

Two-photon fluorescence imaging of DNA in living plant turbid tissue with carbazole dicationic salt

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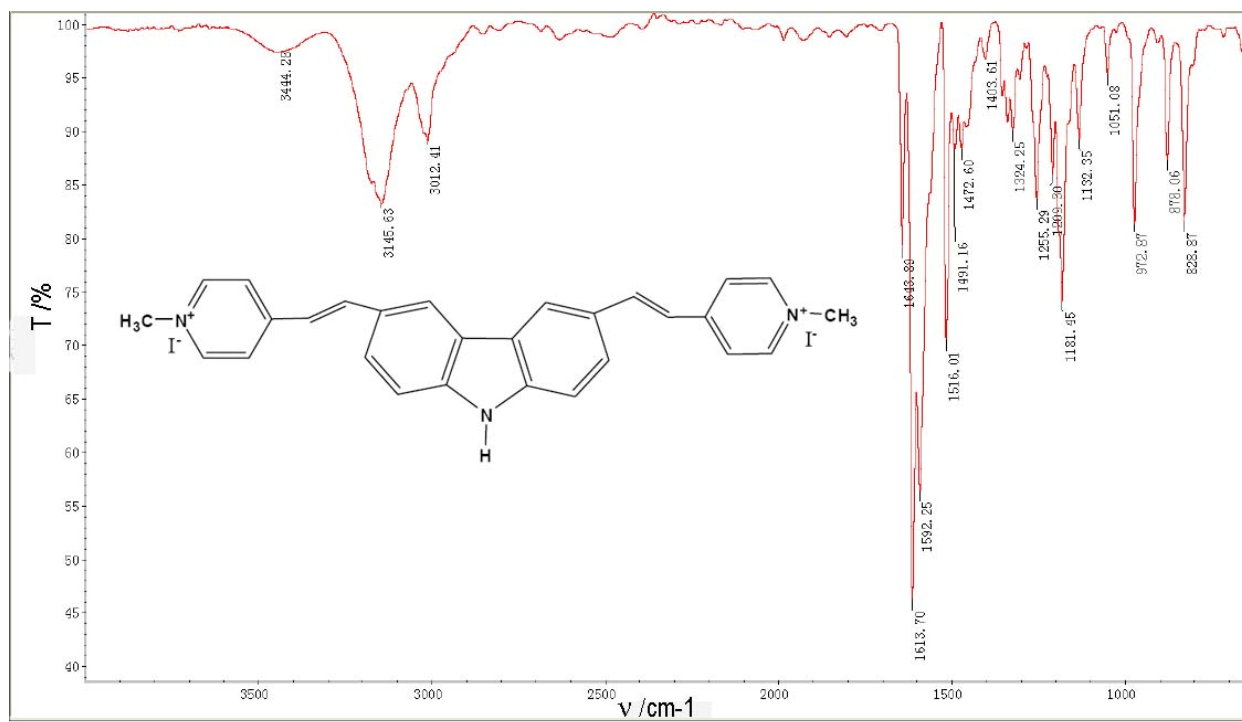
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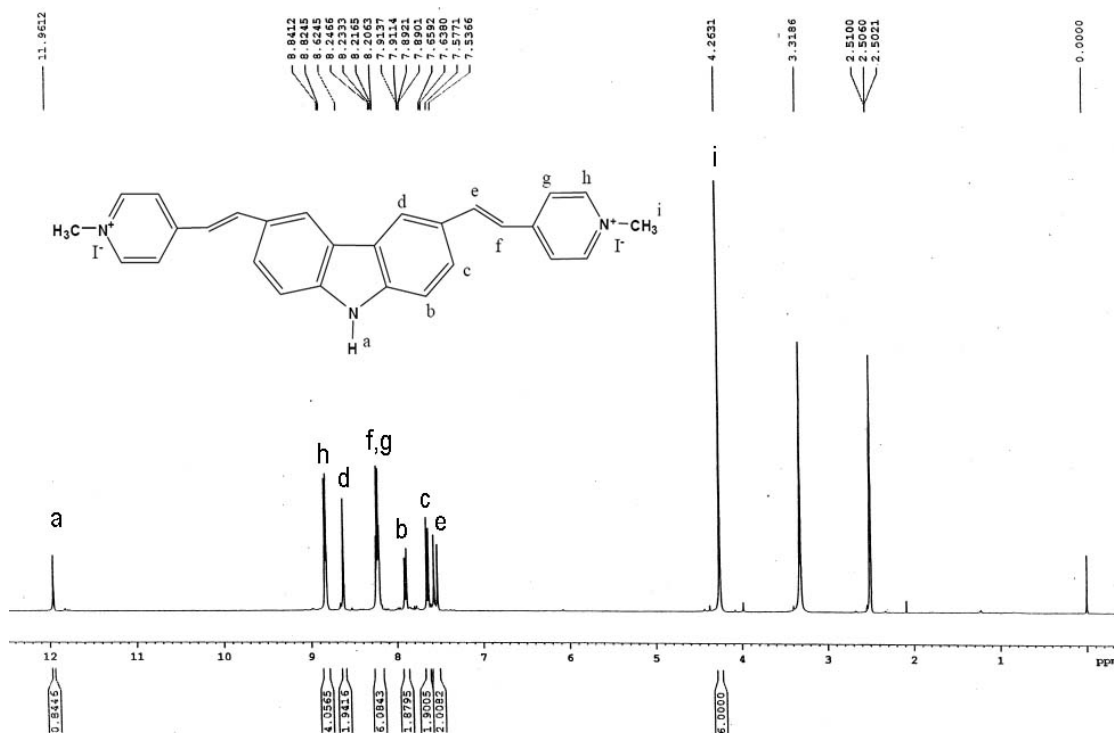
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1. The synthesis and characterization of 3,6-bis(1-methyl-4-vinylpyridinium)carbazole diiodide (BMVC), 9-Ethyl-3,6-bis(1-methyl-4-vinylpyridinium)carbazole Diiodide (9E-BMVC) and 9-Ethyl-3,6-bis(1-hydroxyethyl-4-vinylpyridinium)carbazole Diiodide (9E-BHVC)

a. **BMVC**: This compound was synthesized according to the literature.^[S1] mp>300 °C. IR (cm⁻¹): 972 (ν_{trans}=C-H). ¹H-NMR (400 MHz, DMSO-*d*₆, δ, ppm) δ 11.96(s, 1H, *nitrogen-H*), 8.83(d, *J*=6.7 Hz, 4H, *pyridine-H*), 8.62(s, 2H, *carbazole-H*), 8.20 – 8.25(m, 6H, *pyridine-H* and *trans-vinyl-H*), 7.90 (dd, 2H, *J*₁ = 8.6 Hz, *J*₂ = 0.9 Hz, *carbazole-H*), 7.65(d, *J* = 8.5 Hz, 2H, *carbazole-H*), 7.56(d, *J* = 16.2 Hz, 2H, *trans-vinyl-H*), 4.26(s, 6H, *methyl-H*). ¹³C-NMR (100 MHz, DMSO-*d*₆, δ, ppm) δ = 153.4, 145.3, 142.7, 142.2, 127.4, 127.2, 123.5, 123.4, 121.8, 120.9, 112.7, 47.2. HRMS (m/z): [M-I]⁺ calcd for C₂₈H₂₅N₃I, 530.1088; found, 530.1064. Anal. calcd for C₂₈H₂₅N₃I₂: C 51.16, H 3.83, N 6.39; found: C 50.92, H 3.69, N 6.35.

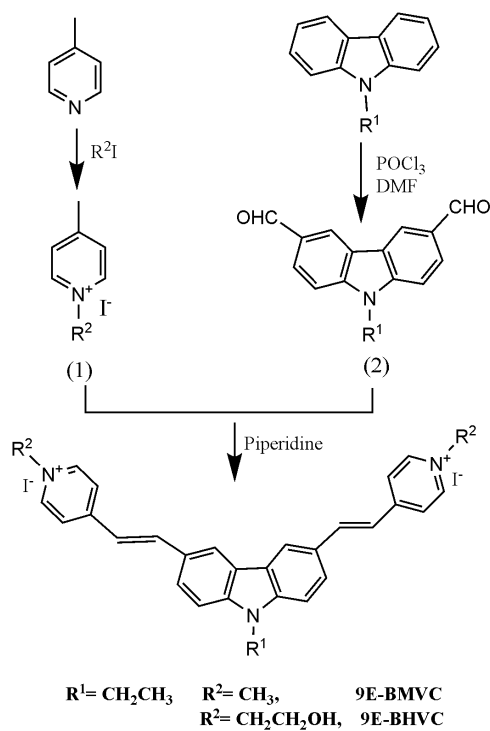


IR spectra of BMVC



^1H NMR spectra of BMVC

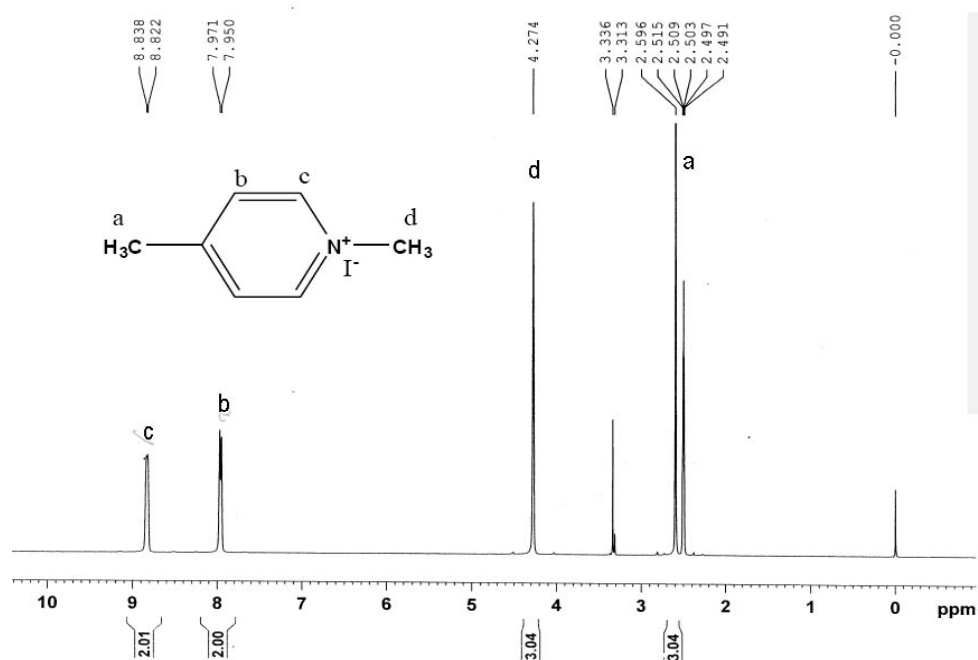
b. 9E-BMVC/9E-BHVC: The synthetic routes are shown in Scheme S1.



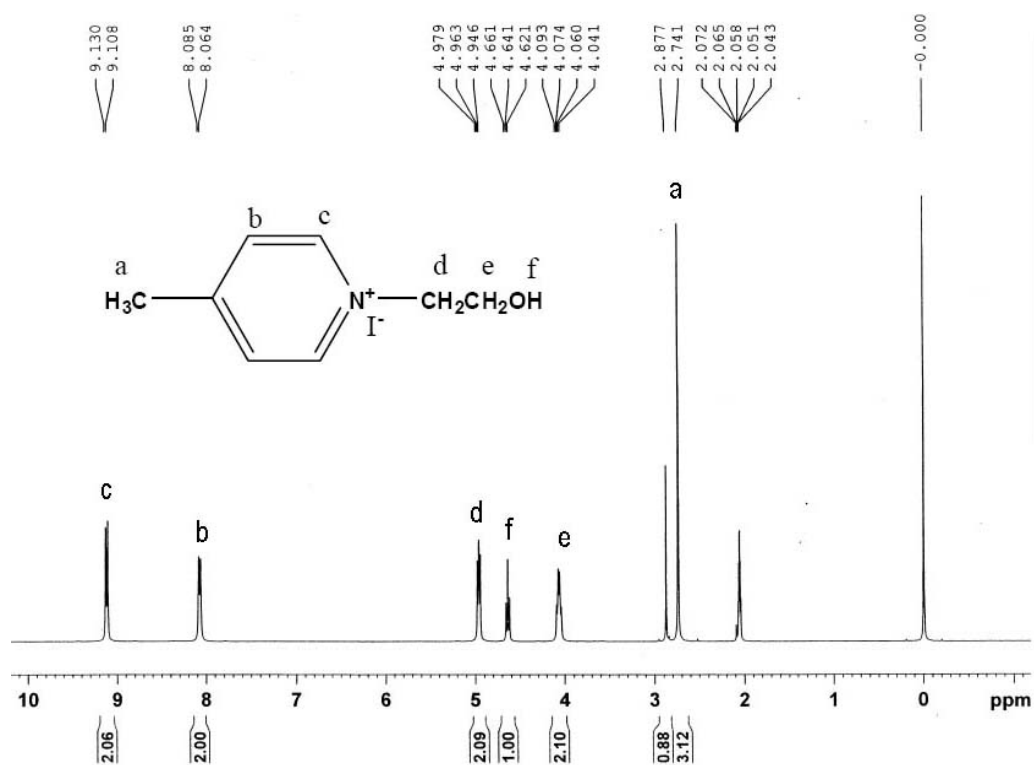
Scheme S1: Synthetic route of 9E-BMVC and 9E-BHVC

***N*-Methyl/hydroxyethyl-4-picoline iodine (1):** 0.05 mol of 4-picoline (4.65 g) and 0.055mol of 2-iodomethane (7.81 g) or 2-iodoethanol (9.50 g) were dissolved in toluene and stirred for 4 hours, thereafter refluxed 30 min. After cooling and filtrating, the resulting solid were washed with ether. White powder was obtained. For *N*-methyl-4-picoline iodine: yield 95%, ^1H NMR (300 MHz, DMSO, δ , ppm) δ 8.84(d, J = 4.8 Hz, 2H, *pyridine-H*), 7.96 (d, J = 6.3 Hz, 2H, *pyridine-H*), 4.28 (s, 3H, *methyl-H*), 2.50 (s, 3H, *methyl-H*). For *N*-hydroxyethyl-4-picoline iodine: yield 90%, ^1H -NMR (300MHz, acetone- d_6 , δ , ppm) δ 9.12 (d, J =6.6 Hz, 2H, *pyridine-H*), 8.07(d, J = 6.3 Hz, 2H, *pyridine-H*), 4.96(t, J = 5.0 Hz, 2H, *methylene-H*), 4.64 (t, J = 6.0 Hz, 1H, *hydroxyl-H*), 4.06 (q, J = 5.2 Hz, 2H, *methylene-H*), 2.74(s, 3H, *methyl-H*).

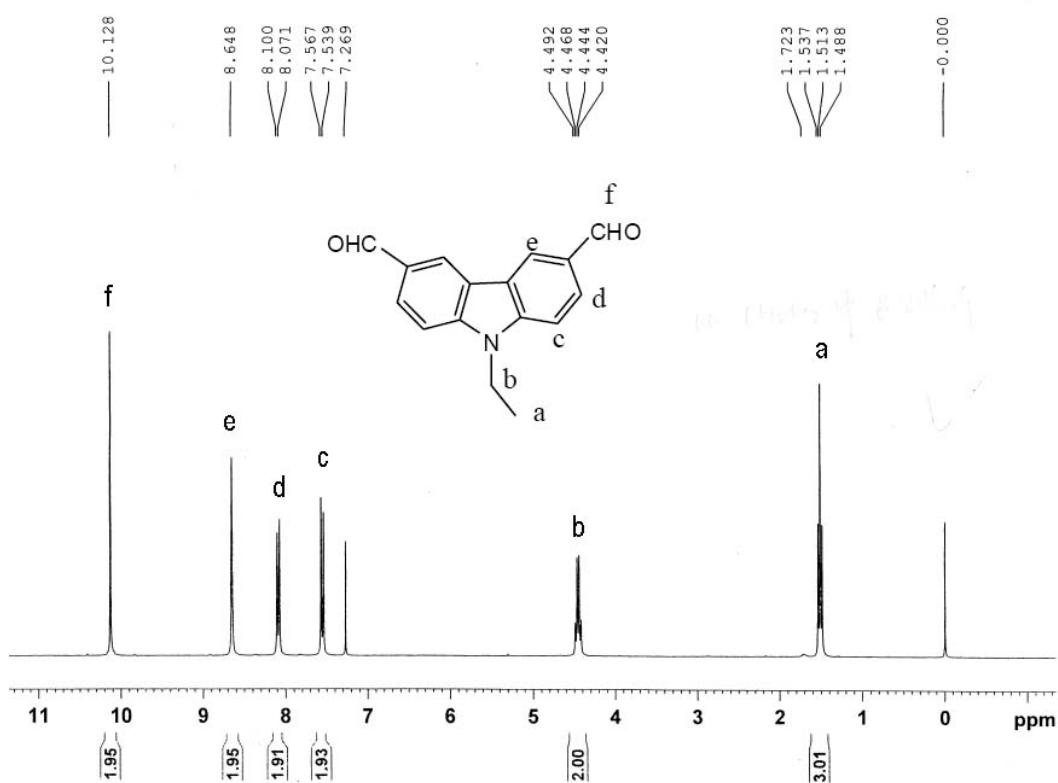
9-Ethyl-3,6-diformyl-9H-carbazole (2): We have synthesized this compound, obtained its single crystal and resolved its crystal data.^[S2] ^1H -NMR (300 MHz, CDCl_3 , δ , ppm) δ 10.12 (s, 2H, *formyl-H*), 8.65 (s, 2H, *carbazole-H*), 8.07 (d, J = 8.7 Hz, 2H, *carbazole-H*), 7.55 (d, J = 8.4 Hz, 2H, *carbazole-H*), 4.45 (q, J = 7.2 Hz, 2H, *methylene-H*), 1.51 (t, J = 7.4 Hz, 3H, *methyl-H*).



^1H NMR spectra of *N*-methyl-4-picoline iodine

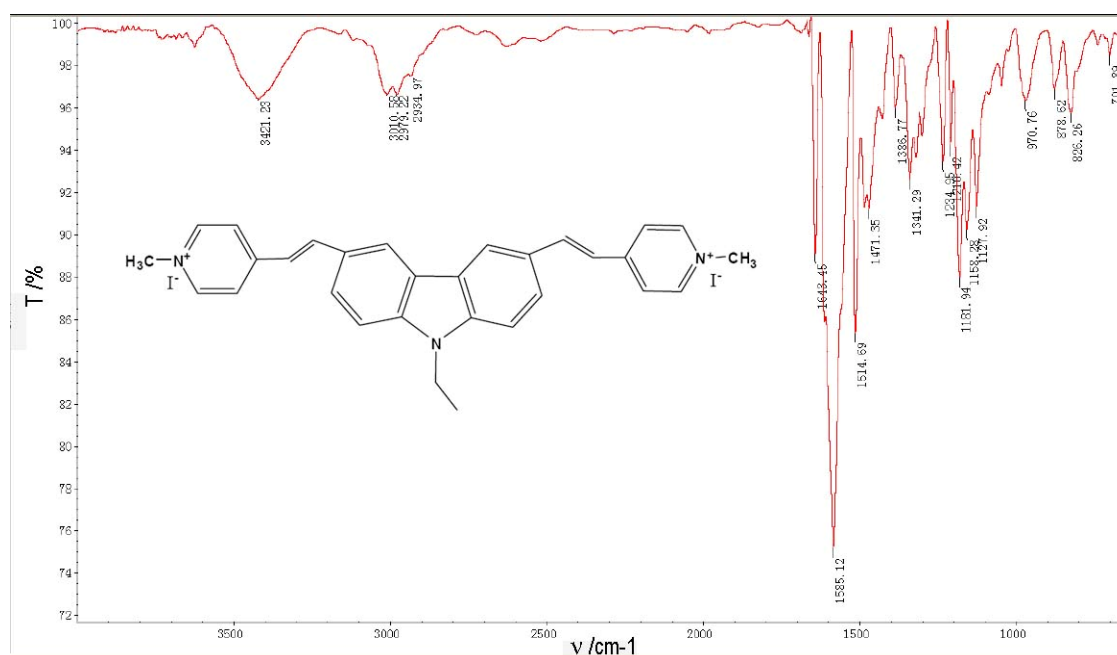


¹H NMR spectra of *N*-hydroxyethyl-4-picoline iodine

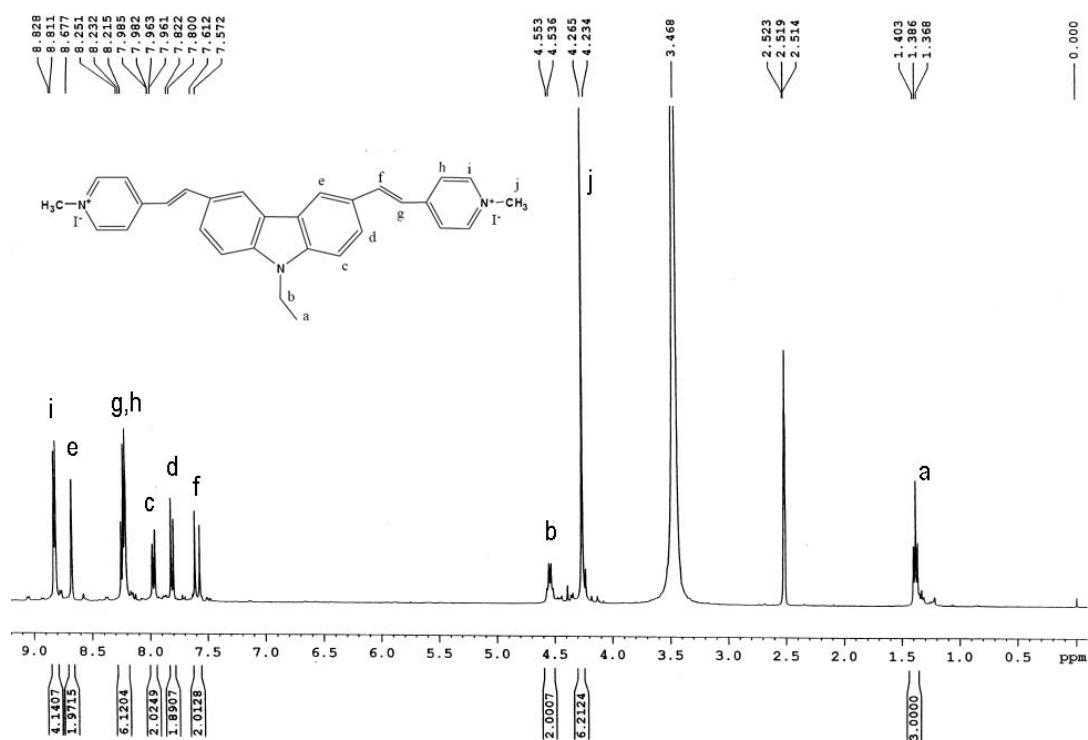


¹H NMR spectra of 9-Ethyl-3,6-diformyl-9H-carbazole

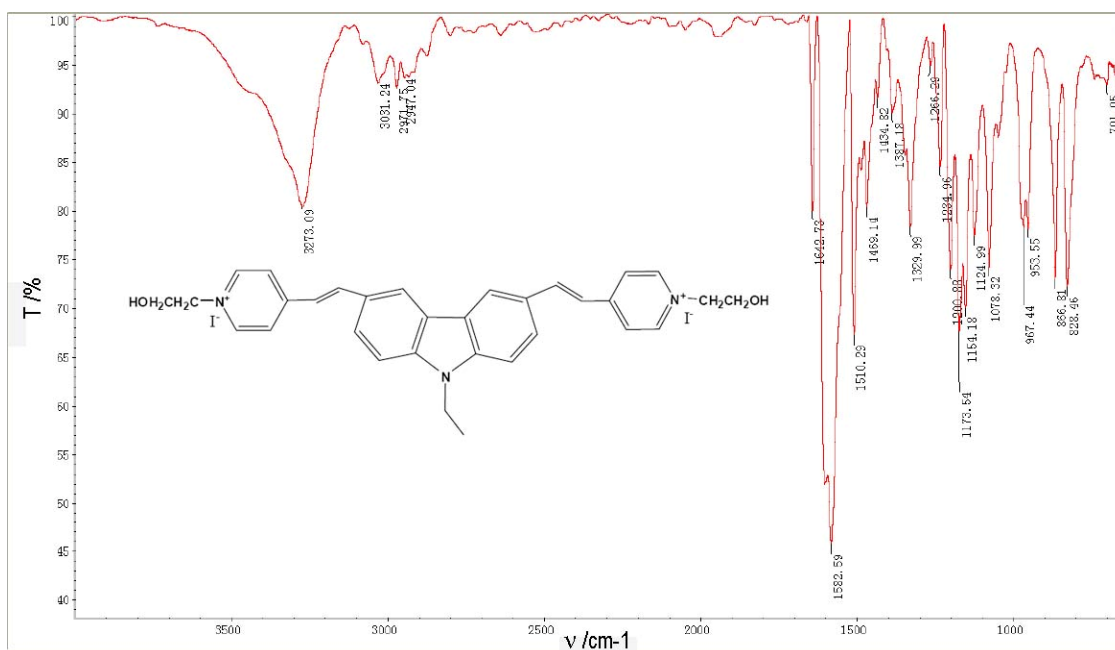
9E-BMVC/9E-BHVC: 2.6 mmol of *N*-methyl-4-picoline iodine (0.61 g) or *N*-hydroxyethyl-4-picoline iodine (0.70 g) and 0.95 mmol of 9-Ethyl-3,6-diformyl-9H-carbazole (0.24 g) were dissolved in 25ml of methanol, then several drops of piperidine was added in the mixture. This solution were stirred and refluxed at 80°C for 4h. The mixture was cooled and the methanol was removed. The residue was washed with ethanol for several times to give red powder. For **9E-BMVC**: yield 68% (mp>300 °C), IR(cm⁻¹): 970 ($\nu_{\text{trans}=\text{C}-\text{H}}$). ¹H-NMR (400 MHz, DMSO-d₆, δ , ppm) δ 8.82(d, J = 6.8 Hz, 4H, *pyridine-H*), 8.67(s, 2H, *carbazole-H*), 8.22–8.25 (m, 6H, *pyridine-H* and *trans-vinyl-H*), 7.97(dd, J_1 = 8.4 Hz, J_2 = 1.2 Hz, 2H, *carbazole-H*), 7.81(d, 2H, J = 8.8 Hz *carbazole-H*), 7.59(d, J = 16.0 Hz, 2H, *trans-vinyl-H*), 4.54 (q, J = 6.8 Hz, 2H, *methylene-H*), 4.26 (s, 6H, *methyl-H*), 1.39(t, J = 7.0 Hz, 3H, *methyl-H*). ¹³C-NMR (100 MHz, DMSO-d₆, δ , ppm) δ = 153.4, 145.3, 142.4, 141.9, 127.6, 127.3, 123.4, 123.2, 121.8, 121.1, 111.0, 47.2, 38.1, 14.3. HRMS (m/z): [M-I]⁺ calcd for C₃₀H₂₉N₃I, 558.1373; found, 558.1401. Anal. calcd for C₃₀H₂₉N₃I₂: C 52.57, H 4.26, N 6.13; found: C 52.35, H 4.23, N 5.92. For **9E-BHVC**: yield 53% (mp>300 °C). IR(cm⁻¹): 967 ($\nu_{\text{trans}=\text{C}-\text{H}}$). ¹H-NMR (400MHz, DMSO-d₆, δ , ppm) δ 8.84(d, J = 6.8 Hz, 4H, *pyridine-H*), 8.65(s, 2H, *carbazole-H*), 8.22–8.26 (m, 6H, *pyridine-H* and *trans-vinyl-H*), 7.97(dd, J_1 = 8.7 Hz, J_2 = 1.2 Hz, 2H, *carbazole-H*), 7.82(d, J = 8.7 Hz, 2H, *carbazole-H*), 7.59(d, J = 16.2 Hz, 2H, *trans-vinyl-H*), 5.25(t, J = 5.2 Hz, 2H, *hydroxyl-H*), 4.56(t, J = 4.8 Hz, 6H, *methylene-H*), 3.88(q, J = 5.0 Hz, 4H, *methylene-H*), 1.38(t, J = 7.1 Hz, 3H, *methyl-H*). ¹³C-NMR (100 MHz, DMSO-d₆, δ , ppm) δ = 153.9, 144.9, 142.6, 142.0, 127.6, 127.4, 123.4, 123.3, 121.8, 121.1, 111.0, 62.4, 60.5, 38.1, 14.4. HRMS (m/z): [M-I]⁺ calcd for C₃₂H₃₃N₃IO₂, 618.1612; found, 618.1588. Anal. calcd for C₃₂H₃₃N₃I₂O₂: C 51.56, H 4.46, N 5.64; found: C 51.27, H 4.39, N 5.55.



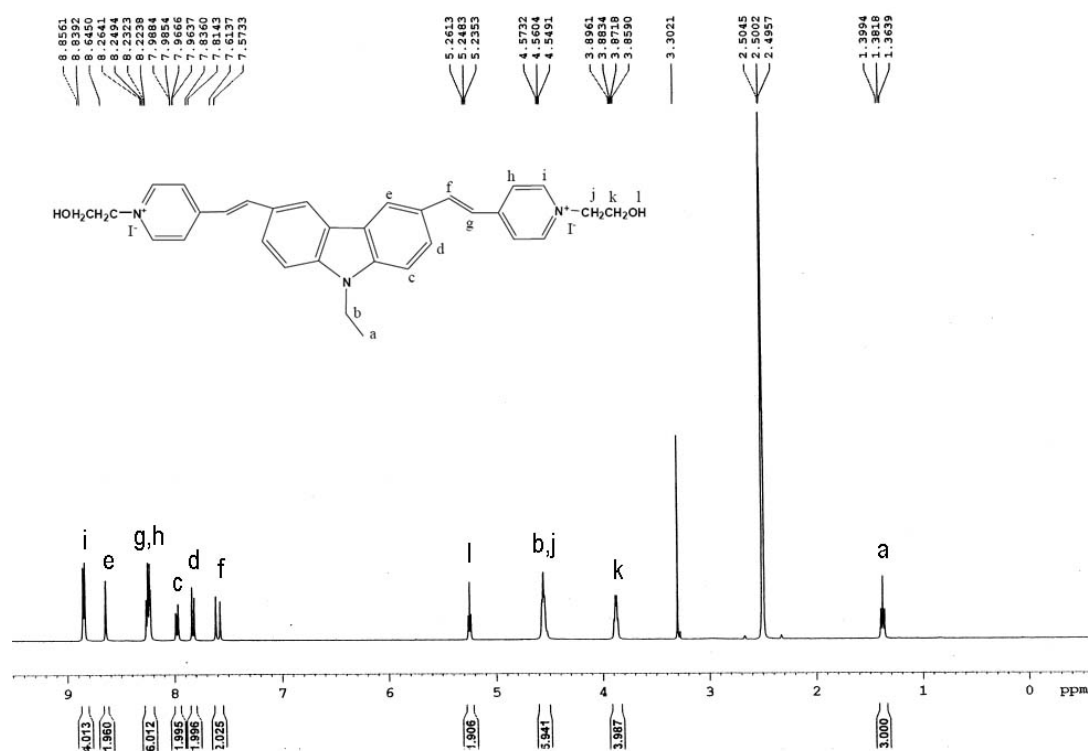
IR spectra of 9E-BMVC



^1H NMR spectra of 9E-BMVC



IR spectra of 9E-BHVC



¹H NMR spectra of 9E-BHVC

2. Preparation and staining of living plant cell and tissue

Arabidopsis protoplasts were isolated from newly expanded callus of Arabidopsis leaves. The protoplasts were generated using a method as reported by Yoo *et al.* [S3] Then the protoplasts were washed three times with protoplast suspension medium. A little amount of mixing aqueous solution of 9E-BHVC/9E-BMVC/BMVC and DAPI was added into medium and incubated 30 min at 25°C in the darkness, and then the protoplasts were dropped on microscope slides for imaging. The Arabidopsis thaliana seeds were germinated and grown on MS medium [S4] containing 3% sucrose and 0.8% agar, and the seedlings were cultured in controlled growth chambers at 22°C under the conditions of 16-h light and 8-h dark. The 2-week-old seedlings were incubated in mixing aqueous solution of 9E-BHVC/9E-BMVC/BMVC and DAPI, and the seedlings were imaged immediately.

3. Measurement equipments and methods

Nuclear magnetic resonance spectra of ^1H and ^{13}C were obtained on a Bruker Avance 300/400 spectrometer. The data of HRMS and elemental analyses were given on LTQ Orbitrap XL and Vario EI III performed, respectively. The UV-visible-near-IR absorption spectra of dilute solutions were recorded on a Perkin Elmer Lambda-35 spectrophotometer using a quartz cuvette having 1 cm path length. IR spectra are measured on Nexus 670. One-photon fluorescence spectra were obtained on a HITACHI F-4500 spectrofluorimeter equipped with a 450-W Xe lamp. Two-photon ones were noted on a SpectroPro300i and the pump laser beam came from a modelocked Ti:sapphire laser system at the pulse duration of 200 fs, a repetition rate of 76MHz (Coherent Mira900-D).

4. TPM experiment

All of microscopic photos were obtained in LSM 510META+NLO of Zeiss. In vivo 2PLSM imaging was performed on a Zeiss (Oberkochen, Germany) LSM 510 equipped with a $40 \times$ [0.75 numerical aperture (NA)] PLAN-NEOFLUOR objective and a photomultiplier tube. A Titanium-Sapphire laser (Coherent) pumped by a 10 W diode laser was used to excite DAPI and 9E-BHVC/9E-BMVC/BMVC at 800 nm. The incident power controlled by changing the output of AOM in Zeiss LSM software on computer interface, and incident power displayed on surface is percent. The total power provided by laser source can maintain stable. Blue fluorescence of DAPI was visualized with a Beam splitter DM510 and BA435–485 nm bandpass emission filter combination, and orange fluorescence of 9E-BHVC/9E-BMVC/BMVC was visualized with BA535–590 nm bandpass emission filter. Both channels were using the BA650nm to block the excitation IR wavelength.

5. Measurement of TPA cross sections

TPA cross sections have been measured using the two-photon induced fluorescence method, and thus cross section can be calculated by means of equation (1) [S5-S7]

$$\delta_s = \delta_r \frac{\Phi_r c_r n_r F_s}{\Phi_s c_s n_s F_r} \quad (1)$$

where the subscripts *s* and *r* refer to the sample and the reference material, respectively. The terms *c* and *n* are the concentration and refractive index of the solution, respectively. *F* is two-photon excited fluorescence integral intensity. Φ is the fluorescence quantum yield. TPA quantum yield measurement is quite difficult compared to the well-established quantum yield measurement of SPF. So, one might suppose that two quantum yields are coincidental. δ_r is the TPA cross-section of the reference molecule. [S8,S9]

6. Measurement of binding parameters of carbazole derivatives to DNA: The intrinsic binding constants (*k*) were obtained by the one-photon fluorescence titration method. The excitation wavelengths of 434 nm for BMVC, 444 nm for 9E-BMVC and 451 nm for 9E-BHVC were used. And the fluorescence intensity monitored at 554 nm for BMVC and 566 nm for 9E-BMVC/9E-BHVC, were analyzed with the Scatchard equation according to equation (2) [S10]

$$r / c_f = kn - kr \quad (2)$$

Where *k* is binding constant, *n* is the number of dye sites of per phosphate, *r* is the ratio of the concentration of the binding dye to the concentration of DNA (in phosphate) and *C_f* is the concentration of free dye. The concentration of the binding compound was calculated using equation (3) [S11]

$$c_b = c_t [(F - F^0) / (F^{\max} - F^0)] \quad (3)$$

where *c_t* is the total compound concentration, *F* is the observed fluorescence intensity at given DNA concentration, *F⁰* is the fluorescence intensity in absence of DNA, and *F^{max}* is the fluorescence intensity of the totally binding compound.

7. Fig. S1

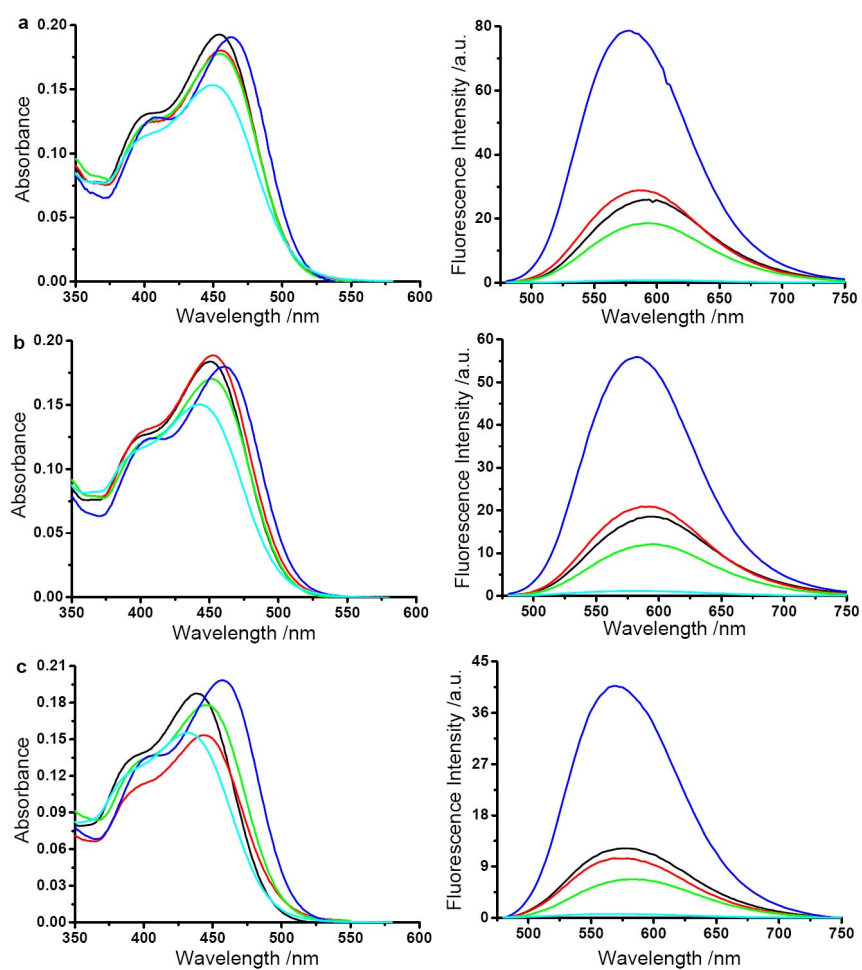


Fig. S1 Electronic absorption (left) and one-photon fluorescence spectra (right) of 9E-BHVC (a), 9E-BMVC (b) and BMVC (c) in various polar solvent. Blue: Ethanol, Red: Acetone, Black: Acetonitrile, Green: DMF and Cyan: water. [Compound] = 2.5 μM .

8. Fig. S2

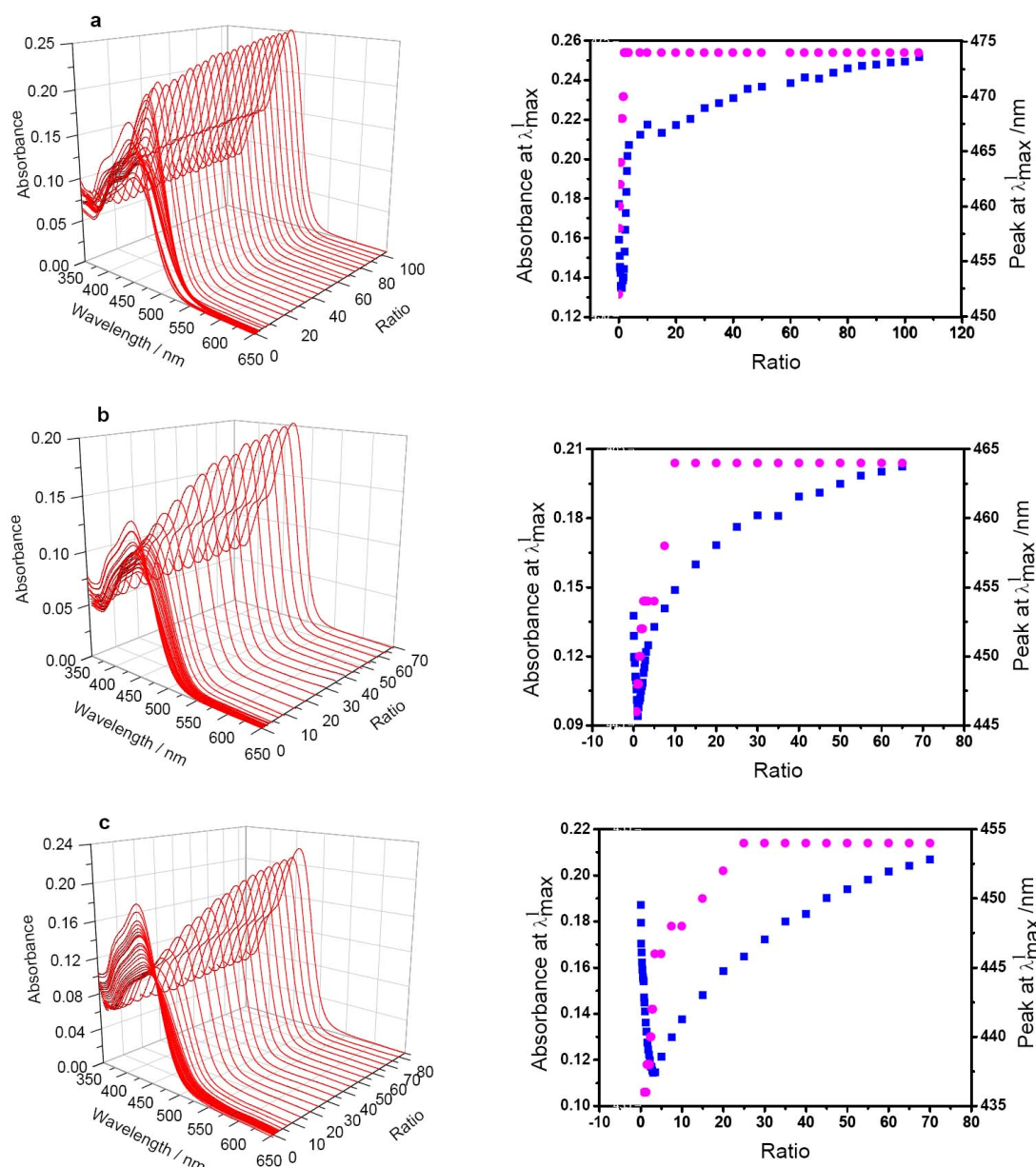


Fig. S2 Electronic absorption spectra of 9E-BHVC (a), 9E-BMVC (b) and BMVC (c) with addition of calf-thymus DNA; tris-HCl buffer solution, 10 mM, pH 7.2, KCl 100 mM. Left: titration curve. Right: change of absorbance (blue) and peak position (magenta) at $\lambda_{\text{max}}^{\text{I}}$. [9E-BHVC] = 4 μM , [9E-BMVC] = [BMVC] = 5 μM . Ratio: [phosphate of DNA]/[dye].

9 . Fig. S3

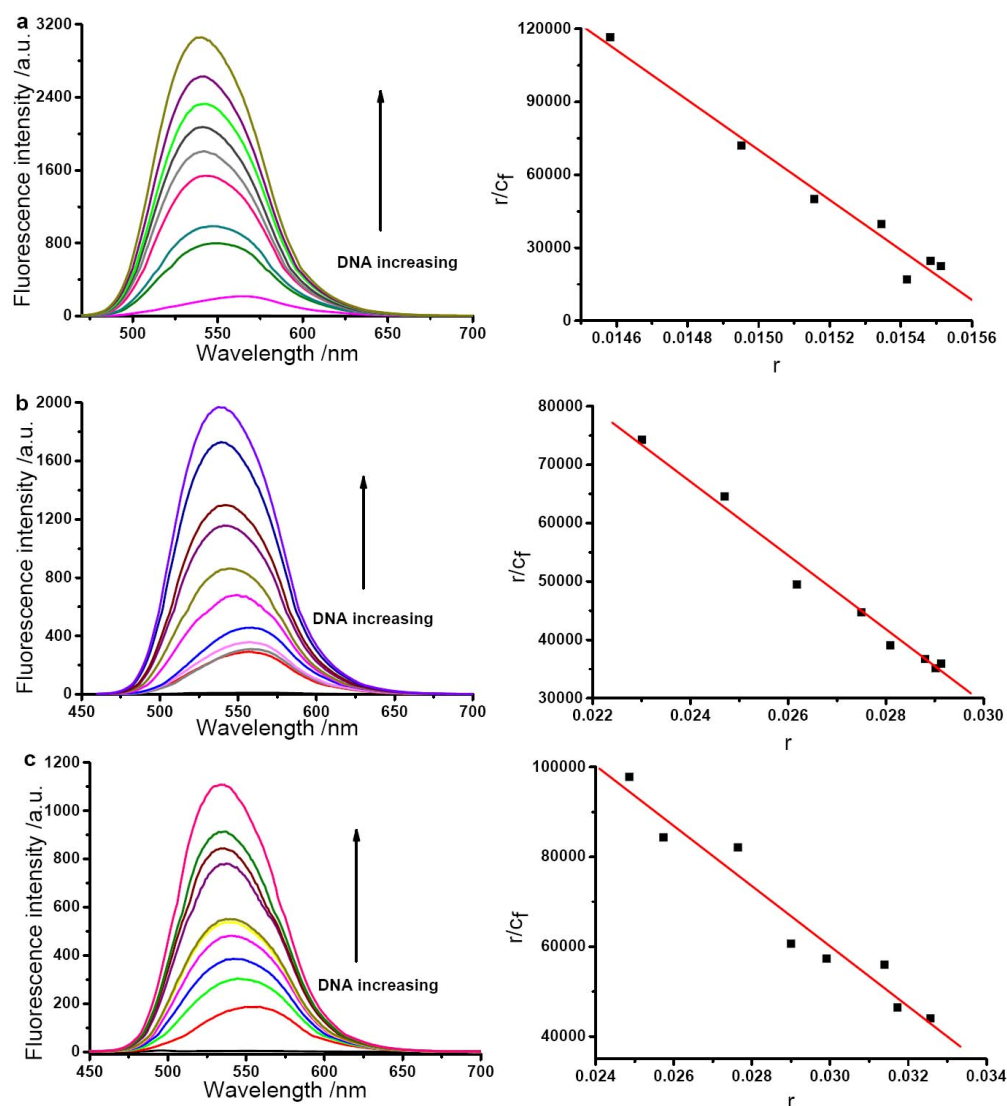


Fig. S3 One-photon fluorescence titration (left) of 9E-BHVC (a)/9E-BMVC (b)/BMVC (c) with DNA and their fitted curve (right) according to Scatchard equation. λ_{ex} = 451 nm and λ_{em} = 566 nm for 9E-BHVC. λ_{ex} = 444 nm and λ_{em} = 566 nm for 9E-BMVC. λ_{ex} = 434 nm and λ_{em} = 554 nm for BMVC. [Compound] = 1 μ M, [phosphate in DNA] = 0 – 70 μ M for 9E-BHVC, 0 – 55 μ M for 9E-BMVC and 0 – 40 μ M for BMVC. Their binding constants are 1.02×10^7 M $^{-1}$ (9E-BHVC), 6.46×10^6 M $^{-1}$ (9E-BMVC) and 6.70×10^6 M $^{-1}$ (BMVC). Corresponding binding sites are 64 (9E-BHVC), 29 (9E-BMVC) and 25.6 (BMVC) phosphates/dye. Goodness-of-fit: R^2 = 0.944, 0.984, 0.972 for BMVC, 9E-BMVC and 9E-BHVC.

10. Fig. S4

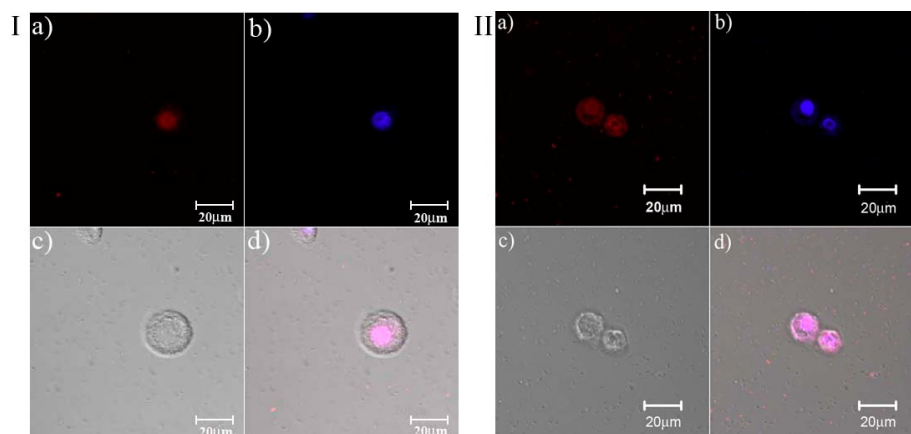


Fig. S4 TPM photos of *Arabidopsis* protoplasts incubated with 9E-BMVC (I) BMVC (II) and DAPI for 30 min. (a) Fluorescence of 9E-BMVC/BMVC (1 μ M) at 3% power, (b) Fluorescence of DAPI (1 μ M) at 5% power, (c) Phase-contrast picture and (d) overlay of phase-contrast and fluorescence. Excitation wavelength: 800 nm. Detection wavelength, 435 – 485 nm (DAPI), 535 – 590 nm (9E-BMVC/BMVC).

11. Fig. S5

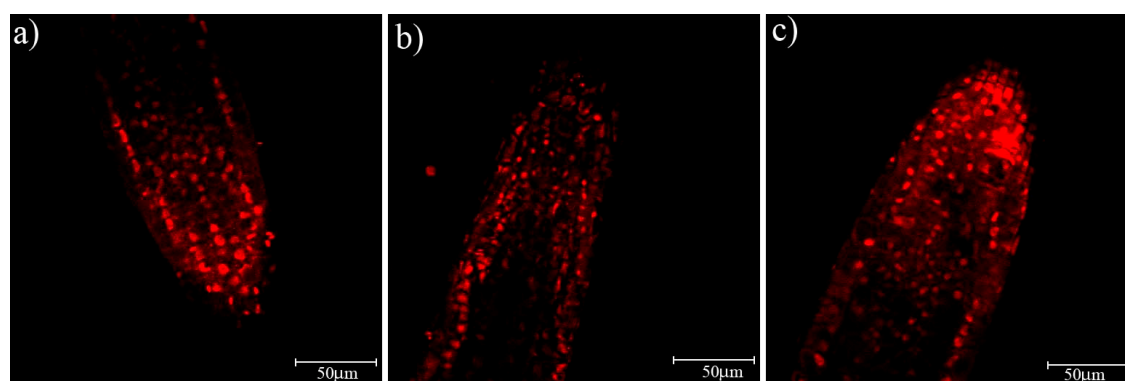


Fig. S5 Fluorescent photos of three *Arabidopsis* root tips incubated with 9E-BHVC for 1 h at 5 μ M. Incident power, 1%. Excitation wavelength, 800 nm. Detection wavelength, 535 – 590 nm.

12. Video S1

Incubated time: 1h. Concentration of 9E-BHVC: 1 μ M, that of DAPI: 2 μ M. Depth range: 0 – 24 μ m. Incident power: 5%. Excitation wavelength: 800 nm. Detection wavelength: 435 – 485 nm (DAPI), 535 – 590 nm (9E-BHVC).

13. Video S2

Incubated time: 1 h. Concentration of 9E-BHVC/DAPI: 3 μ M. Depth range: 0 – 41 μ m. Incident power: 2%.
Excitation wavelength: 800 nm. Detection wavelength: 435 – 485 nm (DAPI), 535 – 590 nm (9E-BHVC).

14. Video S3

Incubated time: 1 h. Concentration of 9E-BHVC/DAPI: 3 μ M. Depth 18 μ m. Incident power range (%): 0.5, 1.0, 1.5, 2.0, 2.5, 3.0. Excitation wavelength: 800 nm. Detection wavelength: 435– 485 nm (DAPI), 535– 590 nm (9E-BHVC).

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