

A comprehensive *in vitro* fluorescence anisotropy assay system for screening ligands of the jasmonate COI1–JAZ co-receptor in plants

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The phytohormone (+)-7-iso-jasmonoyl-L-isoleucine regulates many developmental and stress responses in plants and induces protein–protein interactions between COI1, the F-box component of E3 ubiquitin ligase, and jasmonate ZIM domain (JAZ) repressors. These interactions cause JAZ degradation and activate jasmonate (JA), leading to plant defense responses, growth inhibition, and senescence. Thirteen JAZ subtypes are encoded in the *Arabidopsis thaliana* genome, but a detailed understanding of the physiological functions of these JAZ subtypes remains unclear, partially because of the genetic redundancy of JAZ genes. One strategy to elucidate the complex JA signaling pathways is to develop a reliable and comprehensive binding assay system of the ligands with all combinations of the co-receptors. Herein, we report the development of a fluorescence anisotropy–based *in vitro* binding assay system to screen for the ligands of the COI1–JAZ co-receptors. Our assay enabled the first quantitative analysis of the affinity values and JAZ-subtype selectivity of various endogenous JA derivatives, such as coronatine, jasmonic acid, and 12-hydroxyjasmonoyl-L-isoleucine. Because of its high signal-to-noise ratio and convenient mix-and-read assay system, our screening approach can be used in plate reader–based assays of both agonists and antagonists of COI1–JAZ co-receptors.

The phytohormone (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile³; 1) regulates many developmental and stress responses in plants (1–4). Upon attack by insects or fungal pathogens, JA-Ile (1) is biosynthesized and induces protein–protein interaction (PPI) among COI1, the F-box component of Skip/Cullin/F-box (SCF^{COI1}) E3 ubiquitin ligase, and the JAZ repressors, which are ubiquitinated and then targeted for degradation by the 26S proteasome. Subsequently, a different set of transcription factors targeted by JAZs activate the expression of JA-responsive genes, leading to plant defense and growth inhibition or senescence responses (5–7). COI1 and 13 subtypes of JAZ are encoded in the genome of *Arabidopsis thaliana*, and 1 can induce PPI between COI1 and most JAZ subtypes to cause various physiological responses. However, the detailed physiological functions of these JAZ subtypes remain unclear, mainly due to the complexity of the JA-mediated signaling cascade, the genetic redundancy of JAZ genes, and signaling cross-talk with other phytohormones (8). In addition, functional compensation by other JAZ subtype in *jaz* knockout mutants cause difficulties in the functional analysis of each JAZ subtype (9). Nonetheless, a few examples of specific functions of JAZ proteins have been described, *e.g.* JAZ2 as a controlling factor of stomata dynamics and JAZ9 as a key regulator for defense responses against necrotrophic pathogens (8, 10–13), indicating that specific JAZ proteins regulate distinct transcription factors and downstream responses. The balance between redundant and specific mechanisms of JA signaling likely contributes to fine-tuning of the numerous JA-regulated responses and plant adaptation to the environment. Accordingly, practical chemical tools for the study of JA-mediated signaling cascades such as JAZ subtype–selective ligands are needed (13).

To discover such ligands, reliable and high-throughput *in vitro* assay systems of the ligands for all combinations of COI1–JAZ co-receptors must be prepared. One conventional method is the yeast two-hybrid system. Although capable of screening

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This article contains Figs. S1–S9 and supporting methods and MS charts.

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³ The abbreviations used are: JA-Ile, (+)-7-iso-jasmonoyl-L-isoleucine; JA, jasmonate; JAZ, jasmonate ZIM domain; FA, fluorescence anisotropy; PPI, protein–protein interaction; HTS, high-throughput screening; OG, Oregon Green; COR, coronatine; *ent*, enantiomer; FI, fluorescein; TMR, tetramethylrhodamine; COR-MO, coronatine *O*-methyloxime; SPR, surface plasmon resonance; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; EG, ethylene glycol.

ligands against most combinations of COI1–JAZ co-receptors, false positives and negatives could also be found, in part due to the use of live yeast cells, the performance of which is affected by factors unrelated to binding affinity such as cell-penetration efficiency of the ligands and cell growth inhibition. *In vitro* or semi-*in vivo* pulldown assays can also be used for direct analyses for PPI between phytohormone co-receptors, although they are qualitative in nature and of low-throughput performance. Herein, we disclose a fluorescence anisotropy (FA)-based *in vitro* binding assay system for the ligands of COI1–JAZ co-receptors. FA has been widely used for the quantitative and high-throughput screening (HTS) of PPI inducers or inhibitors (14, 15), and by applying it to the assay system for COI1–JAZ co-receptors, quantitative analyses of the affinity values of endogenous JA derivatives covering almost all the combinations of COI1–JAZ co-receptors were achieved for the first time. Moreover, this system has a sufficiently good signal-to-noise ratio and is capable of mix-and-read assay, and thus is amenable to a plate reader-based assay of COI1–JAZ co-receptor agonists and antagonists.

Results

Fluorescent anisotropy-based binding assay for ligand of COI1–JAZ co-receptors

Short (27-amino-acid) peptide fragments composed of Jas motifs of a JAZ protein were previously found to be sufficient for co-receptor formation, and so we designed a fluorophore-conjugated JAZ peptide for PPI detection of COI1–JAZ co-receptors according to the previously reported crystal structure of COI1–1–JAZ1 complex (7). The conjugated peptide was composed of 27 amino acids (~3,500 Da; Fig. S1), and Oregon Green (OG) was attached to the additional Cys on the N terminus of the Jas motif (OG-Cys-JAZ) (13), a binding domain of JAZ proteins with COI1. The molecular masses of these conjugated peptides were significantly smaller than those of PPI complexes (Fig. 1*b*; ~100 kDa), and thus a dramatic increase in FA can be expected upon formation of the COI1–ligand–JAZ peptide ternary complex (Fig. 1, *b* and *c*). Initially, in a proof-of-principle, positive control experiment, we observed the FA (*r* value) change of OG-Cys-JAZ1 and COI1–GST upon addition of coronatine (COR; 2), a functional mimic of JA-Ile and strong binder of COI1–JAZ co-receptor (16). As shown in Fig. 1*d*, the *r* value was relatively low (~0.092) when OG-Cys-JAZ1 and COI1–GST were dissolved but increased by 1.9-fold upon the addition of 2. In contrast, little or no anisotropy change was observed upon addition of *ent2* as the negative control. Therefore, OG-Cys-JAZ1 can monitor the formation of COI1–2–JAZ ternary complex.

We next sought to enhance the sensitivity of this assay system by examination of the structures of fluorescently labeled JAZ peptides, specifically (i) the position of the fluorophore (N or C terminus of the peptide), (ii) the linker structures between fluorophores and peptides, and (iii) the structures of the fluorophores (Figs. 2*a* and S2). In the case of JAZ1-Cys-OG, in which OG was introduced at the C terminus through Cys (Fig. 2*b*), almost no anisotropy change was observed upon addition of 2 (Fig. S3*a*), although 2 caused PPI between co-receptors,

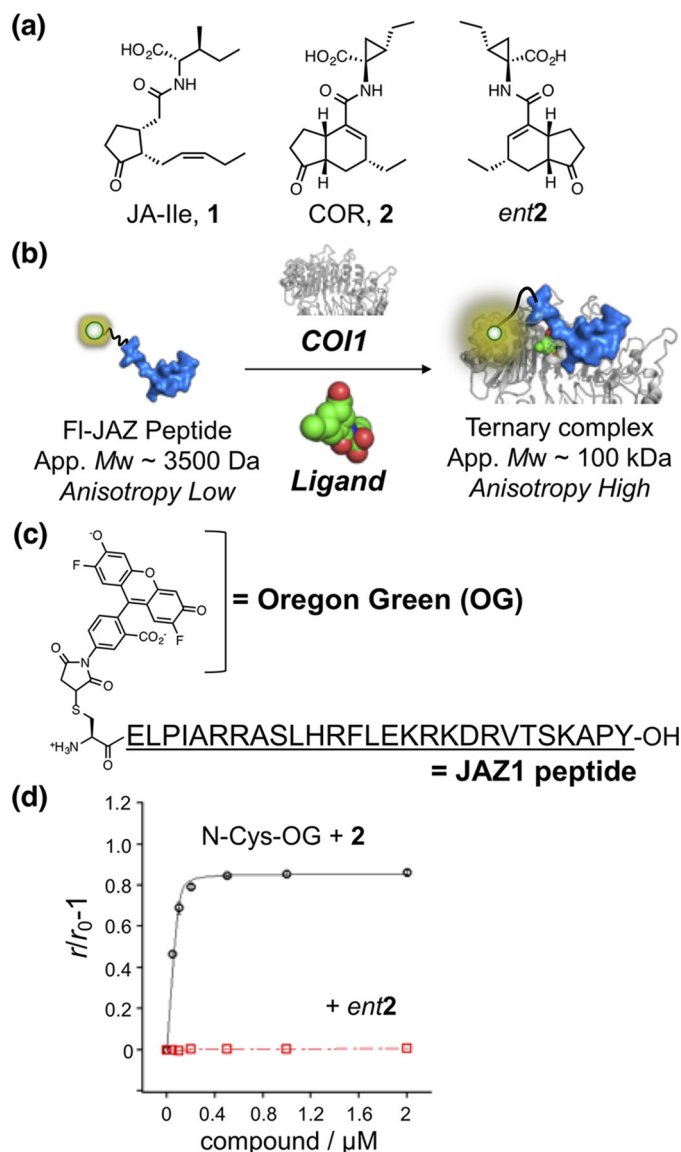


Figure 1. FA-based binding assay for the ligands of COI1–JAZ co-receptors. *a*, chemical structures of JA-Ile (1), COR (2), and enantiomer of COR (*ent2*). *b*, schematic of FA-based binding assay for COI1–JAZ co-receptors. *c*, chemical structure of Oregon Green–conjugated JAZ1 degen peptide (OG-N-Cys-JAZ1). *d*, FA change of OG-N-Cys-JAZ1 with GST-COI1 upon addition of 2 (black circles) or *ent2* (red squares) (0–2 μM). Experiments were performed in triplicate to obtain mean and S.D. (shown as error bars).

similar to the case of OG-Cys-JAZ1 (Fig. S3*b*), indicating that the fluorophore should be conjugated to the N-terminal position of the JAZ peptide. Next, we examined the effect of linker structure/length between OG and each N terminus of JAZ1 (OG-EG4, EG2-JAZ1, and OG-JAZ1) (Fig. 2*b*). It was found that the ratio of anisotropy change ($r/r_0 - 1$) depended upon the linker length (without linker (OG-JAZ1; 1.8) > EG2 (1.0) > EG4 (0.7); Fig. 2*c* and S3, *c* and *d*) despite the fact that all peptides induced PPI with GST-COI1 (Fig. S3*b*). In particular, *r* values of the peptides having various linkers were similar (0.071–0.092), whereas those of the peptides in the presence of 2 and GST-COI1 decreased in proportion to the length of the linkers (OG-JAZ1 (0.22) > EG2 (0.16) > EG4 (0.12); Fig. 2*c* and S3, *c* and *d*). The difference in molecular weights of these peptides is insignificant compared with those of corresponding ter-

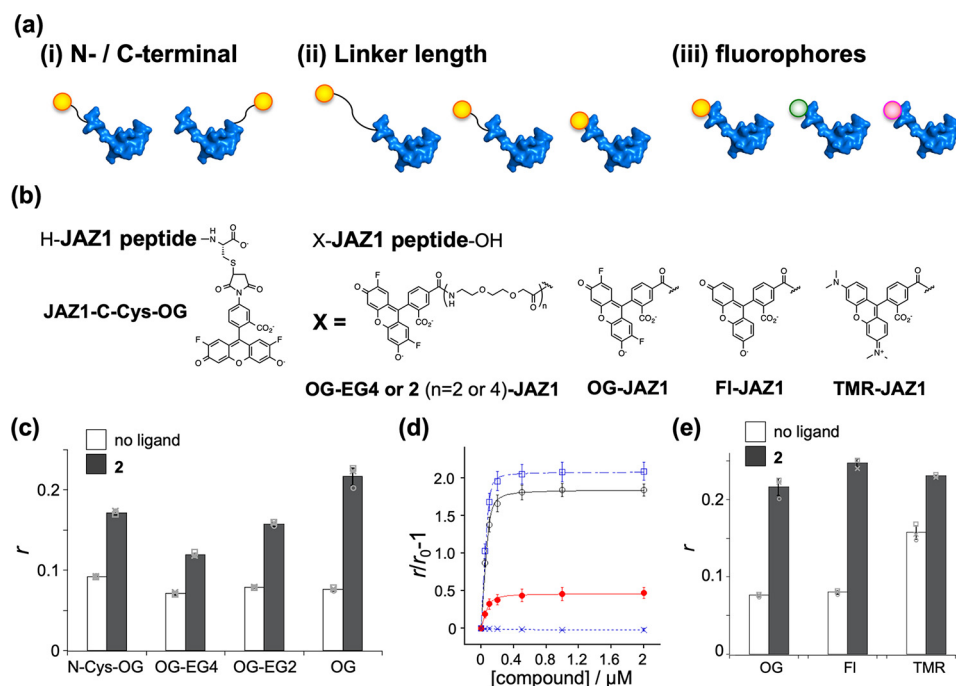


Figure 2. Structural insights of fluorescently labeled JAZ1 peptides for FA-based sensing system of the ligands of COI1-JAZ co-receptors. *a*, *i*–*iii*, schematics of various fluorescently labeled JAZ1 peptides. *b*, chemical structures of fluorescently labeled JAZ1 peptides used in this study; EG, ethylene glycol. *c*, FA values of fluorescently labeled JAZ1 peptides having various linker structures in the absence or presence of 2 (2 μ M). *d*, FA change of OG-JAZ1 (black circles), FI-JAZ1 (blue squares), or TMR-JAZ1 (red filled circles) with GST-COI1 upon addition of 2 (0–2 μ M). The change of FI-JAZ1 with GST-COI1 upon addition of *ent*2 (blue crosses) is also shown as negative control experiments. *e*, FA values of OG-, FI-, and TMR-labeled JAZ1 peptides in the absence or presence of 2 (2 μ M). Experiments shown in *c*–*e* were performed in triplicate to obtain mean and S.D. (shown as error bars).

nary complexes, and thus the differences in the r values of these peptides should be derived from the microenvironmental mobility of the fluorophore in the corresponding ternary complexes. We also designed two peptides bearing fluorescein (FI) and tetramethylrhodamine (TMR) instead of the original OG (FI-JAZ1 and TMR-JAZ1; Fig. 2*b*). As shown in Fig. 2*d*, the r values of all these peptides bearing GST-COI1 increased upon addition of 2 in a dose-dependent manner, and the ratios of anisotropy changes ($r/r_0 - 1$) in FI-JAZ1 and OG-JAZ1 were higher than that in TMR-JAZ1. The FI-JAZ1 with GST-COI1 also showed no change in r values upon addition of *ent*2 as the negative control, and thus FI-JAZ1 as well as original OG-N-Cys-JAZ1 specifically detected the agonist-induced PPI between COI1 and JAZ1. Overall, we found that FI-JAZ1 as well as the original OG-N-Cys-JAZ1 worked well as novel FA-based sensors for COI1-JAZ co-receptor formation. In the following experiments, we used FI-JAZ1 as it is less expensive to acquire.

Quantitative binding analyses of jasmonates with COI1-JAZ co-receptors

We next applied the FA-based sensing system to the quantitative analyses of the binding affinities of various natural jasmonate derivatives bearing COI1-JAZ co-receptors. From the saturation binding curve, the apparent K_d value of 2 with FI-JAZ1 and GST-COI1 was calculated to be 5.0 nM (Fig. 3*a*). A sample of synthetic JA-Ile (3) containing (+)-7-iso-JA-L-Ile (1) and (–)-JA-L-Ile^{pure} diastereomeric isomers in a 95:5 ratio (Fig. 3*b*) and purified bioactive stereoisomer (+)-7-iso-JA-L-Ile (1) also increased the r value in a dose-dependent manner. The K_d value of 1 was 29 nM, which is significantly lower than that of the

mixture, 3 ((–)-JA-L-Ile; 824 nM), and these results are consistent with the previous report (4). In contrast, little or no change was observed upon addition of either jasmonic acid (4) or 12-hydroxy-JA-L-Ile (5) (Figs. 3*b* and S5), which is consistent with previous reports, although the K_d values of 2 and 3 are almost a single order of magnitude lower than the reported values (48 nM for 2 and 18 μ M for 3).

Thirteen JAZ gene subtypes are coded in *Arabidopsis*; the Jas motifs of JAZ5 and -6 are identical, and JAZ7, -8, and -13 are expected to have no or weak affinity with COI1 because they lack a binding sequence (8). Thus, we prepared nine short peptides corresponding to the remaining subtypes (FI-JAZs; Fig. S4) with FI-JAZ13 as a negative control. Next, we applied this sensing system to evaluate the binding affinities of 2, 1, 3, 4, and 5 for all combinations of COI1 and 10 JAZ subtypes. As shown in Fig. 3*c* and with the exception of FI-JAZ13, 2 induced an increase in FA in the presence of GST-COI1 and the corresponding FI-JAZ. The affinity values of 2 are summarized in Table 1 (see also Fig. S5), and those corresponding to JAZ1, -3, -6, and -10 are consistent with previous reports (7, 17–19). Although no affinity values of 2 have been reported for JAZ2, -4, -9, -11, and -12 with COI1, pulldown experiments demonstrated that 2 indeed caused PPI for these COI1-JAZ combinations, similar to the case of JAZ1 (Fig. S6). In the cases of 1, 3, and 5, the anisotropy was also increased in most combinations of COI1-JAZ co-receptors except for JAZ13. In contrast, little change was observed for 4 against all co-receptors (Table 1 and Fig. S5). Affinity values for 1, 3, 4, and 5 with any COI1-JAZ co-receptors were previously unreported, and so our results are the first

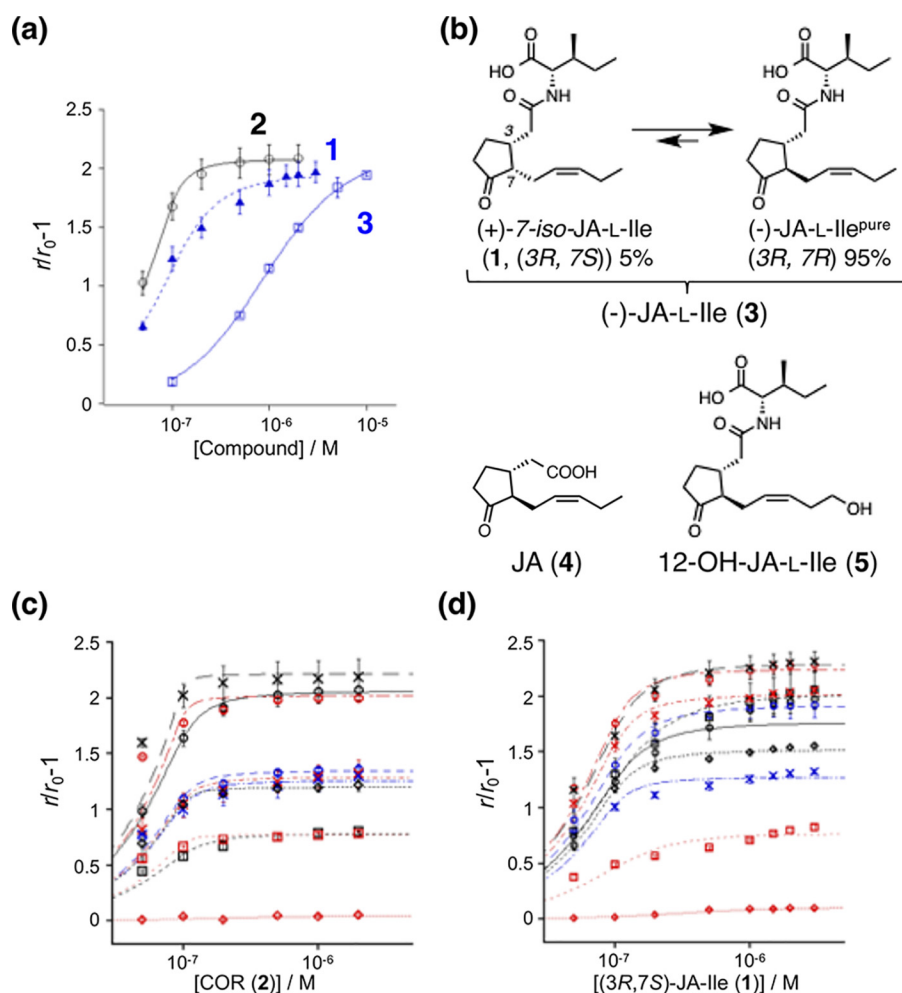


Figure 3. Quantitative binding assay of JA derivatives for all COI1–JAZ co-receptors. *a*, FA change of FI-JAZ1 with GST-COI1 upon addition of **1** (blue triangles; 0–3 μ M), **2** (black circles; 0–2 μ M), or **3** (blue squares; 0–10 μ M). *b*, chemical structures of endogenous JA derivatives: synthetic (–)-JA-L-Ile (**3**; a diastereomeric mixture of (+)-7-iso-JA-L-Ile and (–)-JA-L-Ile^{pure}), JA (**4**), and 12-hydroxy-JA-L-Ile (12-OH-JA-Ile; **5**). *c* and *d*, FA change of FI-JAZs (JAZ1 (black circles), JAZ2 (red circles), JAZ3 (blue circles), JAZ4 (black squares), JAZ5/6 (red squares), JAZ9 (black crosses), JAZ10 (red crosses), JAZ11 (blue crosses), JAZ12 (black diamonds), or JAZ13 (red diamonds)) with GST-COI1 upon addition of **2** (0–2 μ M; *c*) or **1** (0–3 μ M; *d*), monitored by a fluorescence spectrometer. Experiments were performed in triplicate to obtain mean and S.D. (shown as error bars).

Table 1

The K_d values of JA derivatives for all COI1–JAZ co-receptors

	COR (2)		(+)–7-Iso-JA-L-Ile (1)	(–)-JA-L-Ile (3)	JA (4)	12-OH-JA-L-Ile (5)
	Obtained	Literature				
JAZ1	5	^{HM} 48 ^a	29	824	ND ^b	6,461
JAZ2	1	— ^c	7	759	ND	4,824
JAZ3	7	20 ^a	11	681	ND	3,337
JAZ4	6	— ^c	25	1,724	ND	9,234
JAZ5/6	16	68 ^a	34	1,660	ND	24,054
JAZ9	0.5	— ^c	10	608	ND	1,187
JAZ10	6	7 ^a	7	1,471	ND	3,635
JAZ11	4	— ^c	13	616	ND	8,886
JAZ12	4	— ^c	8	768	ND	2,821
JAZ13	ND	— ^d	ND	ND	ND	ND

^a The values have been previously reported (7, 17, 18).

^b ND, the value could not be determined due to the low signal change.

^c Although no value has been reported in the literature, COR-induced PPI was confirmed by another method.

^d PPI induction has not been reported.

quantitative analyses of the affinity of endogenous JA derivatives with most combinations of COI1–JAZ co-receptors. Interestingly, the measured affinity values for **1**, **2**, and **5** against each JAZ subtype were found to be weakly correlated (Fig. S7; correlation factor is 0.55 for **2** and **1**, 0.72 for **2** and **5**, and 0.72

for **1** and **5**), indicating that the JAZ-subtype selectivity of these jasmonates is similar but not identical to each other.

Additionally, as expected, an obvious FA change could be observed with FI-JAZ1 and GST-COI1 upon addition of **2** in a dose-dependent manner with the conventionally used plate

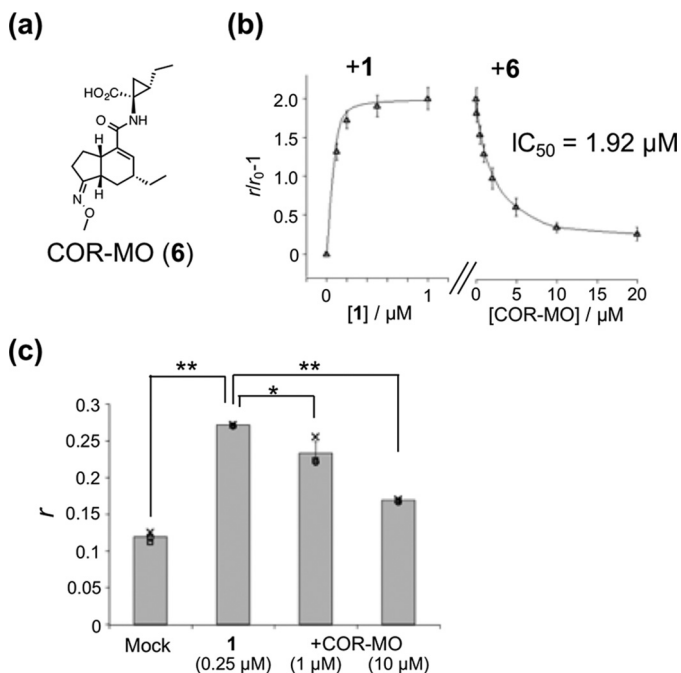


Figure 4. Binding assay for antagonist of COI1-JAZ co-receptors by our FA-based binding assay system. a, chemical structure of COR-MO (6). b, FA change of FI-JAZ1 with GST-COI1 upon addition of (+)-7-iso-JA-L-Ile (1) (0–1 μM ; left) and the following addition of 6 (0–10 μM ; right), monitored by a fluorescence spectrometer. c, FA values of FI-JAZ1 with GST-COI1 in the absence or presence of 1 (0.25 μM) or in the presence of both 1 (0.25 μM) and 6 (1 or 10 μM), monitored by plate reader equipment. Experiments were performed in triplicate to obtain mean and S.D. (shown as error bars). **, $p < 0.01$; *, $p < 0.05$; significant differences between anisotropy values in the absence or presence of the indicated ligands were analyzed by Student's *t* test.

reader equipment (Fig. S8). Therefore, our FA-based binding assay system is amenable to the plate reader-based HTS of COI1-JAZ co-receptor agonists.

Anisotropy-based binding assay systems for antagonist of COI1-JAZ co-receptors

Our FA-based binding assay systems can also evaluate antagonists for COI1-JAZ by monitoring the disassembly of the ternary complex. Previously developed a rationally designed antagonist, coronatine O-methoxime (COR-MO; 6; Fig. 4a), of COI1-JAZ co-receptor based on a crystallographic structure of the ternary complex (20), and thus a proof-of-principle experiment could be carried out using COR-MO as a positive control. Initially, the COI1-(+)-7-iso-JA-L-Ile (1)-FI-JAZ1 ternary complex was prepared in a similar manner to that described above (Fig. 4b). The *r* value of this ternary complex was found to decrease upon addition of 6 in a dose-dependent manner (Fig. 4b), indicating that 6 disrupted the formation of the COI1-1-FI-JAZ1 ternary complex and released the small peptide FI-JAZ1 into solution. The half-maximum inhibitory concentration (IC_{50}) of 6 was 1.92 μM , which was consistent with the literature value (IC_{50}) of 0.43–1.53 μM . An inhibitory effect of 6 on COI1-1-FI-JAZ1 was further confirmed by our HTS system with plate reader equipment (Fig. 4c). These results clearly exemplify the utility of our HTS system for the screening of the COI1-JAZ antagonists.

Discussion

Coronatine (2) has been considered as a structural and biological mimic of JA-Ile (1), but several researchers have claimed it to have a broader range of functions that includes rapid chlorosis and plant defense responses in both COI1-JAZ-dependent and -independent manners (21–23). Our quantitative analyses of 2 and bioactive hormone (+)-7-iso-JA-L-Ile (1) for the co-receptors suggested that their JAZ-subtype selectivities were obviously different; that is, affinity values of 1 and 2 for each JAZ subtype were weakly correlated (Fig. S7; the correlation factor is 0.55), providing a possible explanation for the differences in bioactivities of 1 and 2, at least *in vitro*.

Although several synthetic jasmonates derivatives have been developed, their structure-activity relationship profiles have been mainly collected from whole-plant bioassays (24) and are therefore reflective of many different processes. In contrast, our binding assay system for the quantitative assessment of ligand-subtype selectivity will provide new insights into not only the mode of action of these derivatives but also the biological role of each JAZ subtype.

Other phytohormones such as auxin, gibberellin, and abscisic acid also act as PPI inducers for the corresponding co-receptors, of which there are various subtypes with high genetic redundancy (25–27). Napier and co-workers *et al.* (28) previously reported a pioneering surface plasmon resonance (SPR)-based binding assay system of auxin co-receptors that yielded definitive, quantitative structure-activity relationship profiles by using biotin-conjugated degron peptides with F-box protein counterparts, including TIR1 and AFB5. Alternatively, the FA-based binding assay has been used in HTS analyses of PPI modulators, similar to SPR-based methods (the PPI inhibitor database reports fluorescence polarization or anisotropy assays in the top ranking 51% of their cases) (29). One advantage of the FA-based assay is that it is “mix-and-read”: all the components of the assay can be mixed prior to compound administration and read out directly, allowing for the rapid HTS assay of various ligands simultaneously. Also, fluorescent probes are used in rather low concentrations (50–100 nM in this case, depending on the signal-to-noise ratio or ligand affinity), and its miniaturization into sample uncoated microplates, which saves biological materials (proteins or peptides) and compounds by simple reduction of the assay volume, is relatively straightforward and results in costs per well that are lower than for SPR or other biomolecule immobilization-based PPI detection methods (AlphaScreen or biolayer interferometry).

Recently, Littleson *et al.* (30) have reported a scalable and flexible synthetic strategy with coronatine and successfully created a huge library (~120) of its derivatives, although their JAZ-subtype selectivity has yet to be discussed. We developed a versatile binding assay method for the chemical screening of both agonist and antagonist ligands of COI1-JAZ co-receptors. Our design strategy of the FA-based JAZ sensors is simple and may be applicable to agronomically relevant plant species in addition to *Arabidopsis thaliana* (e.g. rice; three COI1 and 15 JAZ are coded in its genome, and all Jas motifs in JAZ have already been identified) (31). Therefore, the library of coronatine derivatives combined with our screening method should constitute a

useful chemical tool for the elucidation of complex, JA-related signaling cascades as well as molecularly targeted agrochemicals in plants in the future (13).

Experimental procedures

General materials and methods

All chemical reagents and solvents were obtained from commercial suppliers (Wako Pure Chemical Industries Co. Ltd., Nacalai Tesque Co. Ltd., Watanabe Chemical Industries Co. Ltd., Thermo Fisher Scientific K.K., and GE Healthcare) and used without further purification. Ultraviolet (UV)-visible spectra were recorded on a UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). Fluorescence spectra and anisotropy were recorded on a FP-8500 (Jasco, Tokyo, Japan). Plate reader assays were recorded on a SpectraMax-M5 (Molecular Devices, LLC). SDS-PAGE and Western blotting were carried out using a Mini-Protein III electrophoresis apparatus (Bio-Rad). Chemiluminescence signals were detected with a LAS 4000 imaging system (Fujifilm, Tokyo, Japan). Reversed-phase or straight-phase high-performance LC (HPLC) was carried out on a PU-4180 Plus with UV-4075 and MD-4010 detectors (Jasco). UV detection was performed at 220 nm. MALDI-TOF MS analyses were performed on a 4800 Plus MALDI-TOF/TOF Analyzer (AB Sciex, Framingham, MA).

Synthesis

1, **2**, *ent2*, and **3–6** were prepared as described previously (straight-phase HPLC charts of (–)-JA-L-Ile (**3**) and purified (+)-7-iso-JA-L-Ile (**1**) are shown in Fig. S9) (4, 20, 32, 33). All JAZ peptides were prepared by microwave-assisted solid-phase synthesis with NovaSyn® TGA resin (90 μ m) using Initiator⁺ Alstra (Biotage Ltd., Charlotte, NC). A representative protocol for the case of Fl-JAZ peptide is as follows. Solid-phase peptide was mixed with 5-carboxyfluorescein diacetate (3 eq), *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU; 5 eq), 1-hydroxybenzotriazole monohydrate (HOBt·H₂O) (5 eq), and DIPEA (5 eq) in dry DMF at room temperature for 2 h. After the reaction, the peptide was deprotected by stirring with TFA solution at room temperature for 1.5 h (in the case of JAZ9 or -13, deprotection was performed with TFA solution containing thioanisole, anisole, and 1,2-ethanedithiol (34) to avoid methionine oxidation). A representative protocol for the case of OG-conjugated or TMR-conjugated JAZ1 peptide is as follows. Solid-phase peptide was mixed with 5-carboxy-Oregon Green diacetate *N*-succinimidyl ester (3 eq; the synthetic protocol is shown in the supporting information) or 5-carboxytetramethylrhodamine *N*-succinimidyl ester (3 eq; Biotium) and DIPEA (5 eq) in dry DMF at room temperature for 1 h. After the reaction, the peptide was deprotected by stirring with TFA solution at room temperature for 1 h. The reaction mixture was purified by reversed-phase HPLC using a Develosil ODS-HG-5 column (4.6-mm inner diameter \times 250 mm), eluting with a linear gradient (CH₃CN (0.05% TFA):H₂O (0.05% TFA) = 20:80 (5 min) to 50:50 (35 min)) to afford fluorophore-conjugated JAZ peptide. After lyophilization, conjugated JAZ peptide was dissolved in sterilized water to prepare the stock solution. The concentration of the solution was calculated from the UV-visible absorption spectrum as

shown below (see *Fluorescence anisotropy titration experiments* under “Experimental procedures”). The purity of these peptides was confirmed by HPLC analyses (Figs. S2 and S4), and the peptides were characterized by MALDI-TOF MS as follows (MS charts are shown in the supporting information): OG-C-JAZ1: *m/z* [M + H]⁺ calcd for 3803.90, found 3803.90; OG-EG4-JAZ1: *m/z* [M + H]⁺ calcd for 3922.02, found 3922.03; OG-EG2-JAZ1: *m/z* [M + H]⁺ calcd for 3776.95, found 3776.92; OG-JAZ1: *m/z* [M + H]⁺ calcd for 3631.87, found 3631.89; Fl-JAZ1: *m/z* [M + H]⁺ calcd for 3595.89, found 3595.91; TMR-JAZ1: *m/z* [M + H]⁺ calcd for 3649.99, found 3649.97; Fl-JAZ2: *m/z* [M + H]⁺ calcd for 3609.91, found 3609.92; Fl-JAZ3: *m/z* [M + H]⁺ calcd for 3444.84, found 3444.86; Fl-JAZ4: *m/z* [M + H]⁺ calcd for 3514.86, found 3514.90; Fl-JAZ5/6: *m/z* [M + H]⁺ calcd for 3598.90, found 3598.93; Fl-JAZ9: *m/z* [M + H]⁺ calcd for 3521.78, found 3521.80; Fl-JAZ10: *m/z* [M + H]⁺ calcd for 3644.93, found 3644.93; Fl-JAZ11: *m/z* [M + H]⁺ calcd for 3854.01, found 3854.00; Fl-JAZ12: *m/z* [M + H]⁺ calcd for 3748.96, found 3748.96; Fl-JAZ13: *m/z* [M + H]⁺ calcd for 3749.01, found 3749.01.

Fluorescence anisotropy titration experiments

All chemicals were dissolved in ethanol to generate 10 mM stock solutions and diluted with 20% ethanol aqueous for preparation of 100 μ M stock solutions. All fluorescent peptides were dissolved in sterilized water to generate stock solutions. The concentrations of these peptides were determined by their absorbance (at 495 nm for Oregon Green® and 494 nm for fluorescein) in 0.1 N NaOH aqueous using a molar extinction coefficient of 76,000 M^{−1} cm^{−1} for Oregon Green (35) and 75,000 M^{−1} cm^{−1} for fluorescein (36). The concentration of rhodamine-conjugated peptide was determined by the absorbance at 543 nm in methanol using a molar coefficient of 92,000 M^{−1} cm^{−1} (37). The concentration of COI1-GST was estimated by the Bradford assay and the efficiency of pulldown experiments with **2** and OG-N-Cys-JAZ1 (13), allowing stock solutions of known concentration to be prepared. Fluorescence anisotropy titration experiments were performed at 25 °C in 50 mM Tris-HCl buffer (pH 7.8, 100 mM NaCl, 20 mM 2-mercaptoethanol, 10% glycerol, 0.1% Tween20, 100 nM inositol 1,2,4,5,6-pentakisphosphate) using a quartz cell (50 μ l). The ligand was added dropwise to the solution containing COI1-GST (100 nM) and each fluorescent JAZ peptide (100 nM), and anisotropy intensities were measured ($\lambda_{\text{ex}}/\lambda_{\text{em}}$ = 485 nm/522.5 nm for Oregon Green, $\lambda_{\text{ex}}/\lambda_{\text{em}}$ = 485 nm/516.5 nm for fluorescein, $\lambda_{\text{ex}}/\lambda_{\text{em}}$ = 543 nm/573.5 nm for tetramethylrhodamine). Fluorescence anisotropy values (*r*) were calculated using the following equation: $r = (I_{\text{VV}} - G \times I_{\text{VH}}) / (I_{\text{VV}} + 2G \times I_{\text{VH}})$ where *I*_{VV} and *I*_{VH} are the fluorescence intensities observed through polarizers parallel and perpendicular to the polarization of the exciting light, respectively, and *G* is a correction factor to account for instrumental differences in detecting emitted compounds. An average value of three independent measurements was plotted for each point. Anisotropy titration curves were analyzed with nonlinear curve-fitting analysis to evaluate apparent *K_a* and *K_d* values.

Fluorescence anisotropy measurements with plate reader equipment

Fluorescence anisotropy was measured at 25 °C in the same buffer for fluorescence anisotropy titration experiments (pH 7.8) using a 96-well black clear-bottomed plate (100 µl/well). The ligand was added dropwise to the solution containing COI1-GST (50 nM) and each fluorescent JAZ peptide (50 nM), and anisotropy values were measured ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 485 \text{ nm}/516 \text{ nm}$ for fluorescein).

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