Electronic Supplementary Material (ESI) for RSC Chemical Biology. This journal is © The Royal Society of Chemistry 2021

## Supporting Information for

Title: Rational design of a stapled JAZ9 peptide inhibiting protein-protein interaction of plant transcription factor

#### Authors:

Kaho Suzuki,<sup>a</sup> Yousuke Takaoka,\*<sup>a</sup> and Minoru Ueda\*<sup>ab</sup>

# Affiliations:

<sup>a</sup> Ms. Kaho Suzuki, Dr. Yousuke Takaoka, Prof. Dr. Minoru Ueda
Graduate School of Science, Tohoku University, 6-3, Aramaki-Aza-Aoba, Aoba-ku,
Sendai 980-8578, Japan Department
<sup>b</sup> Prof. Dr. Minoru Ueda
Graduate School of Life Science, Tohoku University, 6-3, Aramaki-Aza-Aoba, Aoba-ku,
Sendai 980-8578, Japan Department
E-mail: ytakaoka@tohoku.ac.jp, minoru.ueda.d2@tohoku.ac.jp

Contents	
Scheme S1.	page S2
Figure S1–S10.	pages S3-S12
Table S1.	page S13
Experimental section	pages S14-S19
References	page S19



Scheme S1. Synthesis of double-stapled peptide (4) (R' = side chain of each amino acid).



**Figure S1**. HPLC charts of the reaction mixtures (top) or purified samples (bottom) of the peptides synthesized in this study; (a) TMR-JAZ9wt (1), (b) TMR-JAZ9st1 (2), (c) TMR-JAZ9st2 (3), (d) TMR-JAZ9st3 (4). Asterisks are shown for each target peak. These peptides were purified in a linear gradient condition as follows: A (CH<sub>3</sub>CN with 0.05% TFA) : B (0.05% TFA aq.) = 20 : 80 (5 min) to 50:50 (35 min).



**Figure S2**. MALDI-TOF MS spectra of TMR-conjugated peptides; (a) TMR-JAZ9wt (1), (b) TMR-JAZ9st1 (2), (c) TMR-JAZ9st2 (3), (d) TMR-JAZ9st3 (4).



**Figure S3**. Top-5 model structures which are obtained by conformational searching with MD simulation are superimposed; (a) JAZ9st1, (b) JAZ9st2. The amino acids at the stapling sites (Q221X, A225X, A228X, and E232X) were shown as cyan ball-and-stick models.



**Figure S4**. FA change of TMR-JAZ9 peptides (1; black circle, 2; blue square, 3; green diamond, 4; red cross) upon addition of MYC2 (a,  $0 - 5 \mu$ M) or MYC4 (b,  $0 - 5 \mu$ M). Experiments were performed in triplicate to obtain mean and S.D. (shown as error bars).



**Figure S5**. Sequence alignment MYC3, MYC2 and MYC4 proteins used in this study. Green bar shows the binding site of MYC3 for a JAZ peptide, indicating that it was almost identical to MYC2 or MYC4.



**Figure S6.** (a, b) The reported JAZ9wt-MYC3 structure with interacting residues depicted as stick models; (a) charge interaction networks (R234 of JAZ9 with E142/E143/E148 of MYC3, R229/S226 of JAZ9 with D94/W92 of MYC3, R223 of JAZ9 with M155 of MYC3), (b) hydrophobic interaction networks (L227/L231 of JAZ9 with L152/I122/L125 of MYC3, F230 of JAZ9 with Y97/F151 of MYC3, L227 of JAZ9 with M155 of MYC3). (c, d) The model structure obtained by a docking simulation of JAZ9st3 and MYC3 with interacting residues depicted as sticks; (c) charge interaction networks (R234 of JAZ9 with E143/E148 of MYC3 (E142 was absent, which was different from (a)), R229/S226 of JAZ9 with D94/W92 of MYC3, R223 of JAZ9 with M155 of MYC3), (d) hydrophobic interaction networks (L227/L231 of JAZ9 with L152/I122/L125 of MYC3, F230 of JAZ9 with F151 of MYC3 (Y97 was absent, which was different from (b)), L227 of JAZ9 with M155 of MYC3). As shown in this figure, the stapled hydrocarbon structure (cyan, stick) was not involved in the interaction between JAZ9st3 and MYC3.



**Figure S7**. (a, b) Analysis of JA-responsive gene (a; *JAZ1*, b; *LOX2*) expression by quantitative RT-PCR in 7-d-old *Arabidopsis* seedlings (Col0) with or without chemicals (MeJA, or MeJA and 4, 10  $\mu$ M respectively) (n = 4). The seedlings were treated with chemicals for 2 h (a) or for 8 h (b), respectively. Experiments were repeated at least three times, and significant differences were evaluated by one-way ANOVA/Tukey HSD post hoc test (p < 0.01).



**Figure S8**. Dose dependency of the stapled peptide in the analysis of JA-responsive gene expression by quantitative RT-PCR in 7-d-old *Arabidopsis* seedlings (Col0) with or without MeJA (10  $\mu$ M) in the absence or presence of **4** (5, 10, and 20  $\mu$ M) (n = 4). The seedlings were treated with chemicals for 2 h (a, *AOS*) or for 8 h (b, *VSP2*), respectively. Experiments were repeated at least three times, and significant differences were evaluated by one-way ANOVA/Tukey HSD post hoc test (p < 0.01).



**Figure S9**. Analysis of JA-responsive gene expression by quantitative RT-PCR in 7-d-old *Arabidopsis* seedlings (Col0) with or without chemicals (MeJA, or MeJA and 1, 10  $\mu$ M respectively) (n = 4). The seedlings were treated with chemicals for 2 h (a, *AOS*) or for 8 h (b, *VSP2*), respectively. Experiments were repeated at least three times, and significant differences were evaluated by one-way ANOVA/Tukey HSD post hoc test (p < 0.01).



**Figure S10**. Evaluation of GUS activity in the roots of 4-d-old 35S:JAZ1-GUS plants (n=4). Seedlings were pretreated for 1 h with mock (a), with MeJA only (b), or with MeJA and **4** (c) (10  $\mu$ M respectively), and stained with 5-bromo-4-chloro-3-indolyl glucuronide (n=4). Experiments were repeated at least three times, and significant differences were evaluated by one-way ANOVA/Tukey HSD post hoc test (*p* < 0.01). Scale bar, 1 mm.

Allong orida synthasa	
Allene Oxide Synthuse	5 CICCOTTAATTICICOTC 5
(AOS: AT5G42650)	5' GCAGCAACAGATTATACAAC 3'
Vegetative storage protein 2	5' AGATCAATGGGCTGATTTGG 3'
(VSP2: AT5G24770)	5' GTGTATACAAGGGGACAATGCG 3'
Transcription factor MYC2	5' ATCTATACGCAAGAACAGC 3'
( <i>MYC2</i> : AT1G32640)	5' GACCCCATAACTTTCTAAAC 3'
Protein TIFY 10A	5' GTCTTCAAACCCTCAAAC 3'
(JAZ1: AT1G19180)	5' AGCATGAAGATAGGAGCTT 3'
Lipoxygenase 2	5' GAATGGGTGTTCCTTATAGT 3'
( <i>LOX2</i> : AT3G45140)	5' ATAGGAGACAAAGGAGCATT 3'
Polyubiquitin 10	5' GGCCTTGTATAATCCCTGATGAATAAG 3'
( <i>UBQ10</i> : AT4G05320)	5' AAAGAGATAACAGGAACGGAAACATAGT 3'

 Table S1. Gene sequences of all primers for qRT-PCR used in this study.

# Experimental section 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., GE Healthcare, Sigma-Aldrich Co. Llc., KISHIDA CHEMICAL Co., Ltd., Kanto Chemical Co. Ltd., Nippon Gene Co. Ltd.) and used without further purification. Ultraviolet (UV)-visible spectra were recorded on a UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). Circular dichroism spectra were recorded on a J-820 (JASCO, Tokyo, Japan). Fluorescence spectra and anisotropy were recorded on a FP-8500 (JASCO, Tokyo, Japan). Reverse-phase high-performance liquid chromatography (HPLC) was carried out on a PU-4180 plus equipped with UV-4075 and MD-4010 detectors (JASCO, Tokyo, Japan). Absorbance at 220 nm and 540 nm was monitored by an MD-4010 photodiode array detector (PDA). MALDI-TOF MS analysis was performed on an Autoflex Max (Bruker Daltonics Inc., MA). The 3D structures were constructed using MOE 2016.08 software (Chemical Computing Groups, Montreal, Canada). The AlphaScreen assay was carried out on an EnVision (PerkinElmer, Inc., USA). Seedling photographs were taken with an E-520 digital camera (Olympus Corp., Japan). Quantitative RT-PCR analysis was carried out on a StepOnePlus Real-Time PCR System (Life Technologies, USA). GUS quantification was carried out on the spectrophotometer Infinite M200Pro (TECAN, Switzerland).

#### Syntheses and purification of JAZ peptides

JAZ peptides were prepared by microwave-assisted solid phase synthesis with Fmoc-Leu-Wang resin (90 µm, 15 µmol scale) using Initiator+ Alstra (Biotage Ltd, North Carolina, US). A representative protocol for the case of TMR-JAZ9st3 (4) is as follows. The resin was swollen in DMF at 70 °C for 20 min. The Fmoc protecting group was removed by treating with 20% piperidine in DMF twice. Amino acid coupling was accomplished by mixing the resin with Fmoc protected amino acids (6 eq), 1-(7-aza-*1H*-benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, 6 eq), 1-Hydroxy-7-azabenzotriazole (HOAt, 6 eq), and DIPEA (12 eq) in DMF, and subjecting it to microwave irradiation at 50 °C for either 10 min (natural amino acids) or 30 min (Fmoc-*S*-2-(4-pentenyl)alanine (Fmoc-S<sub>5</sub>-OH) and the following amino acids of Fmoc-S<sub>5</sub>-OH) (2×).<sup>S1</sup> After the peptide had been fully elongated, olefin metathesis was carried

out by treating it with a 10 mM solution of benzylidene-bis(tricyclohexylphosphine)dichlororuthenium (IV) (Grubbs' first-generation metathesis catalyst, 1 eq) in dry 1,2dichloroethane (DCE) for 30 min with microwave irradiation at 50 °C under a N2 atmosphere.<sup>S2</sup> After completion of the reaction, the resin was washed with DCE. Fresh catalyst solution (1 eq) was then added and the reaction repeated with the same conditions. After the second treatment, the resin was washed three times with DCE followed by twice resin DCM. Finally, the Fmoc-deprotected with with was mixed 5carboxytetramethylrhodamine (TMR-COOH, 1.5 eq), HATU (3 eq), and DIPEA (6 eq) in dry DMF, and was shaken vigorously at r.t. for 16 h ( $2\times$ ). The resin was washed with DMF  $(3\times)$ , DCM  $(3\times)$  and mixed solvent of methanol and DCM  $(3\times)$ , and then treated with 1 mL of a cleavage cocktail (95% TFA, 2.5% TIS, 2.5% H<sub>2</sub>O) at r.t. for 1.5 h. The resultant filtrate was precipitated with cold diethylether. The residue was washed twice with cold diethylether, and then dried in vacuo. The crude products were purified by HPLC using a Develosil ODS-HG-5 column ( $\Phi$  4.6×250 mm) eluting with a linear gradient (CH<sub>3</sub>CN (0.05% TFA):H<sub>2</sub>O (0.05% TFA) = 20:80 (5 min) to 50:50 (35 min)) to afford TMR-conjugated stapled JAZ peptide. After lyophilization, the peptide was again dissolved in a sterilized water to prepare the stock solution. The concentration of the solution was determined by the absorbance at 543 nm in methanol using a molar coefficient of 92,000 M<sup>-1</sup>cm<sup>-1</sup>.<sup>S3</sup> The purity of these peptides was confirmed by HPLC analysis, and these were characterized by MALDI-TOF MS as follows: TMR-JAZ9wt (1): m/z [M+H]<sup>+</sup> calcd for 2895.61, found 2895.59; TMR-JAZ9st1 (2): m/z [M+H]<sup>+</sup> calcd for 2946.68, found 2946.68; TMR-JAZ9st2 (3): m/z [M+H]<sup>+</sup> calcd for 2945.70, found 2945.71; TMR-JAZ9st3 (4): m/z [M+H]<sup>+</sup> calcd for 2996.77, found 2996.77.

#### **Protein preparation**

Expression and purification of His6-SUMO-MYCs was performed according to the previous reports as follows with minor modifications.<sup>S4</sup> BL21 (DE3) cells transformed with the expression plasmids (pE-SUMO3-MYCs, LifeSensors) were grown in LB medium at 37 °C to an optical density (OD<sub>600</sub>) of ~0.6 and induced with 0.1 mM IPTG at 16 °C for 24 h. Cells were harvested, resuspended in 10 mL extract buffer (pH 8.0, 20 mM Tris-HCl, 200 mM NaCl, 10% Glycerol, 10% cOmplete<sup>TM</sup> EDTA-Free) per 1 L of cells. The suspension was sonicated on ice for 10 sec (×5). The lysate was centrifuged at 20,000 g at 4 °C for 30 min. The supernatant was loaded on a 2.0 mL Ni Sepharose column and rotated at 4 °C for 30 min. The column was washed with 10 mL wash buffer (50 mM imidazole in extract buffer) for three times, and eluted with 1 mL of elution buffer (250 mM imidazole in extract buffer). The eluted His6-SUMO-MYCs was again purified with TOYOPEARL HW40S (Tosoh Corporation, Japan) and extract buffer containing 10 mM 2-mercaptoethanol. The concentration of the total protein was calculated from Bradford assay, and the purity of His6-SUMO-MYC3 was determined by the analyses of SDS-PAGE and CBB staining. Expression and purification of GST-COI1 was performed according to the previous report.<sup>S5</sup>

## **Circular dichroism (CD) experiments**

Circular dichroism spectroscopy experiments were performed on a J-820 using a quartz cuvette (path length: 1 cm). The peptide stock solution was diluted to 10  $\mu$ M with PBS buffer and CD measurements were recorded at 25 °C. The spectra were averages of 16 successive accumulations. The raw data were converted in terms of per-residue molar ellipticity (deg cm<sup>2</sup>/dmol), as calculated per mole of amide groups present and normalized by the molar concentration of peptides. Smoothing and correction of the background spectra were performed afterwards. The contents of  $\alpha$ -helix of each peptide was calculated as previously reported (<u>http://bestsel.elte.hu/index.php</u>).<sup>S6</sup>

#### In silico MD simulation of the stapled peptides and docking simulation with MYC3

The initial structure of JAZ9wt complexed with MYC3 was obtained from the database of the Protein Data Bank (PDB, https://www.rcsb.org/) using PDB ID: 4RS9.<sup>S4</sup> The stapled JAZ9 peptides were manually modified based on JAZ9wt using the builder tab in the Molecular Operating Environment (MOE) software 2018.01.<sup>S7</sup> All designed peptides and MYC3 protein were optimized by adding hydrogens using MOE software. In case of the peptides, 2D structures of the main chain atoms between each stapled site were constraint as helix. Potential conformation of the designed peptides was searched using LowModeMD<sup>S8</sup> with parameters such as iteration limit: 10000, gradient: 0.005, RMSD limit: 0.25, energy window: 40, conformation limit: 10000. All these peptides were saved in mdb database which was further used for docking studies. Top 10 conformations for each peptide were superimposed as shown in Figure 2ef and S3.

The docking algorithm of the MOE software was used to dock the calculated various conformations of the non-stapled and double stapled peptides (JAZ9wt and

JAZ9st3) as the ligand with optimized MYC3 (44-258) as the receptor. The parameters were set as Re-scoring function: London dG, placement: triangle matcher, Retain: 100, Refinement: Rigid Receptor, and Re-scoring 2: GBVI/WSA dG. The top conformation for each peptide was selected on the basis of S score and further evaluated to study the interaction networks as shown in Figure 3d and S5.

#### AlphaScreen inhibitory assays

AlphaScreen (Perkin Elmer) experiments were performed at 25 °C in 20 mM Tris- HCl buffer (pH 8.0, 200 mM NaCl, 10% glycerol). 15  $\mu$ L of the reaction mixture (50 nM Biotin-JAZ9 peptide,<sup>S4</sup> 5  $\mu$ M His6-SUMO-MYCs, and TMR-conjugated JAZ peptides (0, 0.3, 1, 3, 5, 10  $\mu$ M) in buffer) was added to a 1/2 Area AlphaPlate<sup>TM</sup>-96 (PerkinElmer), and then 10  $\mu$ L of a detection mixture (0.2  $\mu$ L of streptavidin donor beads and 0.2  $\mu$ L of nickel-acceptor beads in buffer) was added to each well. Thereafter, the plate was incubated for 1 h at r.t.. Luminescence signals were detected by using the EnVision 2105 Multimode Plate Reader (PerkinElmer). The experiment was repeated three times, and the data were presented as mean values with standard deviation.

## Fluorescence anisotropy (FA) as quantitative binding assay

For COI1-JAZ binding assay, fluorescence anisotropy titration experiments were performed at 25 °C in 50 mM Tris-HCl buffer (pH 7.8, 100 mM NaCl, 20 mM 2mercaptoethanol, 10% glycerol, 0.1% Tween20, 100 nM inositol-1,2,4,5,6pentakisphosphate, IP5) using a quartz cell (50 µL) as previously described.<sup>S9</sup> The ligand (JA-Ile) was added dropwise to the solution containing COI1-GST (100 nM) and TMRconjugated JAZ peptide (100 nM), and anisotropy intensities were measured ( $\lambda_{ex}/\lambda_{em} =$ 543 nm/573.5 nm for tetramethylrhodamine). For JAZ-MYCs binding assay, the measurement was carried out in 20 mM Tris-HCl (pH 8.0, 200 mM NaCl, 10% Glycerol) using a quartz cell (150 µL). MYC was added dropwise to the solution containing TMRconjugated JAZ peptide (100 nM). Fluorescence anisotropy values (*r*) were calculated using the following equation:  $r = (I_{VV} - G \times I_{VH}) / (I_{VV} + 2G \times I_{VH})$  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 ( $G = I_{HV} / I_{HH}$ ). An average value of three independent measurements was calculated for each point. Anisotropy titration curves were analyzed with the nonlinear curve-fitting analysis to evaluate apparent  $K_a$  and  $K_d$  values.

## Plant materials and growth conditions.

Arabidopsis thaliana seeds were surface-sterilized in 5% sodium hypochlorite with 0.3% Tween20 and vernalized for 2–3 days at 4 °C. All seedlings were grown under a 16 h light (118 µmol m<sup>-2</sup>s<sup>-1</sup>; cool-white fluorescent light)/8 h dark cycle at 22 °C in a Biotron NC-220 growth chamber (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan).  $P_{35S}$ -JAZ1:GUS seedlings<sup>S10</sup> for GUS assays were vertically grown on 1/2 MS plates for 4 days. Col0 seedlings for quantitative RT-PCR analyses were grown in 1/2 MS liquid medium for 7 days.

#### **Quantitative RT-PCR analyses**

In a Biotron NC-220 growth chamber, each compound was treated to sevenday-old plants in 1/2 MS liquid medium. Each peptide and MeJA were treated for 2 h (for *AOS*, *MYC2*, and *JAZ1*) or 8 h (for *VSP2*, and *LOX2*). Then, using an ISOGEN kit (Nippon Gene, Japan), total RNA was isolated and then first-strand cDNA was gained with ReverTra Ace reverse transcriptase (Toyobo, Japan) with oligo-dT primers. A StepOnePlus Real-Time PCR System (Life Technologies, USA) was used for quantitative PCR (all primers sequences for qPCR were shown in Table S1). *Polyubiquitin 10* was used as a reference gene.

#### GUS staining and quantification

Four-day old seedlings of  $P_{355}$ -JAZ1:GUS was transferred in 1/2 MS liquid medium containing 10 µM MeJA or both of 10 µM MeJA and 10 µM 4 for 1 h.<sup>S6, S11</sup> Seedlings were then immersed in GUS staining buffer (50 mM phosphate buffer, pH 7.0, 1 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 1 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 0.1% TritonX-100, 1 mg ml<sup>-1</sup> 5-bromo-4-chloro-3indolyl glucuronide (X-Gluc) at 37 °C. After staining, the solution was exchanged to 90% acetone. Images were taken with an E-520 digital camera (Olympus Corp., Japan). Alternatively, 20 seedlings of  $P_{355}$ -JAZ1:GUS were treated in 1/2 MS liquid medium with 10 µM MeJA or both 10 µM MeJA and 10 µM 4 for 1 h. Then, 20 roots were collected, frozen and was homogenized with extract buffer (50 mM phosphate buffer, pH 7.0; 10 mM EDTA (Ethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid disodium salt dihydrate), 10 mM 2-mercaptoethanol; 0.1% sarcosyl (N-lauroylsarcosine sodium salt; > 94%, Sigma-Aldrich) and 0.1% Triton X-100). Total protein content was quantified by the Bradford method. Then, 10  $\mu$ L of extract was incubated with 40  $\mu$ L of extract buffer containing 1 mM 4-MUG (4-methylumbelliferyl- $\beta$ -D-glucuronide hydrate; > 98%, Sigma-Aldrich) for 1 h at 37 °C. 11  $\mu$ L of the samples were taken at t = 0 and t = 1 h, and 99  $\mu$ L 0.2 M Na<sub>2</sub>CO<sub>3</sub> was used to stop the reaction. Fluorescence was measured at ex/em 365/460 nm with the spectrophotometer Infinite M200Pro (TECAN, Switzerland). Three independent replicates were measured with similar results.

#### References

S1) K. Yamada, I. Nagashima, M. Hachisu, I. Matsuo, H. Shimizu, *Tetrahedron Lett.* **2012**, *53*, 1066.

S2) Y.-W. Kim, P. S. Kutxhukian, G. L. Verdine, Org. Lett. 2010, 12, 3046.

S3) T. Yoshii, K. Mizusawa, Y. Takaoka, I. Hamachi, J. Am. Chem. Soc. 2014, 136, 16635.
S4) F. Zhang, J. Yao, J. Ke, L. Zhang, V. Q. Lam, X. F. Xin, X. E. Zhou, J. Chen, J. Brunzelle, P. R. Griffin, M. Zhou, H. E. Xu, K. Melcher, S. Y. He, Nature 2015, 525, 269.
S5) Y. Takaoka, M. Iwahashi, A. Chini, H. Saito, Y. Ishimaru, S. Egoshi, N. Kato, M. Tanaka, K. Bashir, M. Seki, R. Solano, M. Ueda, Nat Commun 2018, 9, 3654.

S6) Micsonai, A.; Wien, F.; Kernya, L.; Lee, Y.-H.; Goto, Y.; Refregiers, M.; Kardos, J., *Proc. Natl. Acad. Sci. USA* **2015**, E3095.

S7) MOE (The Molecular Operating Environment) software available from Chemical Computing Group Inc. and MOLSIS Inc. (Tokyo, Japan) (http://www.chemcomp.com).
S8) Labute, P. J. Chem. Inf. Model 2010, 50, 792.

S9) Y. Takaoka, K. Nagumo, I. N. Azizah, S. Oura, M. Iwahashi, N. Kato, M. Ueda, J. Biol. Chem. 2019, 294, 5074.

S10) I. Monte, M. Hamberg, A. Chini, S. Gimenez-Ibanez, G. Garcia-Casado, A. Porzel,F. Pazos, M. Boter, R. Solano, *Nat. Chem. Biol.* 2014, *10*, 671.

S11) M. L. Campos, Y. Yoshida, I. T. Major, D. O. Ferreira, S. M. Weraduwage, J. E. Froehlich, B. F. Johnson, D. M. Kramer, G. Jander, T. D. Sharkey, G. A. Howe, *Nat. Commun.* **2016**, *7*, 12570.