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Design and synthesis of biotin-tagged photoaffinity probes of jasmonates

Min Gu [a,b,†], Jianbin Yan [c,†], Zhiyan Bai [c], Yue-Ting Chen [b], Wei Lu [a], Jie Tang [a], Liusheng Duan [c], Daoxin Xie [c,*], Fa-Jun Nan [b,*]

[a] Department of Chemistry and Institute of Medicinal Chemistry, East China Normal University, 3663 North Zhong Shan Road, Shanghai 200062, China
[b] National Center for Drug Screening, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, 189, Guo Shou Jing Road, Shanghai 201203, China
[c] MOE Key Laboratory of Bioinformatics, School of life Sciences, Tsinghua University, Beijing 100084, China

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ABSTRACT

Jasmonates (JAs) are a class of oxylipin compounds that play diverse roles in plant defense and development. The F-box protein coronatine insensitive1 (COI1) plays a crucial role in the JA signaling pathway. To determine whether COI1 binds directly to jasmonates, three biotin-tagged photoaffinity probes for JAs, a jasmonic acid photoaffinity probe (PAJA), a JAIle photoaffinity probe (PAJAIle), and a coronatine photoaffinity probe (PACOR), were designed and synthesized based on analysis of JA structure–activity relationships and molecular modeling of the interaction between COI1 and JAs. Among them, PACOR exhibited the most significant biological activity in inhibiting root growth, promoting accumulation of JA-responsive proteins, and triggering COI1–JAZ1 interaction in *Arabidopsis* seedlings. PACOR is an effective tool for elucidating the interaction between COI1 and JA.

1. Introduction

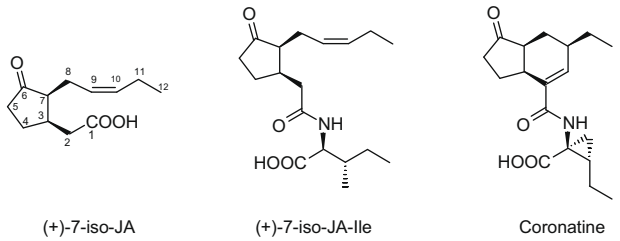
Plant hormones regulate numerous responses in plant growth and development. Elucidation of the mechanisms responsible for hormone perception is important for understanding the effects of plant hormones. Although many hormone receptors, such as the auxin receptor,[1,2] the gibberellin receptor,[3] and the brassinosteroid receptor[4] have been identified, little is known about jasmonate (JA) receptors.

JAs are indispensable in plant defense responses and aspects of plant growth and development.[5–13] An *Arabidopsis coronatine insensitive1* (*COI1*) gene encodes an F-box protein required for JA-regulated plant fertility and defense.[14] We previously demonstrated that COI1 interacts with multiple proteins to form the SCF^{COI1} E3 ubiquitin ligase complex, which is required for all JA responses.[15–17]

Recent studies have shown that the SCF^{COI1} complex recognizes JA transcriptional repressors called jasmonate ZIM-domain (JAZ) proteins and targets them for subsequent degradation by the 26S proteasome in the presence of the hormone.[18–20] In addition, physical interaction of SCF^{COI1} with the JAZ proteins is promoted by a bioactive isoleucine-conjugated form of jasmonic acid (JA-Ile) or its stereochemical mimic, coronatine (COR)[21–23] (Fig. 1). These results suggest that the SCF^{COI1}–JAZ complex is the site of JA-Ile/COR perception.[21]

To determine whether COI1 binds directly with JA-Ile/COR, we used photoaffinity labeling activity-based probe technology, which is very useful for identifying specific receptors for small-molecule ligands and for localizing the ligand binding site within the receptor. In our previous study,[24] we reported a photoaffinity probe PACOR and demonstrated that PACOR physically binds with COI1 protein, which accelerates researches on the mechanism of JA perception. Here, we explain chemical, analytical and biological detail on design, synthesis and action of PACOR and two other similar probes PAJA and PAJAIle, and comparison of these compounds in terms of bioactivity and binding.

To get active photoaffinity probes for JAs, we firstly analyzed the interaction between COI1 and several common jasmonates through molecular modeling. We found that the jasmonate moiety in JA-Ile could bind to the P1 and P2 pockets and the bottle neck region of COI1, which suggests that the jasmonate moiety is



(+)-7-iso-JA (+)-7-iso-JA-Ile Coronatine

Figure 1. Structures of JA, JA-Ile, coronatine.

* Corresponding authors. Tel./fax: +86 10 62781575 (D.X.); tel./fax:+86 21 50800954 (F.-J.N.).
E-mail addresses: daoxinlab@mail.tsinghua.edu.cn (D. Xie), fjnan@mail.shcnc.ac.cn (F.-J. Nan).
† These authors contributed equally to this work.

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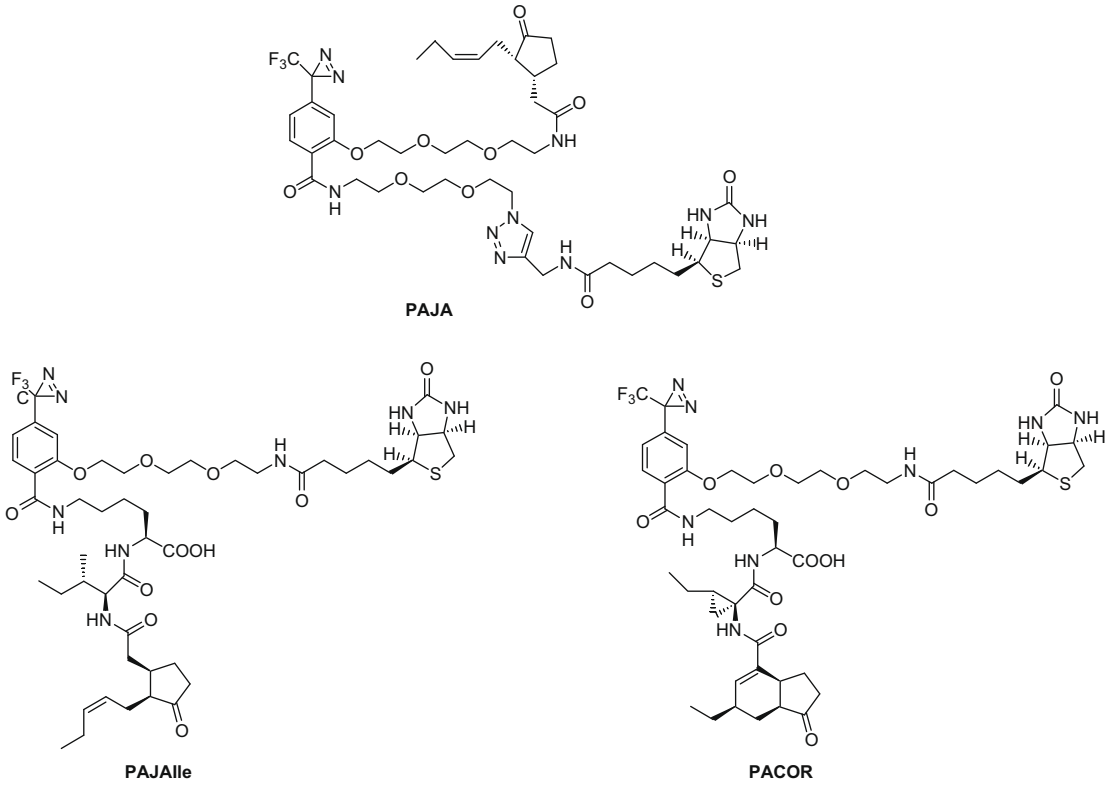


Figure 2. Structures of jasmonate probes.

essential to stabilize its binding with COI1 and it is difficult to modify at these positions for an active probe. However, the isoleucine moiety of JA-Ile extends outward from the COI1 surface pocket, which makes the free carboxyl group become a possible site to modify. Then, we designed and synthesized three biotin-tagged photoaffinity probes based on different jasmonates including PAJA, PAJAIle, and PACOR (Fig. 2), in the latter two probes, a free carboxylic acid group was introduced through a lysine linker to mimic the carboxylic acid group existed in JAIle and coronatine.

By comparing the bioactivities of PAJA, PAJAIle, and PACOR, PACOR was found to exhibit the highest biological activity in respect of inhibiting root growth, accumulating JA-responsive proteins, and triggering the COI1–JAZ1 interaction, while PAJAIle. PAJA just showed weak biological activity. Therefore, PACOR was selected as an useful tool for studying the interaction between COI1 and JAs.

2. Preparation of the probes

Scheme 1 shows the synthesis of the PAJA probe. Preparation of the probe was commenced with compound **1**, to which *tert*-butyl 2-(2-(2-bromoethoxy)ethoxy)ethylcarbamate was attached, resulting in **2**. Oxidization of **2** with $(C_4H_9)_4NMnO_4$ in pyridine resulted in **3**, which was then condensed with succinimide using EDC in dichloromethane to produce **4**. Compound **4** was condensed with 2-(2-(2-azidoethoxy)ethoxy)ethylcarbamate to produce **5**, and removal of the *tert*-butyloxycarbonyl protecting group of **5** and subsequent condensation with JA-OSu resulted in **7**. Click reaction of **7** with alkynylethylamide biotin produced the PAJA probe.

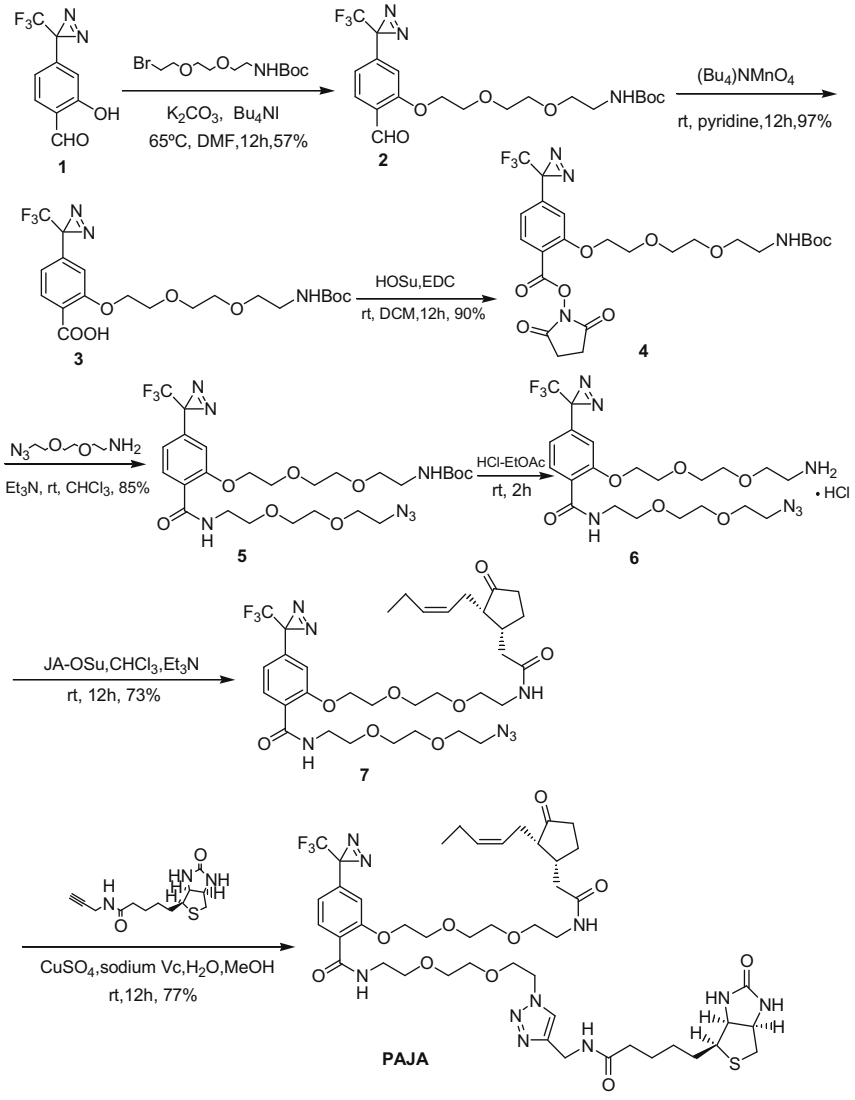
Scheme 2 shows the synthesis of the PAJAIle and PACOR probes. First, Fmoc-Lys(*Z*)-OH was condensed with 2-(trimethylsilyl)ethanol using DCC and DMAP in dichloromethane to produce **8**. Then **9** was obtained by reducing **8** with H_2/Pd in methanol to remove the CBZ group. At the same time, a few drops of acetic acid were added to protect the Fmoc group. Compound **9** was condensed with **4** under basic conditions to produce **10**. Compound **10** was rapidly reacted with piperidine in DMF, removing the Fmoc group to produce **11**. Compound **11** was condensed with JA-Ile/coronatine using DCC, HOBT, and Et_3N in dichloromethane to obtain **12** and **13**. Removal of the *tert*-butyloxycarbonyl protecting groups of **12** and **13** with 2 N HCl–EtOAc and subsequent incorporation of the biotin tag in dichloromethane produced probes **14** and **15**. Deprotection of the 2-(trimethylsilyl)ethyl groups of **14** and **15** using tetrabutylammonium fluoride in THF yielded the photoaffinity probes, PAJAIle and PACOR.

3. Biological activities of the probes

The biological activities of the three JA photoaffinity probes were examined in *Arabidopsis* using three bioassays: root growth inhibition, accumulation of JA-responsive proteins, and promotion of the COI1–JAZ1 interaction.

One of the first biological activities observed for JAs was the root growth inhibition.[25,26] JAs supplemented to the growth medium cause a significant inhibition of seedling root growth. Therefore, we first investigated whether the effect of the probes on the root growth of *Arabidopsis* seedlings was similar to that of the JAs. Seedlings were grown on MS medium containing 100 μM of PAJA, PAJAIle, PACOR, or MeJA for nine days, after which the root lengths of the seedlings were measured. Treatment without JAs or with BP (a negative control consisting of a photoreactive group and a biotin tag) alone, did not inhibit root growth (Fig. 3). In contrast, treatment with MeJA had the strongest effect on root growth, followed by PACOR, which indicates that PACOR had the best bioactivity for JAs of the three probes developed (Fig. 3).

JAs are thought to play an important role in the defense reaction of plants against phytopathogens. One of important ways that help protect plants from fungal pathogens damage is the activation of the expression of antifungal proteins, such as thionin, osmotin



Scheme 1. Synthesis of PAJA.

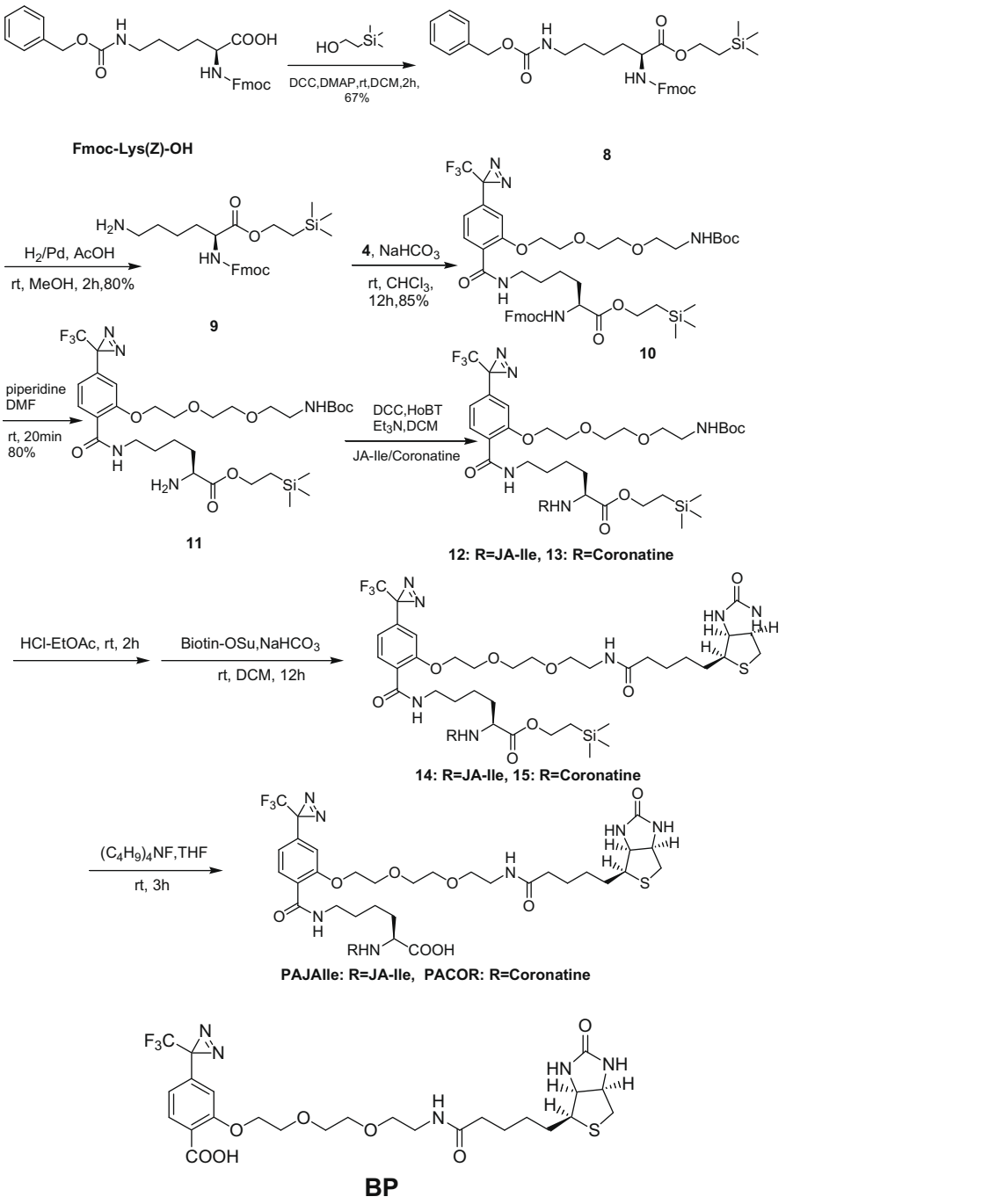
and defensin. *Arabidopsis* thionin gene *Thi2.1* can be used as a molecular marker to detect the biological activity of jasmonate.[27] To determine whether the probes induced expression of Thi2.1 proteins, we generated plants transgenic for a *thionin 2.1*-GUS (β-glucuronidase) reporter construct,[28] in which the *GUS* gene was driven by the promoter of *thionin 2.1* that is induced by JA treatment. Seedlings carrying the *thionin 2.1*-GUS reporter construct were treated with 100 μM PAJA, PAJAIle, PACOR, or MeJA and were then subjected to histochemical staining. As expected, the seedlings treated with MeJA showed strong blue staining, whereas there were no blue staining in the seedlings without treatment (Mock). The PAJA probe did not show any significant blue staining. The PAJAIle probe induced slight GUS expression. In contrast, the PACOR probe showed clear GUS activity, indicating that the PACOR probe was the most active of the three probes (Fig. 4).

Finally, we examined whether the probes facilitated formation of the COI1–JAZ1 interaction. An in vitro pull-down assay was performed using recombinant *Arabidopsis* JAZ1–His and supernatants of plant leaf extracts prepared from *coi1–1* mutant plants with transgenic expression of the HA-tagged COI1 protein (COI1-HA).[15] As shown in Figure 5, the ranking of the three probes in promoting the interaction between COI1 and JAZ1 was the same as those for root growth inhibition and *thionin 2.1*-GUS protein accumulation. The PACOR probe had the strongest promoting effect, followed by PAJAIle. The PAJA probe was inactive.

4. Discussion

The activity-based protein profiling (ABPP) strategy was developed in recent years for protein discovery. Typical ABPP probes comprise a bifunctional moiety that binds to and covalently modifies the active site of a specific protein target. This enables efficient and direct detection of the target protein through specific binding, physical attachment of a chemical probe to the active site of the target protein, and formation of a permanent covalent bond between the probe and its receptor via photolysis.[29] For subsequent retrieval of the bound complex, the chemical probe often contains another moiety that can either be pre-attached or react with a reporter group such as a fluorescent tag or a biotin tag.

To develop a biotin-tagged photoaffinity probe for JA that has biological activity, we analyzed the structure–activity relationship between COI1 and JAs[30–32] and performed molecular modeling of the interaction between COI1 and JAs.[24] We found that the JA moiety of JA-Ile and the CFA moiety of COR are very important for biological activity, which suggests that modification of the carboxylic groups of JAs and COR would be tolerated.[24] Therefore,



Scheme 2. Synthesis of the PAJAIle and PACOR probes.

the photoaffinity group was introduced in the carboxylic groups of JAs and COR. A polyethylene glycol linker with rational length was introduced to link the photoaffinity group and the biotin tag, ensuring a large degree of conformational freedom. Based on these analyses, the probe PAJA was designed and synthesized first. Unfortunately, PAJA just showed very weak activity, one possible reason is lack of a free carboxylic acid group, which also indicated that jasmonic acid itself is not the endogenous bioactive form of JAs.

According to the proposed model of JA-Ile binding to COI1, the P3 pocket mainly contains hydrophilic residues,[24] whereas the P4 pocket contains hydrophobic residues. These features of the P3 and P4 pockets may imply that both hydrophobic and electrostatic interactions are involved in the interaction with JAZs. This information indicated that the carboxylic acid group might be important due to form electrostatic interaction. Therefore, a lysine link was introduced between the JAs/COR motifs and the photoaffinity group. The two amino groups of lysine were used to connected the JAs/COR motifs and the photoaffinity group, therefore provided a free carboxyl acid group near the JAs/COR motifs. The probe PAJAIle was found to have much better activity than PAJA, but still was not satisfied for further mechanism studies. Finally, we chose COR to replace the initial JA-Ile moiety. The new probe PACOR was proved to has strong biological activity in several bioassays, which is consistent with previous results that COR has stronger biological activity than JA-Ile.[22]

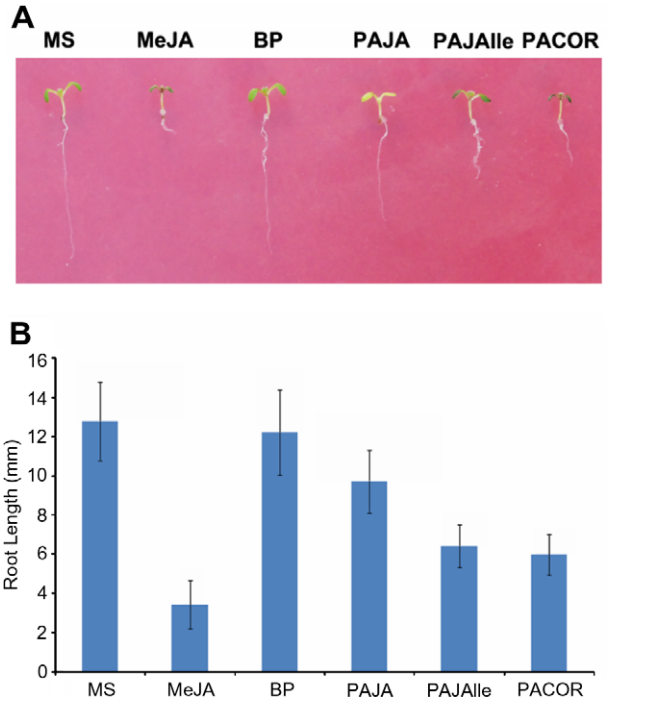


Figure 3. Comparison of the three probes in terms of root growth inhibition of *Arabidopsis* seedlings. Wild-type (Col-0) *Arabidopsis* seedlings were grown on MS medium without JAs (control) or containing BP, MeJA, PAJA, PAJAIle, or PACOR at 100 μM for nine days. (A) Photographic image of the seedlings. (B) Primary root lengths of 9-day-old seedlings. Error Bars indicate SE (n >30).

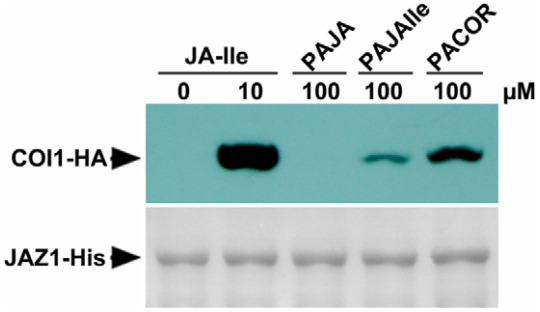


Figure 5. Comparison of the three probes in terms of promotion of the interaction between COI1 and JAZ1. Pull-down reactions were carried out using recombinant JAZ1-His and total plant extracts prepared from *coi1–1[COI1-HA]* seedlings. JAZ1-His containing pull-down assays were supplemented with buffer (indicated by 0) or JA-Ile, PAJA, PAJAIle, or PACOR at the indicated concentrations. Recovery of COI1 was detected using an anti-HA antibody (upper panel). The PVDF membrane was stained to visualize the recovery of JAZ1 by the Ni-NTA affinity resin (lower panel).

The PACOR probe was useful for revealing the direct binding of JA to COI1.[24] Studies are under way to elucidate the mechanism of JA perception in greater detail using photoaffinity labeling technology.

5. Experimental section

5.1. Synthetic materials and methods

Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification. (±)-Jasmonic acid (J2500) were purchased from Sigma. JA-Ile was ordered commercially from BioDuro Beijing and used as a mixture of isomers. Coronatine (C8115) was purchased from Sigma. Compounds **2**, **3** and **4** were synthesized as reported.[33] Dichloromethane (DCM) and tetrahydrofuran (THF) were distilled from sodium, DMF was distilled in a vacuum, triethylamine (TEA) was distilled under normal pressure. The reaction flasks containing all diazirine derivatives were protected from light by wrapping with aluminum foil. ^{1}H (300 MHz) NMR spectra were recorded on a Varian Mercury-Vx 300 M Fourier transform spectrometer. The chemical shifts were reported in δ (ppm) using the δ 7.26 signal of $CDCl_3$ (^{1}H NMR) as internal standards. Low-resolution mass data were obtained on an Agilent 6110 Single Quadrupole LC/MS System. High-resolution mass data were obtained on a MICROMASS Q-Tof UltimaTM spectrometer. HPLC analyses data were performed on an HP 1100 series LC system (HP ChemStation A.06.03), system A: column, Zorbax SB-C18, 4.6 mm × 150 mm, 5 μ; mobile phase, CH_3CN/H_2O; flow rate, 1.0 ml/min; UV wavelength, 220 nm; temperature, ambient temperature; injection volume, 10 μl. System B: column, phenomenex Gemini C18, 4.6 mm × 150 mm, 5 μ; mobile phase, CH_3OH/H_2O (pure H_2O or H_2O with 0.1% TFA); flow rate, 1.0 ml/min; UV wavelength, 220 nm; temperature, ambient temperature; injection volume, 10 μl.

5.2. Synthesis of probes

5.2.1. Compound 5

2-[2-(2-Azido-ethoxy)-ethoxy]-ethylamine (44 mg, 0.253 mmol) and **4** (85 mg, 0.148 mmol) were dissolved in chloroform (5 ml) at 0 °C, then TEA (62 μl, 0.444 mmol) was added. The reaction was stirred for 12 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3, the reaction mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography ($CH_3OH/CHCl_3$, 1:100) to afford **5** as a pale yellow gum (80 mg, 85%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.21 (s, 1H), 8.15 (d, J = 8.4 Hz, 1H), 6.86 (d, J = 8.4 Hz, 1H), 6.68 (s, 1H), 5.02 (s, 1H), 4.24 (t, J = 4.5 Hz, 2H), 3.88 (t, J = 4.5 Hz, 2H), 3.61 (m, 12H), 3.49 (t, J = 5.1 Hz, 2H), 3.32 (m, 6H), 1.39 (s, 9H); ESI-LRMS (m/z): $C_{26}H_{38}F_3N_7NaO_8$: 656.2 $[M+Na]^+$.

5.2.2. Compound 7

Compound **5** (60 mg, 0.095 mmol) was dissolved in dichloromethane (5 ml), 2 ml HCl–EtOAc (2 N) was added to the solution at 0 °C. The reaction was stirred for 2 h at room temperature. The

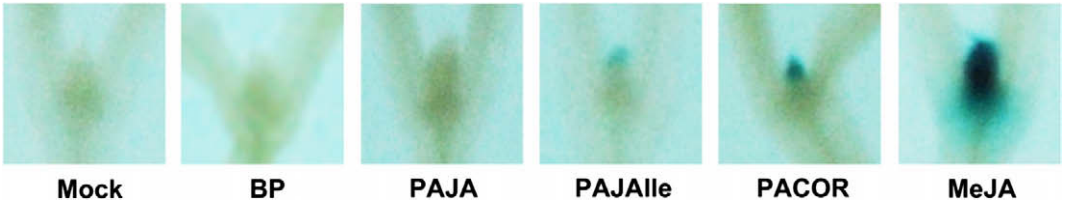


Figure 4. Comparison of the three probes in terms of induction of the *Thionin2.1::GUS* reporter construct in *Arabidopsis* seedlings. Eight-day-old *Thionin2.1::GUS Arabidopsis* seedlings were treated with 100 μM BP, PAJA, PAJAIle, PACOR, or MeJA or were untreated (Mock), and were then stained for GUS activity.

solvent was removed to afford **6**, **6** was dissolved in chloroform (3 ml) at 0 °C, then JA-OSu (35 mg, 0.114 mmol) and TEA (68 μl, 0.50 mmol) was added. The reaction was stirred for 12 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3, the reaction mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography ($CH_3OH/CHCl_3$, 1:50) to afford **7** as a pale yellow gum (50 mg, 73%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.18 (s, 1H), 8.14 (d, J = 8.1 Hz, 1H), 6.87 (d, J = 8.4 Hz, 1H), 6.70 (s, 1H), 6.18 (s, 1H), 5.41 (m, 1H), 5.26 (m, 1H), 4.26 (t, J = 4.5 Hz, 2H), 3.88 (t, J = 4.5 Hz, 2H), 3.67 (m, 12H), 3.51 (t, J = 4.8 Hz, 2H), 3.42 (q, J = 5.1 Hz, 2H), 3.33 (t, J = 4.5 Hz, 2H), 2.50 (m, 1H), 2.31 (m, 4H), 2.05 (m, 7H), 1.82 (m, 1H), 1.36 (m, 1H), 0.93 (t, J = 7.8 Hz, 3H); ESI-LRMS (m/z): $C_{33}H_{46}F_3N_7NaO_8$: 748.3 $[M+Na]^+$.

5.2.3. PAJA

MeOH (3 ml) was added to a reaction bottle containing **7** (28 mg, 0.039 mmol), then prop-2-ynylamide-biotin (12 mg, 0.041 mmol) and 1 ml 10 mM $CuSO_4$ water solution (0.01 mmol) were added in. Under the protection of nitrogen, 30 mg/ml sodium ascorbic solution (280 μl, 0.042 mmol) was added at last. The reaction was stirred for 12 h at room temperature. After MeOH was removed, the mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography ($CH_3OH/CHCl_3$, 1:20) to afford probe PAJA as a pale yellow gum (30 mg, 77%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.23 (s, 1H), 8.15 (d, J = 8.1 Hz, 1H), 7.72 (s, 1H), 7.34 (s, 1H), 6.90 (d, J = 7.5 Hz, 1H), 6.71 (s, 1H), 6.50 (m, 1H), 6.39 (s, 1H), 5.73 (s, 1H), 5.42 (m, 1H), 5.28 (m, 1H), 4.48 (m, 2H), 4.26 (m, 2H), 3.84 (m, 2H), 3.60 (m, 8H), 3.48 (m, 2H), 3.42 (m, 2H), 3.12 (m, 1H), 2.90 (m, 1H), 2.74 (m, 1H), 2.52 (m, 1H), 2.33 (m, 4H), 2.22 (m, 2H), 2.13 (m, 2H), 2.06 (m, 2H), 1.87 (m, 1H), 1.64 (m, 4H), 1.47 (m, 4H), 1.25 (m, 8H), 0.95 (m, 5H); ESI-HRMS (m/z): calcd for $C_{46}H_{65}F_3N_{10}NaO_{10}S$: 1029.4456 $[M+Na]^+$; found 1029.4460. HPLC purity system A: 95.25%; system B: 95.12%.

5.2.4. Compound 8

Fmoc-Lys(*Z*)-OH (502 mg, 1 mmol), 2-trimethylsilanyl-ethanol (180 mg, 1.53 mmol), *N*′,*N*′-dicyclohexylcarbodiimide (DCC) (310 mg, 1.50 mmol) and 4-dimethylamino-pyridine (DMAP) (100 mg, 0.82 mmol) were dissolved in DCM (10 ml), The reaction mixture was stirred for 3 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3, the reaction mixture was extracted with EtOAc (3 × 50 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography (EtOAc/petroleum ether, 1:3) to afford **8** as a pale yellow gum (395 mg, 67%). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.76 (d, J = 7.2 Hz, 2H), 7.60 (d, J = 6.9 Hz, 2H), 7.33 (m, 9H), 5.37 (m, 1H), 5.08 (s, 1H), 4.78 (m, 1H), 4.39 (m, 3H), 4.22 (m, 3H), 3.20 (d, J = 6.3 Hz, 2H), 1.85 (m, 1H), 1.65 (m, 2H), 1.56 (m, 2H), 1.39 (m, 2H), 1.01 (m, 2H), 0.06 (s, 9H); ESI-LRMS (m/z): $C_{34}H_{42}N_2NaO_6Si$: 625.3 $[M+Na]^+$.

5.2.5. Compound 9

Compound **8** (80 mg, 0.13 mmol) was dissolved in methanol (5 ml), Pd/C (8 mg) and a few drops of acetic acid were added in the solution, the air in the reaction bottle were replaced by hydrogen. The reaction was stirred for 3 h at room temperature. The solvent was removed to afford **9** as a gum (50 mg, 80%). ^1H NMR (300 MHz, $CDCl_3$): δ = 7.72 (d, J = 7.2 Hz, 2H), 7.53 (d, J = 7.2 Hz, 2H), 7.35 (m, 4H), 4.27 (m, 6H), 2.98 (s, 2H), 1.78 (s, 4H), 1.46 (s, 2H), 1.25 (m, 3H), 0.98 (m, 2H), 0.02 (s, 9H); ESI-LRMS (m/z): $C_{26}H_{36}N_2NaO_4Si$: 491.2 $[M+Na]^+$.

5.2.6. Compound 10

Let **9** (25 mg, 0.05 mmol) and **4** (30 mg, 0.05 mmol) dissolved in chloroform (2 ml) at 0 °C, then added $NaHCO_3$ (10 mg, 0.12 mmol). The reaction mixture was stirred for 12 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3, the reaction mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography ($CH_3OH/CHCl_3$, 1:80) to afford **10** as a pale yellow gum (42 mg, 85%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.19 (d, J = 8.4 Hz, 1H), 8.03 (t, J = 5.1 Hz, 1H), 7.76 (d, J = 7.5 Hz, 2H), 7.59 (t, J = 6.3 Hz, 2H), 7.39 (t, J = 7.5 Hz, 2H), 7.29 (t, J = 7.2 Hz, 2H), 6.75 (d, J = 7.8 Hz, 1H), 6.64 (s, 1H), 5.67 (d, J = 7.8 Hz, 1H), 4.92 (s, 1H), 4.42 (t, J = 7.5 Hz, 2H), 4.18 (m, 7H), 3.76 (m, 2H), 3.58 (m, 2H), 3.43 (m, 4H), 3.23 (m, 2H), 1.84 (m, 1H), 1.75 (m, 1H), 1.60 (m, 2H), 1.41 (s, 9H), 1.25 (m, 3H), 0.99 (m, 2H), 0.03 (s, 9H); ESI-LRMS (m/z): $C_{46}H_{60}F_3N_5NaO_{10}Si$: 950.4 $[M+Na]^+$.

5.2.7. Compound 11

Compound **10** (42 mg, 0.05 mmol) was dissolved in DMF(0.5 ml), piperidine (25 μl, 0.25 mmol) was added to the solution, the reaction mixture was stirred for 0.5 h at room temperature. The reaction solution was diluted by water, mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with water, saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography ($CH_3OH/CHCl_3$, 1:20) to afford **11** as a pale yellow gum (26 mg, 80%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.16 (d, J = 8.1 Hz, 1H), 8.02 (t, J = 5.4 Hz, 1H), 6.85 (d, J = 8.4 Hz, 1H), 6.65 (s, 1H), 5.13 (s, 1H), 4.23 (m, 2H), 4.15 (m, 2H), 3.88 (m, 2H), 3.67 (m, 2H), 3.58 (m, 2H), 3.45 (m, 2H), 3.37 (m, 3H), 3.26 (m, 2H), 1.73 (m, 2H), 1.63 (m, 6H), 1.39 (s, 9H), 0.95 (m, 2H), 0.02 (s, 9H); ESI-LRMS (m/z): $C_{31}H_{50}F_3N_5NaO_8Si$: 728.3 $[M+Na]^+$.

5.2.8. Compound 12

Compound **11** (24 mg, 0.034 mmol), JA-Ile (10 mg, 0.031 mmol), DCC (20 mg, 0.097 mmol) and 1-hydroxybenzotriazole (HOBt) (10 mg, 0.074 mmol) were dissolved in DCM (5 ml), then TEA (10 μl, 0.08 mmol) was added, The reaction mixture was stirred for 12 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3, the reaction mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography ($CH_3OH/CHCl_3$, 1:100) to afford **12** as a pale yellow gum (19 mg, 61%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.21 (d, J = 8.1 Hz, 1H), 8.13 (t, J = 4.8 Hz, 1H), 6.91 (d, J = 8.4 Hz, 1H), 6.86 (m, 1H), 6.69 (s, 1H), 6.35 (m, 1H), 5.42 (m, 1H), 5.28 (m, 1H), 4.95 (s, 1H), 4.43 (m, 2H), 4.25 (m, 2H), 4.19 (m, 2H), 3.90 (m, 2H), 3.68 (m, 2H), 3.51 (m, 2H), 3.39 (m, 2H), 3.28 (m, 2H), 2.62 (m, 1H), 2.37 (m, 5H), 2.17 (m, 3H), 2.07 (m, 4H), 1.87 (m, 4H), 1.60 (m, 4H), 1.47 (s, 9H), 1.25 (m, 2H), 0.91 (m, 11H), 0.03 (s, 9H); ESI-LRMS (m/z): $C_{49}H_{77}F_3N_6NaO_{11}Si$: 1033.4 $[M+Na]^+$.

5.2.9. Compound 13

Compound **11** (15 mg, 0.021 mmol), coronatine (5 mg, 0.016 mmol), DCC (20 mg, 0.097 mmol) and 1-hydroxybenzotriazole(HOBt) (10 mg, 0.074 mmol) were dissolved in DCM (5 ml), then TEA (10 μl, 0.08 mmol) was added, The reaction mixture was stirred for 12 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3,

the reaction mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography (CH_3OH/$CHCl_3$, 1:100) to afford **13** as a pale yellow gum (10 mg, 63%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.16 (d, J = 8.1 Hz, 1H), 8.00 (t, J = 5.7 Hz, 1H), 7.39 (d, J = 5.1 Hz, 1H), 6.88 (d, J = 8.4 Hz, 1H), 6.70 (s, 1H), 6.65 (d, J = 5.1 Hz, 1H), 6.32 (s, 1H), 4.97 (t, J = 4.8 Hz, 1H), 4.51 (m, 1H), 4.26 (m, 2H), 4.18 (m, 2H), 3.88 (m, 2H), 3.68 (m, 2H), 3.61 (m, 2H), 3.51 (m, 2H), 3.41 (m, 2H), 3.28 (m, 2H), 3.18 (m, 2H), 2.37 (m, 4H), 1.93 (m, 2H), 1.72 (m, 2H), 1.62 (m, 4H), 1.56 (m, 3H), 1.42 (s, 9H), 1.23 (m, 7H), 0.94 (m, 6H), 0.01 (s, 9H); ESI-LRMS (m/z): $C_{49}H_{73}F_3N_6NaO_{11}Si$: 1029.4 $[M+Na]^+$.

5.2.10. Compound 14

Compound **12** (9 mg, 0.009 mmol) was dissolved in dichloromethane (3 ml), 2 ml HCl–EtOAc (2 N) was added to the solution at 0 °C. The reaction was stirred for 2 h at room temperature. The solvent was removed, then the residue was dissolved in dichloromethane (2 ml) at 0 °C, then Biotin-OSu (6 mg, 0.018 mmol) and NaHCO3 (10 mg, 0.12 mmol) was added. The reaction was stirred for 12 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3, the reaction mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography (CH_3OH/$CHCl_3$, 1:50) to afford **14** as a pale yellow gum (8 mg, 80%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.17 (d, J = 8.1 Hz, 1H), 8.10 (t, J = 5.1 Hz, 1H), 7.55 (d, J = 5.7 Hz, 1H), 7.33 (d, J = 5.7 Hz, 1H), 6.90 (d, J = 8.1 Hz, 1H), 6.69 (s, 1H), 6.58 (s, 1H), 5.40 (m, 1H), 5.32 (m, 1H), 5.07 (s, 1H), 4.50 (m, 2H), 4.31 (m, 3H), 4.23 (m, 2H), 4.18 (m, 3H), 3.88 (m, 2H), 3.78 (m, 2H), 3.68 (m, 2H), 3.64 (m, 2H), 3.53 (m, 2H), 3.38 (m, 3H), 3.15 (m, 1H), 2.90 (m, 1H), 2 .65 (m, 2H), 2.32 (m, 4H), 2.11 (m, 7H), 1.80 (m, 2H), 1.64 (m, 4H), 1.45 (m, 4H), 1.25 (m, 6H), 0.94 (m, 10H), 0.02 (s, 9H); ESI-LRMS (m/z): $C_{54}H_{83}F_3N_8NaO_{11}SSi$: 1159.4 $[M+Na]^+$.

5.2.11. Compound 15

Compound **13** (9 mg, 0.009 mmol) was dissolved in dichloromethane (3 ml), 2 ml HCl–EtOAc (2 N) was added to the solution at 0 °C. The reaction was stirred for 2 h at room temperature. The solvent was removed, then the residue was dissolved in dichloromethane (2 ml) at 0 °C, then Biotin-OSu (6 mg, 0.018 mmol) and $NaHCO_3$ (10 mg, 0.12 mmol) was added. The reaction was stirred for 12 h at room temperature. The solvent was concentrated and HCl (1 N) was added to acidify the mixture to pH 2–3, the reaction mixture was extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with saturated brine, dried over anhydrous Na_2SO_4. The solvent was concentrated and the residue was purified with silica gel chromatography (CH_3OH/$CHCl_3$, 1:50) to afford **15** as a pale yellow gum (7 mg, 70%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.17 (d, J = 8.1 Hz, 1H), 8.03 (t, J = 4.8 Hz, 1H), 7.67 (s, 1H), 7.45 (d, J = 5.7 Hz, 1H), 6.89 (d, J = 8.1 Hz, 1H), 6.69 (s, 1H), 6.63 (s, 1H), 6.44 (s, 1H), 6.09 (s, 1H), 4.76 (s, 1H), 4.45 (m, 2H), 4.26 (m, 3H), 4.15 (m, 3H), 3.89 (m, 2H), 3.68 (m, 2H), 3.63 (m, 2H), 3.52 (m, 2H), 3.41 (m, 4H), 3.13 (m, 2H), 2.92 (m, 1H), 2.72 (m, 1H), 2.36 (m, 3H), 2.15 (m, 3H), 1.83 (m, 5H), 1.70 (m, 7H), 1.44 (m, 6H), 1.22 (m, 6H), 0.93 (m, 6H), 0.01 (s, 9H); ESI-LRMS (m/z): $C_{54}H_{79}F_3N_8NaO_{11}SSi$: 1155.4 $[M+Na]^+$.

5.2.12. PAJAIle

Compound **14** (8 mg, 0.007 mmol) was dissolved in THF (1 ml), a few drops of tetrabutylammonium fluoride was added to the solution. The reaction was stirred for 3 h at room temperature. The solvent was concentrated and the residue was purified with Reversed-Phase C_{18} chromatography (H_2O/CH_3OH, 50:50) to afford PAJAIle as a pale yellow gum (6 mg, 82%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.17 (d, J = 8.1 Hz, 1H), 7.95 (t, J = 5.1 Hz, 1H), 7.53 (d, J = 5.7 Hz, 1H), 7.43 (d, J = 6.3 Hz, 1H), 6.90 (d, J = 8.1 Hz, 1H), 6.69 (s, 1H), 6.58 (s, 1H), 5.41 (m, 1H), 5.32 (m, 1H), 5.07 (s, 1H), 4.50 (m, 2H), 4.31 (m, 3H), 4.23 (m, 2H), 3.88 (m, 2H), 3.78 (m, 2H), 3.68 (m, 2H), 3.64 (m, 2H), 3.53 (m, 2H), 3.38 (m, 3H), 3.15 (m, 1H), 2.90 (m, 1H), 2.64 (m, 2H), 2.33 (m, 4H), 2.12 (m, 7H), 1.79 (m, 2H), 1.66 (m, 4H), 1.44 (m, 4H), 1.24 (m, 6H), 0.92 (m, 10H); ESI-HRMS (m/z): calcd for $C_{49}H_{71}F_3N_8NaO_{11}S$: 1059.4813 $[M+Na]^+$; found 1059.4805. HPLC purity system A: 95.69%; system B: 96.03%.

5.2.13. PACOR

Compound **15** (6 mg, 0.005 mmol) was dissolved in THF (1 ml), a few drops of tetrabutylammonium fluoride was added to the solution. The reaction was stirred for 3 h at room temperature. The solvent was concentrated and the residue was purified with Reversed-Phase C_{18} chromatography (H_2O/CH_3OH, 50:50) to afford PACOR as a pale yellow gum (4 mg, 73%). ^1H NMR (300 MHz, $CDCl_3$): δ = 8.16 (d, J = 8.1 Hz, 1H), 8.02 (t, J = 5.1 Hz, 1H), 7.62 (s, 1H), 7.45 (d, J = 5.7 Hz, 1H), 6.89 (d, J = 8.1 Hz, 1H), 6.69 (s, 1H), 6.62 (s, 1H), 6.42 (s, 1H), 6.13 (s, 1H), 4.88 (s, 1H), 4.48 (m, 2H), 4.29 (m, 3H), 3.89 (t, 2H), 3.68 (m, 2H), 3.63 (m, 2H), 3.53 (m, 2H), 3.41 (m, 4H), 3.13 (m, 2H), 2.88 (m, 1H), 2.67 (m, 1H), 2.36 (m, 3H), 2.13 (m, 3H), 1.79 (m, 5H), 1.61 (m, 7H), 1.41 (m, 6H), 1.24 (m, 6H), 0.93 (m, 6H); ESI-HRMS (m/z): calcd for $C_{49}H_{67}F_3N_8NaO_{11}S$: 1055.4484 $[M+Na]^+$; found 1055.4500. HPLC purity system A: 96.81%; system B: 97.20%.

5.3. Root length assay

Arabidopsis seeds (Col-0) were plated on MS medium or MS containing 100 μM MeJA, PAJA, PAJAIle, PACOR and BP, respectively. They were incubated for 3 d at 4 °C and then grown for 9 d at 22 °C. For each treatment, about 30 seedlings were grown on a small plate (Φ35 mm) containing 2 ml of medium. The experiment was repeated three times.

5.4. Induction of JA-responsive gene in *Arabidopsis* seedlings

Arabidopsis seedlings (Col-0) carrying *Thionin 2.1*-GUS (β-glucuronidase) reporter construct,[28] in which the *GUS* gene was driven by the promoter of *thionin 2.1* that is induced by JA treatment, were grown on MS plate for eight days, transferred to a 1.5 ml tube containing liquid MS medium (Mock) or MS with 100 μM of BP, PAJA, PAJAIle, PACOR, or MeJA, and incubated for 8 h in dark at 22 °C. These seedlings then were stained in staining solution (0.05 M sodium phosphate buffer pH 7.2, 0.2% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 2 mM X-Gluc) for 16 h in dark at 37 °C. The seedlings were finally washed and fixed in ethanol.

5.5. Pull-down assays

Protein pull-down assays were performed as described.[34] The seedlings with transgenic expression HA-tagged COI1 protein were ground in liquid nitrogen to fine powder. Soluble proteins was extracted from seedlings in a buffer containing 50 mM Tris-Cl (pH 7.8), 100 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 20 mM 2-mercaptoethanol, 10 mM MG132 and the EDTA-free complete miniprotease inhibitor cocktail according to manufacturer's instructions (Roche). Recombinant JAZ1-His was expressed in *Escherichia coli* and purified by Ni affinity chromatography. Fifty micrograms JAZ1-His were incubated with 5 mg of total plant extract, supplemented where indicated with the probes

and incubated for 1 h at 4 °C. After washing, pull-down mixtures were loaded on SDS–polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membrane and detected with an anti-HA antibody (Sigma).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.059.

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