent system to give (2,5-dimethoxyphenyl) 2-nitrobenzyl alcohol **10** (420 mg, Yield 86%) as a yellow prism crystal: m.p. 69–70 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H = 7.87 (dd, J = 8.3, 1.2, 1H), 7.56 (qd, J = 7.9, 1.5, 2H), 7.40 (td, J = 8.3, 1.3, 1H), 6.88 (d, J = 2.4, 1H), 6.80–6.76 (m, 2H), 6.59 (d, J = 4.0, 1H), 3.74 (s, 3H), 3.68 (s, 3H) 3.23 (d, J = 4.3, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 153.7, 150.6, 148.8, 137.7, 133.0, 131.4, 129.4, 128.2, 124.1, 113.4, 113.1, 111.5, 66.6, 55.8, 55.7; UV (MeOH): λ_{max} (log ε) = 204 (4.43), 292 nm (3.66); positive FAB MS *m/z* 289 $[M]^+$, HR-FAB MS: calcd for $C_{15}H_{15}NO_5$: 289.0950 $[M]^+$, found for 289.0926 (Δmmu 2.4).

The alcohol **10** (70 mg, 0.24 mmol) and *N,N′*-dicyclohexylcarbodiimide (75 mg, 0.36 mmol) were added to the solution of indole 3-acetic acid (85 mg, 0.49 mmol) and 4-dimethylaminopyridine (46 mg, 0.38 mmol) in dry THF (3 mL), and then stirred for 2 h at room temperature. The reaction mixture was poured into sat. NH_4Cl solution (10 mL) and then extracted with EtOAc (3×30 mL). The EtOAc layer was washed with brine (10 mL) and dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. This residue was purified by silica gel column chromatography ($CHCl_3$/EtOAc = 95:5) to give DMPNB-caged IAA **3a** (95 mg, Yield 88%) as a yellow powder: m.p. 101–102 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.11 (br s, 1H), 7.88–7.93 (m, 1H), 7.74 (s, 1H), 7.59 (d, J = 8.0, 1H), 7.36–7.43 (m, 2H), 7.35 (d, J = 8.2, 1H), 7.29–7.33 (m, 1H), 7.19 (t ,J = 7.0, 1H), 7.16 (d, J = 2.2, 1H), 7.09 (td, J = 1.0, 8.0, 1H), 6.75 (d, J = 1.6, 2H), 6.49 (s, 1H), 3.87 (d, J = 2.4, 2H), 3.66 (s, 3H), 3.49 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.2, 153.4, 151.0, 148.3, 136.1, 134.3, 132.8, 129.3, 128.6, 128.0, 127.2, 124.6, 123.2, 122.2, 119.7, 119.0, 113.6, 113.2, 111.7, 111.1, 108.1, 68.1, 55.9, 55.4, 31.3; UV (60% aqueous EtOH): λ_{max} (log ε) = 223 (4.85), 281 nm (3.90); Positive FAB MS *m/z* 446 $[M]^+$, HR-FAB MS: calcd. for $C_{25}H_{22}N_2O_6$: 446.1478 $[M]^+$, found for 446.1499 (Δmmu 2.1); molecular extinction coefficient at 365 nm = 368 $M^{-1}cm^{-1}$; the quantum yield ($\Phi_{reactant}$ in 60% aqueous EtOH) = 0.04.

(2,5-Dimethoxyphenyl)(2-nitrobenzyl) 2,4-dichlorophenoxy acetate, DMPNB-caged 2,4-D (3b): The same procedure described for **1a** was employed for the caging of 2,4-dichlorophenoxy acetic acid (90 mg, 0.48 mmol) with **10** (70 mg, 0.24 mmol). The title compound (102 mg, Yield 86%) was obtained by silica gel column chromatography (*n*-hexane/EtOAc = 7:3) as a yellow powder: m.p. 117–118 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.96 (dd, J = 1.2, 8.3, 1H), 7.83 (s, 1H), 7.55 (td, J = 1.3 7.7, 1H), 7.47 (td, J = 1.5, 7.9, 1H), 7.42 (dd, J = 1.2 7.7, 1H), 7.36 (d, J = 2.8, 1H), 7.12 (dd, J = 2.5, 8.6, 1H), 6.82 (d, J = 2.5, 1H), 6.81 (s, 1H), 6.77 (d, J = 8.9, 1H), 6.64 (d, J = 2.2, 1H), 4.78 (s, 2H), 3.73 (s, 3H), 3.71 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 166.6, 153.5, 152.2, 151.0, 148.2, 133.5, 133.0, 130.2, 129.3, 129.0, 127.6, 127.2, 127.0, 124.8, 124.1, 114.5, 114.1, 113.4, 111.8, 68.9, 66.1, 56.1, 55.7; UV (60% aqueous EtOH) λ_{max} (log ε): 226 (4.18), 285 nm (3.72); Positive FAB MS *m/z* 491 $[M]^+$, HR-FAB MS: calcd for $C_{23}H_{19}Cl_2NO_7$: 491.0539 $[M]^+$, found for 491.0527 (Δmmu 1.1); molecular extinction coefficient at 365 nm = 313 $M^{-1}cm^{-1}$; the quantum yield ($\Phi_{reactant}$ in 60% aqueous EtOH) = 0.05.

(2,5-Dimethoxyphenyl)(2-nitrobenzyl) naphthalene 1-acetate, DMPNB-caged NAA (3c): The same procedure described for **1a** was employed for the caging of naphthalene 1-acetic acid (90 mg,

0.48 mmol) with **10** (70 mg, 0.24 mmol) The title compound (107 mg, Yield 97%) was obtained by silica gel column chromatography (*n*-hexane/EtOAc = 7:3) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.99$ (dd, $J = 2.5, 6.7$, 1H), 7.90 (dd, $J = 1.9, 7.6$, 1H), 7.86 (dd, $J = 2.4, 6.1$, 1H), 7.80 (dd, $J = 1.8, 7.3$, 1H), 7.72 (s, 1H), 7.40–7.51 (m, 4H), 7.35–7.39 (m, 2H), 7.14 (dd, $J = 1.6, 7.4$, 1H), 6.74 (d, $J = 1.9$, 2H), 6.37 (s, 1H), 4.16 (d, $J = 6.7$, 2H), 3.63 (s, 3H), 3.47 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 169.7$, 153.3, 151.0, 148.2, 134.1, 133.8, 132.7, 132.1, 130.2, 129.1, 128.6, 128.5, 128.2, 128.1, 127.8, 126.4, 125.8, 125.5, 124.6, 124.0, 113.7, 113.2, 111.7, 68.3, 55.9, 55.5, 39.2; UV (60% aqueous EtOH) λ_{max} ($\log \varepsilon$) = 224 (4.78), 282 nm (3.95); Positive FAB MS *m/z* 457 $[M]^+$, HR-FAB MS: calcd for $C_{27}H_{23}NO_6$: 457.1525 $[M]^+$, found for 457.1516 (Δmmu −0.9); molecular extinction coefficient at 365 nm = 358 $M^{-1}cm^{-1}$; the quantum yield ($\Phi_{reactant}$ in 60% aqueous EtOH) = 0.05.

Synthesis of 1-(2-nitrophenyl)ethyl IAA, 2,4-D and NAA, NPE-caged auxins (1a–c): Indole 3-acetic acid and 2,4-dichlorophenoxy acetic acid were caged with 1-(2-nitrophenyl)ethanol **8** by same synthetic procedure described at **3a** to give NPE-caged IAA (**1a**) and NPE-caged 2,4-D (**1b**) as yellow oil (53% yield and 81% yield, respectively). NPE-caged NAA was synthesized according to the published procedure. The molecular extinction coefficients at 365 nm of **1a**, **1b** and **1c** were 204, 158 and 180 $M^{-1}cm^{-1}$, respectively. The quantum yields ($\Phi_{reactant}$ in 60% aqueous EtOH) of **1a**, **1b** and **1c** were 0.14, 0.16 and 0.16, respectively. The detailed synthetic procedures and spectroscopic data for these compounds were described in the Supporting Information.

(Naphthalen-1-yl)(2-nitrobenzyl) auxins [NNB-caged auxins 2a–c], ((2-(benzyloxy)phenyl) (2-nitrobenzyl) auxins [BOPNB-caged auxins 4a and 4b] and (phenyl)(2-nitrobenzyl) auxins [PNB-caged auxins 5a and 5b]: The alcohols **9**, **11** and **12** , caging groups were synthesized by Grignard reaction of *o*-nitroacetophenone and the corresponding aryl bromide. Auxins (IAA, NAA and 2,4-D) were caged with the alcohols (**9**, **11** and **12**) by the same procedures described for 3a. The detailed synthetic procedures and spectroscopic data of **2a** to **5b** were described in the Supporting Information.

Quantum yield determination: The quantum yield for the photoconversion of caged auxins was determined by a direct comparison with that of NPE-caged benzonate, 1-(2-nitrophenyl)ethyl benzoate, under the same photolysis conditions. The photochemical properties and quantum yield of 2-nitrophenylethyl benzoate (Φ = 0.19) have been well characterized by Zhu et al.[19] NPE-caged benzoate was synthesized and used as a standard. Caged auxin solution (60% aqueous EtOH) was irradiated by UV light (365 nm) with a fluorophotometer (Shimadzu RF-1500, Kyoto, Japan). Caged compounds were photolyzed at identical optical densities at the irradiated wavelength. At regular intervals during photolysis, aliquots of the photolyzed solutions were analyzed by reversed-phase HPLC, using the Inertsil ODS-3 column (5 μm, 4.6 X 150 mm, GL Science, Tokyo, Japan), to determine the photolytic conversion. The HPLC analysis conditions and chromatograms were described in Supporting Information.

Plant materials: *Arabidopsis thaliana* ecotype Columbia was used for all experiments. The transgenic Arabidopsis reporter line *DR5::GUS* was previously described.[14] Suspension-cultured transgenic tobacco BY-2 cells, DR5::GFP and GFP-Talin lines[15,17] were maintained in modified MS medium (4.3 g L^{-1} Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands), 30 g L^{-1} sucrose, 200 mg L^{-1} KH_2PO_4, 100 mg L^{-1} inositol, and 1 mg L^{-1} thiamine, pH 5.8) as described previously[18] on a rotary shaker (150 rpm) at 25 °C in the dark.

In vitro photolysis of caged auxin in culture media: Caged auxin was dissolved in GM liquid medium (GM, 0.5 x Murashige and Skoog salts [Gibco BRL, Gaithersburg, MD], 1% sucrose, 1 x B5 vitamins, and 0.2 g L^{-1} 2-(4-morpholino)-ethane sulfonic acid (MES), pH 5.8). 20 μM caged auxin medium was then irradiated for 20 min by a 16 W UV hand lamp equipped with a band pass filter (350–400 nm) at distance of 5 cm (1820 μW cm^{-12}) from the solution. The DR5::GUS seedlings ($n = 10$) were incubated in the photolyzed caged auxin medium or nonirradiated medium at 24 °C for 5 h under dark. After incubation, the seedlings were immediately frozen and kept frozen until GUS reporter measurement. The auxin-induced GUS (β-glucuronidase) reporter activity was measured by a fluorophotometer.

In vivo photolysis of intracellular caged auxins: To load the caged auxins into cells, the seedlings were immersed in a liquid GM medium containing 20 μM of caged auxins for 30 min. After washing with fresh GM medium, the seedlings were placed on GM agar solidified on slide glass. The roots were irradiated with UV light for 3 s with a fluorescence microscope (Olympus BX50) with a UV-band pass filter (360–370 nm), and then incubated for 5 h in a culture dish in the dark (Supporting Information). For control treatment, the seedlings were treated with same procedures without photoirradiation. After incubation, the induced GUS reporter enzyme was histochemically stained and microscopic pictures were recorded with a digital camera (Olympus SZX9).

Light-control of intracellular auxin level: The seedlings were soaked in a liquid GM medium containing DMPNB-caged auxins (20 μM) for 30 min to load caged auxin into cells. After washing with fresh GM medium, the seedlings were placed on GM agar plates. The UV light was irradiated with a 16 W UV hand lamp at distance of 5 cm. At indicated periods of photoirradiation, the irradiated seedlings were cultured 24 °C for another 5 h under dark and then immediately frozen. The auxin-induced GUS reporter activity was fluorometrically determined.

Histochemical and quantitative measurements of GUS reporter activity: For histochemical GUS enzyme staining, the transgenic *DR5::GUS* seedlings 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) after hormonal induction and transferred to a staining buffer 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc, 1 mM), the substrate for histochemical staining. The seedlings were then incubated at 37 °C until sufficient staining developed (3–4 h). For quantitative measurements, after reporter gene induction ($n = 10$) the seedlings were homogenized in an extraction buffer as previously described.[20] After centrifugation, the GUS activity of the supernatant was measured by a fluorophotometer (λ_{ex}: 365 nm, λ_{em}: 455 nm) by 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, Tokyo, Japan). The experiments were repeated at least two times with three replications.

Photocontrolled root growth: For the primary root growth assay, four-day-old seedlings were immersed in GM liquid media containing DMPNB-caged auxin (5 μM) or (2,5-dimethoxyphenyl)(2-nitrobenzyl) acetate (5 μM), DMPNB-caged acetate, for 30 min. After washing with fresh medium, seedlings were placed vertically on a GM agar plate. The seedlings were then irradiated for 20 min with a 16 W UV hand lamp (350–400 nm) at distance of 5 cm from the plate. After photolysis, the seedlings were vertically cultured at

24 °C in the dark. The pictures were taken at indicated times. The synthesis of DMPNB-caged acetate was described in the Supporting Information.

Auxin manipulation in a single cell: Aliquots (0.5 mL) of three-day-old transgenic BY-2 suspension cells were preloaded with DMPNB-caged IAA (**3a** at a final concentration of 0.5 μM) and incubated for 1 h. Next, the cells were washed four times in MS medium to remove **3a** and were examined under an AxioImager Z.1 microscope (Zeiss) equipped with an ApoTome microscope slider for optical sectioning and a cooled digital CCD camera (AxioCam MRm, Jena, Germany). The active IAA was released by irradiating individual cells with a short 2 s lasting pulse of UV light using filter set 49 (Zeiss, (λ_{ex}: 365 nm, beam-splitter at 395 nm, λ_{em}: 450 nm. Control cells were treated with the same procedures, but without photoirradiation. GFP and YFP fluorescence were recorded through the filter sets 38 HE (Zeiss; λ_{ex}: 470 nm, beam-splitter at 495 nm, and λ_{em}: 525 nm) and 46 HE (λ_{ex}: 500 nm, beam-splitter at 515 nm, and λ_{em}: 535 nm), respectively. Stacks of optical sections were acquired at step sizes of 0.8 μm. Images were processed using the AxioVision software (Rel. 4.6; Zeiss).

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