The alkyne-tag Raman imaging of coronatine, a plant pathogen virulence factor, in *Commelina communis* and the possible mode of action

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The alkyne-tag Raman imaging of coronatine, a plant pathogen virulence factor, in *Commelina communis* and the possible mode of action

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We previously reported that coronatine, a virulence factor of plant bacteria, facilitates bacterial infection through an ER (endoplasmic reticulum)-mediated, non-canonical mechanism in the model dicot plant, *Arabidopsis thaliana*. Here, we report that this same ER-mechanism is ubiquitous among dicots and monocots, and works by affecting the ethylene signaling pathway widely found in plants. The subcellular localization of coronatine by alkyne-tag Raman imaging (ATRI) approach provided the convincing clue.

Crop production worldwide is severely damaged by pathogenic bacteria and herbivorous insects.[1, 2] Two plant hormones, 7-*iso*-jasmonoyl-L-isoleucine (**1**, Figure 1a) and salicylic acid (SA), are implicated in the plant defense response.[3, 4] However, they function antagonistically: defense responses mediated by **1** against insect damage weaken defense responses mediated by SA against pathogenic bacteria, and vice versa.[5] Thus, defenses against pathogenic infection and insect damage cannot be concomitantly upregulated.

Plant pathogenic bacteria take advantage of this in order to infect plants. For example, the plant pathogenic bacteria *Pseudomonas syringae* facilitates its entry into the plant by secreting coronatine (**2**, Figure 1a), a small molecular virulence factor, which hijacks the signaling cascade of **1**. Coronatine (**2**) is a structural and functional mimic of **1**, which antagonistically weakens the SA-mediated defense against bacterial infections,[6, 7] causing the host plant to open its stomata – the main route of bacterial entry into the plant body.[8] A recent study by Panchal *et al.* reported that **2**-mediated stomatal opening facilitates bacterial infection at night when the stomata are closed, and the plant is otherwise more resistant to bacterial infection than it is during the day.[9] The COI1-JAZ co-receptor of **1**,[10-12] which is composed of the F-box protein CORONATINE INSENSITIVE 1 (COI1) and Jasmonate ZIM-Domain (JAZ) repressor, is believed to control both functions of **2**.[8, 13-15] Thus, the suppression of bacterial infection by inhibiting **2** will suppress defense responses against insect damage mediated by **1**.

Recently, we proposed a possible strategy to circumvent this dilemma. The involvement of a non-canonical mechanism for stomata closure was discovered in the opening of the dark-closed stomata of a dicot plant, *Arabidopsis thaliana*, mediated by **2**

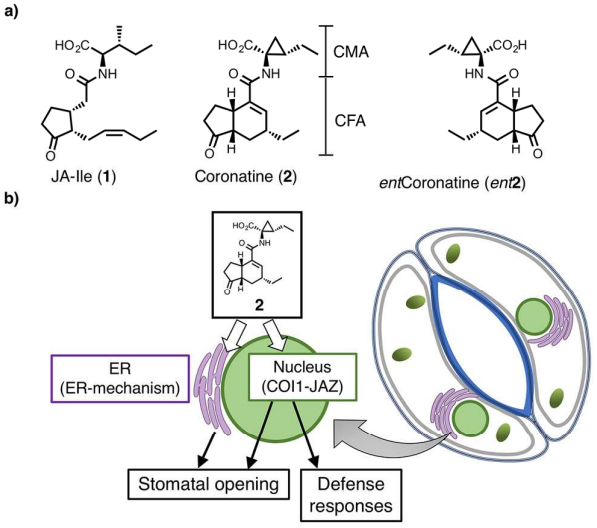

Figure 1. a) Chemical structures of JA–Ile (**1**), coronatine (**2**) and *ent*coronatine (*ent***2**). b) Coronatine (**2**) promotes bacterial infection through two independent mechanisms: COI1-JAZ dependent inhibition of SA-mediated defense and a COI1-JAZ independent ER-mechanism.

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(Figure 1b).[16] The re-opening of dark-induced closure was not impaired in *coi1-1* mutant *Arabidopsis* in which COI1 protein was knocked out (Figure S1).[16] In addition, alkyne-tag Raman imaging (ATRI)[17] revealed that **2** was localized in the endoplasmic reticulum (ER) of the *Arabidopsis* guard cells. Since the COI1-JAZ co-receptor is located exclusively in the nucleus,[18] this result suggested the involvement of a COI1-JAZ- independent mode of action for **2**, inhibition of which should close the stomata and suppress bacterial infection, without affecting the COI1-JAZ-dependent insect defense response. Here, we report that this ER-mechanism would be ubiquitous among plants, including monocot. Using ATRI, we confirmed the ER-localization of a **2**-based probe in the monocot, *Commelina communis*, which has been previously validated in studies of stomatal movements.[19-21] Furthermore, a photoaffinity probe developped on the structure of **2** revealed the corresponding binding protein in a microsomal fraction of *C. communis*. These results enable us to propose a possible mode of action for **2** in the ER-mechanism.

In previous work,[16] we developed alkyne-tagged **2** (**3**) as a Raman probe for ATRI in *Arabidopsis* guard cells (Figure 2). Herein, we used the previously developed di-yne Raman probes **3** and *ent***3**, and the newly developed benzyl ester di-yne Raman probe **4** (Figure 2a and Scheme S1). Probes **3** and **4** gave rise to Raman signals at 2258 and 2256 cm^{-1}, respectively (Figure S2).

First, we conducted a biological assessment of **3** and **4** (Figures 2b-c). To be of use in studies of the ER mechanism, a Raman probe should cause stomatal opening without affecting the conventional COI1-JAZ mechanism; and so we examined the stomatal opening activities of **3** and **4** on *C. communis* stomata (Figure 2b). Fortunately, both **3** and **4** were effective at opening of dark-closed *Commelina* stomata. Then, their affinities for the COI1-JAZ2 co-receptor were examined (Figure 2c). Recently, Gimenez-Ibanez *et al.* reported that JAZ2 is constitutionally expressed in *Arabidopsis* guard cells, and is predominantly responsible for stomatal movement during infection by pathogenic bacteria, among the 13 JAZ subtypes present in Arabidopsis.[22] Accordingly, we carried out a pull-down assay using maltose-binding protein-tagged JAZ2 (MBP-JAZ2) and glutathione-*S*-transferase-tagged COI1 (GST-COI1).[10] As shown in Figure 2c, only **2** caused co-receptor formation between GST-COI1 and MBP-JAZ2; whereas **3**, *ent***3**, and **4** were

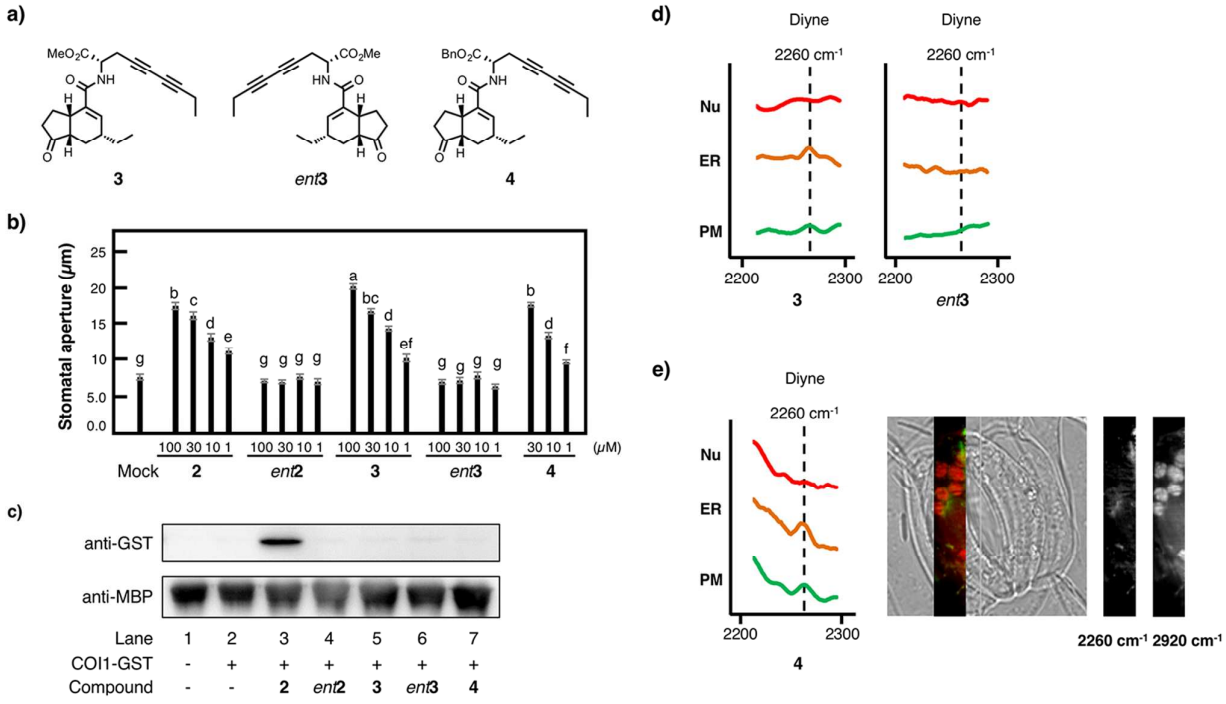

Figure 2. a) Chemical structures of di-yne Raman probes (**3**, *ent***3**, **4**). b) Stomatal opening assay using closed stomata of *Commelina communis* treated with **2**, *ent***2** or di-yne Raman probes (**3**, *ent***3**, **4**). Bars represent the mean stomatal aperture with SE (n = 20 stomata). Different letters indicate significant differences between means (ANOVA: $P < 0.05$). c) Pull-down assay using GST-COI1 (5 nM)/MBP-JAZ2 (ca. 100 nM) in the presence of **2**, *ent***2** or di-yne Raman probes (**3**, *ent***3**, **4**) (100 nM). d) Raman spectra of living guard cells of *Commelina communis* stained with 100 μM of **3** or *ent***3**. e) Raman spectra (left) and images (center and right) of living guard cells of *Commelina communis* stained with 100 μM of **4**. The Raman image (center) was constructed with green (2260 cm^{-1} for di-yne probes) and red (2920 cm^{-1} for C-H bond vibration) channels, which is overlaid with bright field image of the guard cell. Raman images at 2260 cm^{-1} and 2920 cm^{-1} are shown separately in right. Raman spectra in d) and e) shows each subcellular area (average spectra of 6 pixels × 6 pixels: nuclear region in red, perinuclear region in orange, and plasma membrane in green) of the guard cell.

ineffective. These results confirmed that Raman probes **3** and **4** cause opening of *Commelina* stomata without affecting the COI1-JAZ2 mechanism.

In the ATRI experiments, probe **3** caused a weak Raman signal in the ER region around the nucleus of the *Commelina* guard cell; whereas the enantiomer *ent***3** did not give rise to any Raman signals (Figures 2d and S3). This result was further confirmed using probe **4**, which gave rise to a signal that was sufficiently strong to enable the imaging of **4** in *Commelina* guard cells (Figure 2e). In our previous study,[16] ATRI of Raman probe **3** in *Arabidopsis* guard cells could be achieved using *arc6*-mutant *Arabidopsis,* in which the formation of chloroplasts was impaired.[23, 24] This is because the fluorescence from chloroplasts in non-mutant Arabidopsis was too strong for ATRI to be possible. Unfortunately, we cannot use such mutant of *C. communis* beause no mutant is available for *C. communis*. However, we were surprised to discover that **4** enabled the Raman imaging in *Commelina* guard cell without using a mutant plant. These contrasting results between *Arabidopsis* and *Commelina* can be partly attributed to the weaker background fluorescence of *Commelina* guard cells (Figure S4). However, a complete explanation is unclear because **3** and **4** are similarly effective for stomatal opening (Figure 2b). However, our results do confirm that **2** localizes in the ER of a monocot, *C. communis*, as well as a dicot, *A. thaliana* (Figure 1b), and strongly suggest that a COI1-JAZ independent mechanism might be involved in stomatal movement mediated by **2**.

Having established that some target protein of **2** does exist in the ER of *Commelina* guard cells, we designed and synthesized the chemical probe for the detection. Compact photoaffinity-labeling probes (CPAL) **5** and *ent***5**, which bear an trifluoromethyldiazirine (TFMD) moiety as a photoaffinity group, and an azide handle for the introduction of a detection tag (Figure 3a) were designed for this end. In our previous study, CPAL technology was effective for reducing nonspecific binding in the living cell.[25]

As for the Raman probes, the photoaffinity-labeling probe should cause stomatal opening without binding the COI1-JAZ2 co-receptor. Coronatine (**2**) consists of coronafacic acid (CFA) and coronamic acid (CMA) moieties (Figure 1a). Previous structure-activity relationship studies revealed that the CMA moiety is unimportant for stomatal opening.[26, 27] On the other hand, the crystal structure of COI1-**2**-JAZ1 ternary complex showed that **2** fit precisely into the binding pocket formed by the complexation of COI1 and JAZ1, in which the carboxylate functionality of **2** was tightly bound with JAZ1 by hydrogen bonding (Figure S5a).[28] Thus, any structural modifications made to the CMA-moiety of **2** were expected to hinder the formation of a ternary complex with COI1 and JAZ. We also carried out an *in silico* docking study between **5** and COI1-JAZ2. The results showed that **5** could not cause formation of COI1-**5**-JAZ2 ternary complex (Figures 3b and S5b-d).[27,

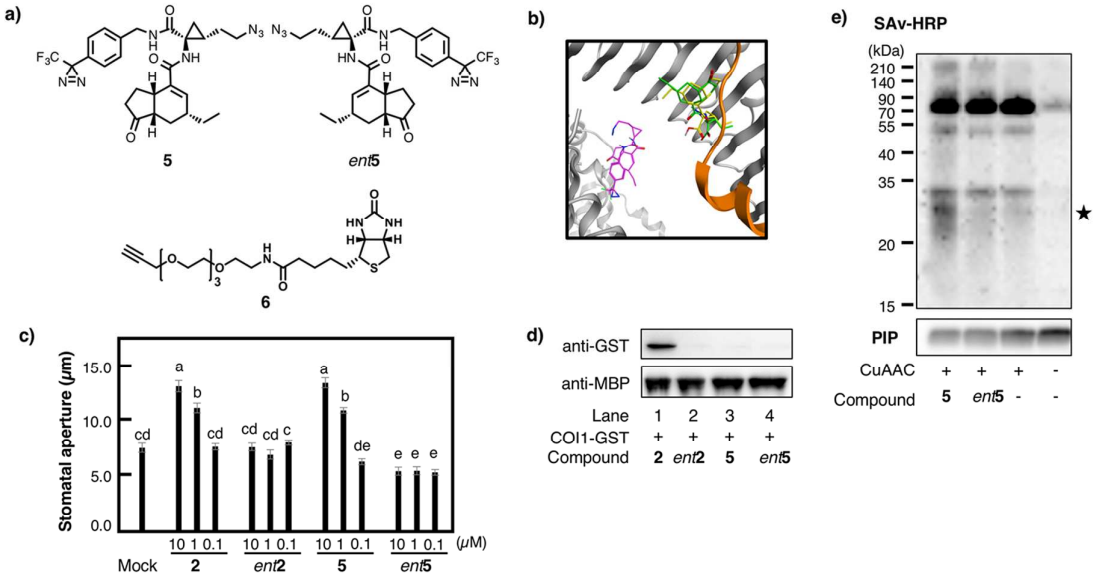

Figure 3. a) Chemical structures of compact photoaffinity probe (**5**, *ent***5**) and alkyne-tethered biotin tag (**6**). b) *In silico* docking model between **2** or **5** and COI1-JAZ2 by MOE 2016.08 software. **2** is shown as green stick, COI1 as gray ribbon, JAZ2 as orange ribbon, calculated **2** as yellow stick and calculated **5** as magenta stick, respectively. c) Stomatal opening assay using closed stomata of *Commelina communis* treated with **2**, *ent***2** or photoaffinity probes (**5**, *ent***5**). Bars represent the mean stomatal aperture with SE (n = 20 stomata). Different letters indicate significant differences between means (ANOVA: $P < 0.05$). d) Pull-down assay of **2**, *ent***2** or photoaffinity probes (**5**, *ent***5**) using GST-COI1 (5 nM)/MBP-JAZ2 (ca. 100 nM). e) Chemiluminescence detection of labeled protein with **5** and *ent***5** from microsomal fraction of *Commelina* guard cell. Aquaporin PIP in each microsomal fraction was shown as input. The specific labeling by **5** is shown as star.

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The modified CMA **S3** bearing TFMD and azide moieties was synthesized as shown in Scheme S2, and then coupled with separately synthesized CFA [26, 27] using COMU® to afford **5**, in moderate yield (Scheme S2). Compound *ent***5** was also prepared according to the same procedure (Scheme S2). Biological assessments of **5** and *ent***5**, stomatal opening on *C. communis*, and affinity with COI1-JAZ2 co-receptor (Figures 3c-d) were then carried out. Natural-type **5** was effective for the opening of dark-closed *Commelina* stomata, whereas the enantiomer *ent***5** was not. Then, the induction of COI1-JAZ2 complex formation was examined by a pull-down assay using MBP-JAZ2/GST-COI1. As predicted by the *in silico* docking studies, no formation of COI1-JAZ2 was observed by the addition of either **5** or *ent***5** (Figure 3d). These results confirm the utility of **5** and *ent***5** in *C. communis* for the detection of the binding protein, independent of the conventional COI1-JAZ2 pathway.

We collected *Commelina* stomata from the plant body by stripping the epidermis of the leaflet. This epidermis predominantly contained stomata, and a membrane fraction containing ER membrane was easily prepared for further photoaffinity labeling (PAL) experiments. PAL was carried out using **5** with *ent***5** as negative control. A copper-catalyzed, azide-alkyne cycloaddition (CuAAC)[30, 31] with the biotin tag **6** gave the labeled membrane proteins, which were detected by chemiluminescence. The difference in the labeling results of **5** and *ent***5** revealed that **5**-specific binding was observed for one band around 30 kDa (Figure 3e).[32] This result using structurally modified **2** suggested that a binding protein of **2** which can recognize the stereochemistry of the ligand would be present in the membrane of *Commelina* guard cells.

These results strongly suggest that the ER-mechanism is ubiquitous among dicots and monocots, and based on a signaling pathway widely found in plants. They also provide an important clue for further studies on ER-mechanism. ER-localization of important components is well known for ethylene (ET) signaling in the plant cell.[33-36] ET is a gaseous plant hormone that is involved in the development and environmental responses of plants, including inhibition of stomatal closure.[37, 38] Considering the ubiquity of ET-signaling among plants, **2** could upregulate the ET-signaling pathway in ER to affect the stomatal movement. Accordingly, the participation of ET-signaling in the ER-mechanism was further examined using the *A. thaliana* mutants *etr1-1*[39] and *ein2-1*,[40] in which the critical, ER-localized components of ET-signaling ETHYLENE RESPONSE1 (ETR1) and ETHYLENE INSENSITIVE2 (EIN2) are impaired, respectively. Normally, ET is perceived by the ET receptor ETR1, which causes dephosphorylation of EIN2, allowing signal transduction to the nucleus to upregulate ET-responses.[37] As shown in Figure 4a, **2**-mediated opening of dark-closed *Arabidopsis* stomata was completely disabled in *etr1-1* and *ein2-1*. This suggests that **2** affects ET signaling through an ER-localized target protein (Figure 4b).

Conclusions

Stomatal pores serve as security gates that protect plants from bacterial invasion, and thus bacterium-triggered stomatal closure is an important tactic used by the immune system to prevent bacterial infection. Bacterial virulence factor **2** facilitates bacterial invasion in the plant body partly by causing stomatal opening through an ER-mechanism. We demonstrated the possibility that this ER-mechanism would be ubiquitously involved among dicots and monocots, and thus its inhibition could be an effective strategy for improving the bacterial resistance of monocots, which include the three major crops corn, rice, and wheat. Based on the ubiquity of this ER-mechanism, we proposed the possible involvement of an ET-signaling pathway. Further studies on this ER-mechanism should inform the development of a

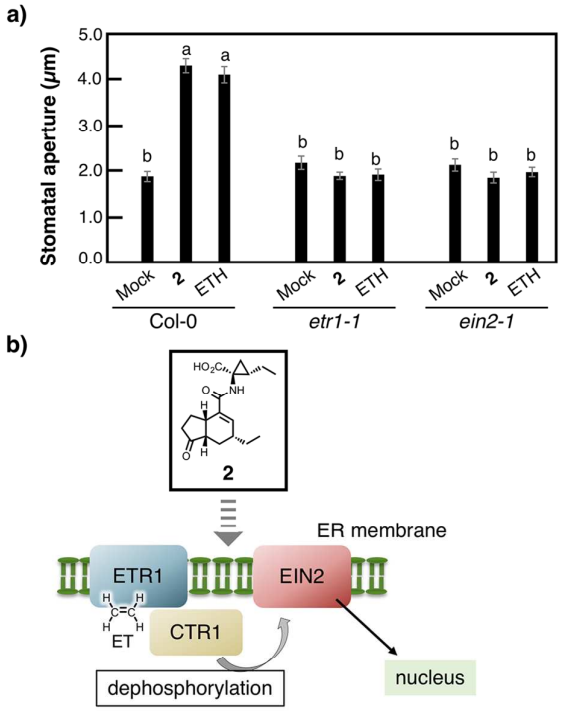

Figure 4. a) Stomatal opening assay using closed stomata of *Arabidopsis thaliana* (Col-0 and *ent2-1*) treated with **2** (10 μM) or ethephon (ETH, 100 μM). Ethephon, a functional mimic of ET, was used as control. b) Schematic representation of ethylene signaling. ETR1, an ET-receptor, dephosphorylate EIN2 through CTR1 on ER.

COI1-JAZ independent inhibitor of **2**, which could serve as an anti-infective chemical effective for wide variety of important crops.

Conflicts of interest

There are no conflicts to declare.

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Arabidopsis thaliana ETR1 mutant (*etr1-1*) and EIN2 mutant (*ein2-1*) were kindly provide by Dr. R Solano and Dr. A. Chini (National Center for Biotechnology, Madrid, Spain). This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas (no. 23102012) 'Chemical Biology of Natural Products (no. 2301)' for MU from MEXT, Japan, a Grant-in-Aid for Scientific Research (no. 26282207, no. 17H06407, and no. 17H00885 to MU), JSPS A3 Foresight Program (to MU), Naito Foundation (to MU), JSPS Asian Chemical Biology Initiative (to MU and MS), JST (JPMJPR16Q4 to YT), and AMED-CREST, AMED (No. JP17gm0710004 to MS).

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