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Caged gene-inducer spatially and temporally controls gene expression and plant development in transgenic Arabidopsis plant

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Abstract—Two new types of caged gene-inducers, caged 17β-estradiol and caged dexamethazone, were synthesized. Caged gene-inducers were applied to transgenic Arabidopsis plants carrying a steroid hormone-inducible transactivation system. Light uncaged caged gene-inducers and controlled spatial and temporal expression of transgene in the transgenic plant. Furthermore, caged gene-inducers enabled the control of root development by light.

In multicellular organisms, the expression of individual genes is temporally and spatially regulated to maintain proper biological processes. The control of specific gene expression in transgenic organism is a powerful technology to assess the biological function of specific proteins, which have greatly contributed to biology. Chemical-inducible gene expression systems have been widely used to control temporal expression of the transgene by chemicals.[1,2] The spatial control of gene expression was partially achieved using tissue-specific promoter to activate the transgene expression.[3–5] However, the resolution of the spatial control and the expression level depend on the nature of each native promoter.

Caged compounds are inactivated bioactive molecules with the functional group blocked by a photo-removable protecting group (caging group). The original biological activity of a caged compound can be readily recovered by photo-irradiation (normally 350–360 nm, ultraviolet light). In previous studies, caged macromolecules related to transcription machinery, such as caged DNA,[6] caged mRNA,[7] and caged GAL4/VP16 (transactivator protein),[8] were developed as a direct approach to control the temporal and spatial expression of specific protein. These caged macromolecules were activated by photo-irradiation and allowed the temporal and spatial control of specific protein expression, because light is easily controllable in terms of time, area, and intensity. However, these approaches require complicated experimental manipulations, such as caging the target molecules and incorporating them into cells, in order to study the protein of interest.

An alternative approach is the combination of caged chemical-inducer and chemical-inducible gene expression system, namely a caged chemical gene-inducer system. In transgenic cell harboring a chemical-inducible transgene, a caged chemical-inducer like caged steroid hormone can be uncaged by a light irradiation and the uncaged inducer can then drive transgene expression within the irradiated area. Previous studies demonstrated that caged estrogen agonist/antagonists,[9,10] caged β-ecdyson,[11] and caged thyroid hormone agonists[12] can control the reporter gene expression in mammal cell culture upon their uncaging by light. These studies demonstrate the feasibility of the system in transiently transfected mammal cell cultures. However, there is no report on the spatial and temporal control of gene expression at the level of individual organisms. In such caged chemical-inducer systems, the design of the caged inducer is crucial for the control of spatial and temporal gene expression, because the chemical and biological properties, such as solubility, stability inside cell, and cell permeability, depend on the nature of caged molecules.

Keywords: Caged hormone; Gene expression; Light.
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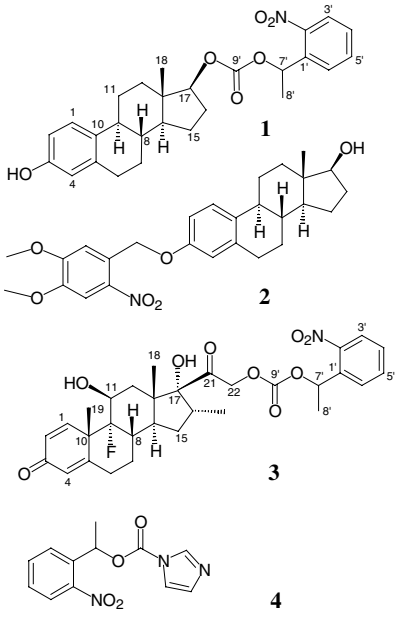
We have designed new caged steroid hormones, caged 17-β-estradiol and caged dexamethazone, as caged gene-inducers with carbonate linker and applied them to transgenic Arabidopsis plants carrying two types of chemical-inducible transactivation system. Arabidopsis plants are ideal transgenic organisms for caged gene-inducer system, since UV light could easily penetrate plant tissues and two chemical-inducible systems have been widely used for the molecular biological studies in Arabidopsis. Furthermore, in contrast to mammal cell, steroid hormones do not appear to affect plant growth.

In this letter, we describe the spatial and temporal control of gene expression by light at individual organism level and demonstrate light-regulated developmental process in transgenic Arabidopsis plant.

To cage the 17-β-estradiol (ER) or dexamethazone (DEX) with 1-(2-nitrophenyl)ethyl carbonate group, we used the 1-(2-nitrophenyl)ethylcarbonyl imidazole (**4**). This activated caging reagent is easy to be synthesized and handled in comparison with a conventional chlorocarbonate-caging reagent, because toxic phosgene is usually used for the synthesis of chlorocarbonate from the corresponding alcohol. 2-Nitrophenylacetophenone was reduced with $NaBH_4$ to yield a 1-(2-nitrophenyl)ethanol. This alcohol was reacted with 1,1′-carbonyldiimidazole to give a caging reagent (**4**).[13] This activated caging reagent was mixed with cesium carbonate and ER or DEX at room temperature for an hour to afford a caged ER (**1**) or caged DEX (**3**), respectively.[14] Caged steroids (**1**) and (**3**) were a mixture of C-7′ epimers in caging moiety. We used caged steroids as a mixture of epimers for further studies because the two epimers could not be separated even by HPLC. The position of 2-nitrophenylethyl carbonate was confirmed to be C-17 position of ER and C-22 position of DEX by the HMBC correlations (H-17/C-9′ carbonyl carbon in **1** and H-22/C-9′ carbonyl carbon in **3**) and downfield shifts of proton signals (H-17 δ_H 3.71 for ER; δ_H 3.73 for **2**; δ_H 4.54 for **1**, H-22 δ_H 4.51 for DEX; δ_H 4.86 for **3**) attributable to carbonate linkage in ^1H NMR spectrum. Previously reported caged ER (**2**) was synthesized according to the literature.[9]



To confirm the release of original hormones from caged hormones **1** and **3** by UV light in vitro, UV-irradiated solutions of **1** or **3** were analyzed by HPLC.[15] After 1-min irradiation by a 6 W-UV lamp, the release of ER and DEX was detected. A time course study indicated that UV-irradiation caused the reduction of caged steroids and consequently released the original steroids in a time-dependent manner. After 40-min exposure, the yields for the release of original steroids from the caged compounds were 45% for the caged ER (**1**) and 56% for the caged DEX (**3**), respectively. The quantum yield ($\phi_{reactant}$) of caged ER (**1**) and DEX (**3**) was determined to be 0.19 and 0.16, respectively.[15] In addition, caged steroids **1** and **3** were stable in dark after 24-h incubation in a medium at 20 μM (data not shown). These results indicate that caged steroids were uncaged by UV light and the original hormones were released in a light-intense-dependent manner, suggesting that the release of hormones could be controlled by light intensity (Fig. 1).

To assess the spatial and temporal control of gene expression by light in vivo, we applied caged ERs **1** and **2** to transgenic Arabidopsis *pER8::GFP* line.[1] This transgenic line strongly expresses a GFP reporter gene under the control of the estrogen-inducible XVE transactivation system.[1] In this system, the expression of the transgene can be tightly regulated by estrogen in a dose-dependent manner. Six-day-old *pER8::GFP* plants were immersed in a medium containing 20 μM caged ERs **1** or **2** for 30 min and seedlings were then washed three times with a medium lacking caged ERs. The irradiation of UV light was carried out with a fluorescent microscope.[16] GFP expression could not be observed without light irradiation (Figs. 2B and C, 1st panel). By contrast, uniform GFP fluorescence was observed in the whole root when light was irradiated over the root after treatment with **1** or **2** (Figs. 2B and C, 2nd panel). UV light was then irradiated as a spot in the middle or the end region of a root (Figs. 2A–C). Figure 2C shows that spot illumination on a root failed to elicit spatial control of GFP expression, when transgenic plants were treated with previously reported caged ER (**2**). GFP fluorescence was uniformly observed over the whole root, even when light was irradiated as a spot. This might be due to the transport of ER from **2** that was bounded to the cell wall after washing. Namely, **2** could not be washed out from the cell wall after loading of **2** into cells. In addition to the photolysis of intracellular **2**, the absorbed **2** on the cell wall was also uncaged by light. The released ER outside cell would then diffuse through the entire root via transport system such as the vessel and sieve tube. In contrast to **2**, spot illumination after the incorporation of our caged ER (**1**) induced spatial GFP expression at only the irradiated area of a root (Fig. 2B), suggesting **1** can be used to control in vivo temporal and spatial expression of transgene by light.

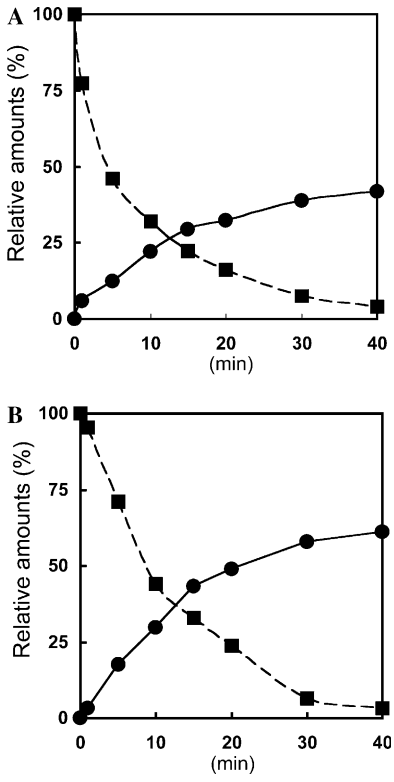


Figure 1. Light uncages caged gene-inducers in a time-dependent manner. Solutions of caged compound were irradiated by UV light and released hormones were analyzed by HPLC. (A) Closed square, caged ER (**1**); closed circle, ER. (B) Closed square, caged DEX (**3**); closed circle, DEX. Relative amounts (%) for ER and DEX indicate released rates from the original caged compounds.

We have applied caged dexamethazone (DEX) to another chemical-inducible gene expression system in vivo. In the absence of an appropriate steroid hormone, the glucocorticoid receptor binds with heat shock proteins (HSPs) through its hormone binding domain located at the carboxy-terminus of glucocorticoid receptor (GR) to form an inactive heterodimeric complex in cytoplasm.[17] The steroid hormone, DEX, dissociates HSPs from the heterodimeric complex and allows the glucocorticoid receptor anchored in the cytosol to translocate into the nucleus. Similarly, the fusion protein of a transcription factor and GR is also inactivated by its interaction with HSPs in the absence of steroid and can be activated in the presence of steroid hormone.[18,19] This GR-fusion system has been shown to work in plants and has been widely applied to studies for functions of plant transcription factor.[20,21] Arabidopsis *pIAA14::GFP-mIAA14-GR* line expresses the translationally fused protein, a mutated *IAA14* fused with GFP and GR at the N- and C-termini, respectively, under the control of *pIAA14* promoter.[22] *IAA14/SLR* is primary auxin-responsive gene that encodes short-lived nuclear repressor regulating the development of lateral root and root hair.[23] Gain-of-function mutation of *IAA14/SLR* confers the resistance of repressor protein to degradation via the ubiquitin–proteasome pathway and consequently the development of root hair and lateral root is repressed in the mutant.[23] Previous report demonstrated that the treatment of this line with DEX causes the accumulation of the fusion protein in nuclei and blocked root hair development as shown in Figure 3B.[22] In wild-type plants, treatment with DEX or **3** with/without light did not affect root hair formation (Fig. 3A).[16] In the transgenic line, treatment of caged DEX (**3**) without light irradiation showed the same root hair formation as observed in control (Fig. 3B). By contrast, no root hair was formed in the presence of **3** when the root was irradiated by UV light (Fig. 3B). In addition, the UV-light irradiation enhanced GFP fluores-

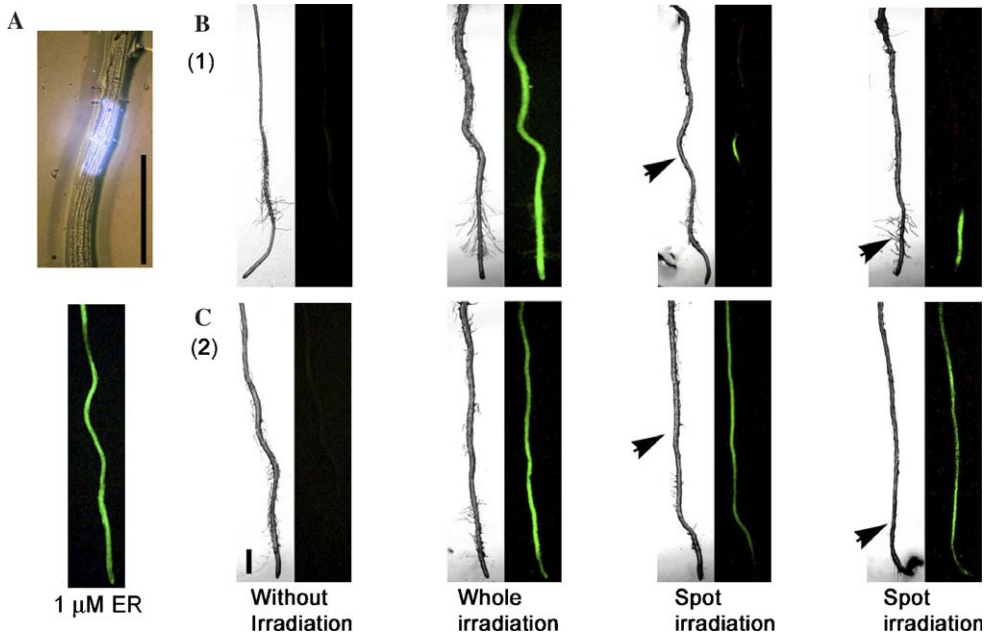


Figure 2. Spatial and temporal control of GFP reporter gene expression by light in root of Arabidopsis *pER8::GFP* plant. (A) UV light was irradiated as a spot on roots by fluorescent microscopy for 3 s. Bar represents 1 mm. ER (1 μM) was used as a positive control. (B and C) Light-activated GFP expression by caged ER (**1**) or previously reported caged ER (**2**). In the second panel, UV light was irradiated on the whole root. In the third and fourth panels, UV light was irradiated as a spot. Arrow indicates the irradiated point. Bar represents 1 mm.

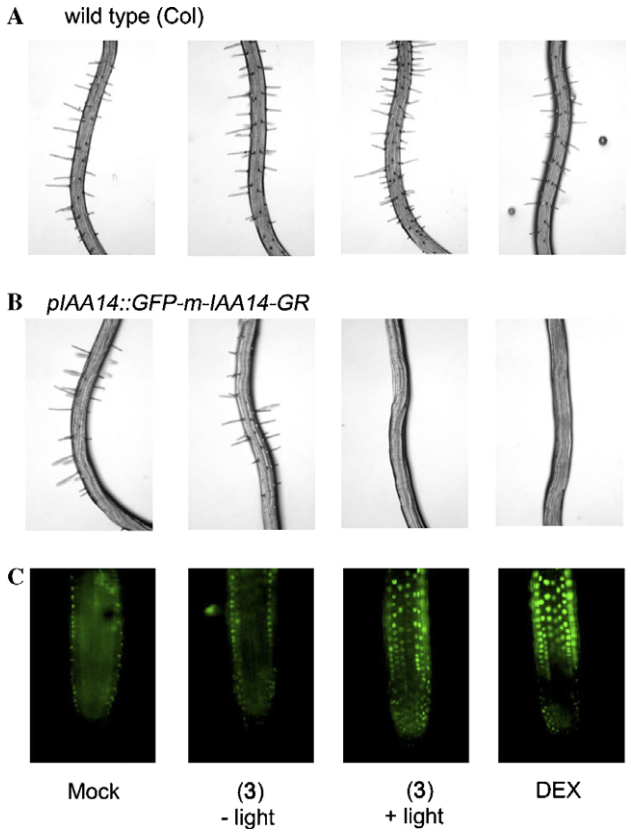


Figure 3. Developmental control of root hair by light in *pIAA14::GFP-mIAA14-GR* Arabidopsis transgenic plants. (A and B) Wild-type (Col) or transgenic seedlings were treated with 0 or 50 μM of caged DEX (**3**) for 30 min. DEX (10 μM) was used as a positive control. UV light was irradiated on the indicated part for 3 s. (C) Accumulation of mIAA14 repressor fused with GFP and GR in nuclei by light. The root tip of the seedling was irradiated by UV light for 3 s. DEX (10 μM) was used as a positive control.

cence in nuclei (Fig. 3C).[16] These observations indicate **3** was uncaged in vivo by light leading to the control of root developmental process by activating the GR-fused repressor.

Here, we demonstrate the spatial and temporal control of gene expression by light at the organism level. This study also shows that light-activated caged gene-inducers can be used to control developmental process in Arabidopsis plants. Our caged gene-inducers are potentially powerful tools to assess protein functions at specific cells and at specific developmental stage in plants.

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14. Synthesis of **1** and **3**. 17β-Estradiol (50 mg, 0.18 mmol) or dexamethazone (50 mg, 0.13 mmol), $CsCO_3$ (2 equiv), and **4** (1 equiv) were dissolved in THF and stirred for an hour at room temperature in darkness. The reaction mixture was extracted with EtOAc after the addition of brine. The organic layer was evaporated. The mixture was subjected to silica gel column and eluted with mixture solvents ($CHCl_3$/acetone 6:1 for **1** and $CHCl_3$/acetone 4:1 for **3**) to yield epimeric mixture of **1** as a light yellow powder (62 mg, 72%) and epimeric mixture of **3** as light yellow powder (54 mg, 73%).
Compound **1** (7′-epimeric mixture): UV λ_{max} (MeOH) nm (log ε): 202 (4.47), 257 (3.56); IR (KBr) V_{max} 3400, 2947, 1750 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.80/0.81 (3 H, s, CH_3-18), 1.18–2.32 (13H, m), 1.69/1.71 (3H, d, J = 6.5, CH_3-8′), 2.49 (2H, m), 4.54 (1H, t, J = 7.5 Hz, H-17), 4.85 (1H, br s, OH), 6.22 (1H, q, J = 6.5 Hz, H-7′), 6.55 (1H, s, H-4), 6.61 (1H, d, J = 8.7 Hz, H-2), 7.12 (1H, d, J = 8.7 Hz, H-1), 7.46 (1H, dd, J = 7.2, 7.1 Hz, H-4′), 7.67 (1H, dd, J = 7.1, 8.0 Hz, H-5′), 7.72 (1H, d, J = 8.0 Hz, H-6′), 7.98 (1H, d, J = 7.2 Hz, H-3′); ^{13}C NMR ($CDCl_3$): δ 11.90/11.97, 22.05/22.17, 23.01/23.03, 26.08/26.11, 27.05, 27.22/27.30, 29.51, 36.71/36.78, 38.47, 42.99/43.02, 43.65, 49.53, 71.59, 86.53/86.60 (C-17), 112.66, 115.20, 124.52/124.55, 126.50/125.54, 126.91/127.0, 128.47/128.50, 132.43, 133.78/133.78, 137.97, 138.13, 147.48/147.51, 153.31, 154.13; HRFABMS *m/z* 488.2029 (calcd for $C_{27}H_{31}O_6Na$, 488.2049).
Compound **3** (7′-epimeric mixture): UV λ_{max} (MeOH) nm (log ε): 205 (4.08), 240 (4.06); IR (KBr) V_{max} 3200, 2932, 1743 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.84/1.03 (3H, s, CH_3-18), 0.87/0.89 (3H, *d*, J = 7.3 Hz, CH_3-20), 1.21 (1H, m), 1.53/1.54 (3H, s, CH_3-19), 1.55 (2H, m), 1.71/1.72 (3H, d, J = 6.4 Hz, CH_3-8′), 1.72 (1H, m), 1.78 (1H, m), 2.05 (1H, m), 2.32 (3H, m), 2.58 (1H, m), 3.05/3.08, (1H, m, H-16), 4.19 (1H, br s, H-11), 4.86 (2H, s, H-22), 6.08 (1H, s, H-4), 6.23, (1H, q, J = 6.4 Hz, H-7′), 6.31 (1H, d, J = 10.0 Hz, H-2), 7.21 (1H, d, J = 10.0 Hz, H-1), 7.46 (1H, dd, J = 7.2, 7.3 Hz, H-4′), 7.71 (1H, dd, J = 7.2, 7.3 Hz, H-5′), 7.82 (1H, dd, J = 7.3 Hz, H-6′), 7.95 (1H, dd, J = 7.3 Hz, H-3′); ^{13}C NMR($CDCl_3$): δ 14.58, 16.17/16.39, 21.82/21.93, 22.83/22.88, 27.31, 32.08, 34.01/34.16 (d, J_{CCF} = 19 Hz), 35.99/36.08, 36.31/36.40, 43.93, 48.15/48.33 (d, J_{CCF} = 23 Hz), 48.45, 48.47, 71.16, 71.73/72.03 (d, J_{CCF} = 38 Hz), 72.30 (C-22), 91.05/91.12, 99.47/100.87 (d, J_{CF} = 175 Hz), 124.27/124.41, 124.93, 127.21/127.26, 128.16/128.71, 129.62, 133.95/133.96, 136.99/137.04, 147.71, 152.44, 154.05, 171.22, 186.73,

204.40/204.46; HRFABMS *m/z* 608.2247 (calcd for $C_{31}H_{36}FNO_9Na$, 608.2272).

15. Photo-conversion of caged steroid hormones to steroid hormones. Fifty micromolar solution (50% aqueous MeOH) of caged steroid hormones was irradiated by a 6 W UV lamp equipped with a band pass filter (350–400 nm) at a distance of 10 cm from solution. The irradiated solution was analyzed by HPLC at regular intervals (t_R = 8.84 min for ER, 16.49 min for **1**: ODS-80T 200 mm × 4.6 mm, MeOH/H_2O 9:1, flow rate 0.5 ml/min. UV detection at 210 nm. t_R = 14.31 min for DEX, 43.93 min for **3**: ODS-80T, MeOH/H_2O 75:25, flow rate 0.5 ml/min. UV detection at 210 nm). The quantum yield of caged compounds was determined by a direct comparison with that of caged benzonate, 2-nitrophenylethyl benzoate, in same photolysis condition. 2-Nitrophenylethyl benzoate was synthesized and used as a standard. The photochemical properties and quantum yield of 2-nitrophenylethyl benzoate have been well characterized by Zhu et al.[24] Caged compound solution (50% aqueous MeOH) was irradiated by UV light (365 nm) with a fluorophotometer (Shimadzu RF-1500). The residual amount of caged compound in the photolyzed solution was analyzed by HPLC. The molecular extinction coefficient of **1** and **3** at 365 nm was determined to be 182 and 188 (M^{-1} cm^{-1}), respectively.
16. Light-regulation of gene expression and development in transgenic plants. Arabidopsis transgenic seedlings with *pER8::GFP* or *pIAA14::GFP-mIAA14-GR* were grown for 5 days on vertically oriented germination medium agar plates (GM, 0.5× Murashige and Skoog salts [Gibco-BRL, Gaithersburg, MD], 1% sucrose, 1× B5 vitamins, and 0.2 g/L 2-(4-morpholino)-ethane sulfonic acid (Mes), pH 5.8). The seedlings were immersed in a liquid GM containing 20 μM caged ERs (**1** or **2**) or 50 μM of caged DEX (**3**) for 30 min. After washing with a fresh GM, the seedlings were placed on GM agar solidified over slide glass. The UV light was irradiated to root on a spot for 3 s by a fluorescent microscope (Olympus BX50) with a UV-band pass filter (360–370 nm). The irradiated seedlings were transferred to a GM agar plates and then incubated vertically for an additional 12 h in darkness. For control treatment, 5-day-old transgenic or wild-type seedlings were transferred to GM agar plate containing 0, 1 μM ER or 10 μM DEX and grown vertically for 12 h. The GFP expression was monitored by a fluorescent microscope (Olympus SZX12 or Olympus BX50: *Ex* 460–490 nm, *Em* > 510 nm) and recorded with a digital camera.
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