DOI: 10.1002/asia.201100392

VIP *Cassia obtusifolia* MetE as a Cytosolic Target for Potassium Isolespedezate, a Leaf-Opening Factor of *Cassia* plants: Target Exploration by a Compact Molecular-Probe Strategy

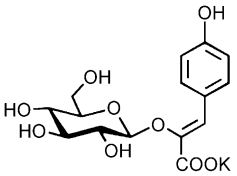
Minoru Ueda,*[a] Yoshiyuki Manabe,[a] Yuki Otsuka,[b] and Nobuyuki Kanzawa[b]

Abstract: Affinity chromatography by using ligand-immobilized bead technology is generally the first choice for target exploration of a bioactive ligand. However, when a ligand has comparatively low affinity against its target, serious difficulties will be raised in affinity-based target detection. We report here that the use of compact molecular probes (CMP) will be advantageous in such cases; it enables the retention of moderate affinity between the ligand and its target in contrast to immobilizing the ligand on affinity beads that will cause a serious drop in affinity to preclude target detection. In the CMP strategy, a CMP containing an azide handle is used for an initial affinity-based labeling of target, and subsequent tagging by CuAAC with a large FLAG tag will give a tagged target protein. By using the CMP strategy, we succeeded in the identification of *Cassia obtusifolia* MetE as a cytosolic target protein of potassium isolespedezate (**1**), a moderately bioactive ligand.

Keywords: affinity chromatography · *Cassia obtusifolia* · molecular probes · natural products · proteins

Introduction

Target identification of bioactive metabolites is an emerging field of natural product chemistry and bioorganic chemistry.[1] In particular, target identification of biologically active metabolites that induce a biologically intriguing phenomena have the possibility of opening a new aspect of chemical biology based on natural product chemistry.



1

Potassium isolespedezate (**1**) was isolated as a leaf-opening factor of *Cassia* plants.[2] Observed diurnal change in the endogenous content of **1** suggested that it might be involved in triggering circadian rhythmic leaf opening.[3] Previous studies suggested that **1** affects motor cells in pulvini of *Cassia mimosoides*[4] through binding membrane proteins of molecular weights 180 and 210 kDa.[5] In this paper, we report on the identification of *Cassia obtusifolia* MetE as a cytosolic target protein of **1** by using our compact molecular probe approach (CMP). Additionally, a hypothetical mechanism including the involvement of identified membrane proteins will be discussed.

[a] Prof. M. Ueda, Y. Manabe
Department of Chemistry, Tohoku University
6-3 Aramaki-aza Aoba, Aoba-ku
Sendai 980-8578 (Japan)
Fax: (+81) 22-795-6553
E-mail: ueda@m.tohoku.ac.jp

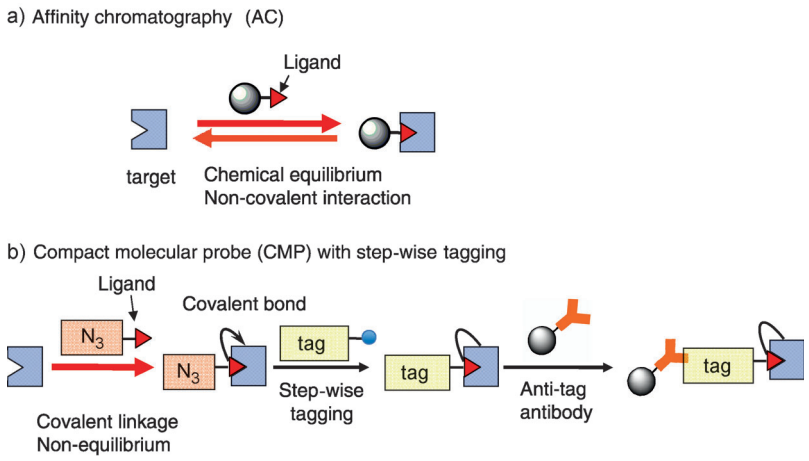
[b] Y. Otsuka, Prof. N. Kanzawa
Department of Material & Life Science
Faculty of Science & Technology, Sophia University
7-1, Kioi-cho, Chiyoda-ku, Tokyo 102-8554 (Japan)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201100392.

Results and Discussion

Target Identification of Potassium Isolespedezate: Issues to be Solved

Affinity chromatography (AC) by using ligand-immobilized bead technology is generally the first choice for target exploration.[6] This potent technology has been applied to many target explorations.[7] However, it is dependent on the extremely high bioactivity of the ligand, as strong as 10^{-9} M,[8a] because the bioactivity of the ligand is tightly correlated with its affinity to the target. AC is based on the noncovalent interaction between ligand and target, and thus is dependent on a chemical equilibrium (Scheme 1a). The affinity would dramatically decrease when the ligand is immobilized on beads. Thus, successful target exploration requires both a high affinity ligand and an abundant target



Scheme 1. Concepts of a) the AC separation of target protein and b) the CMP approach to target protein.

protein.[8] Ligands of much lower affinity cannot give a clear result because of nonspecific binding.

On the other hand, our ligand **1** showed moderate bioactivity (10^{-5}–10^{-6} M) on *Cassia* plants and was presumed to have moderate affinity with the corresponding target. Such a comparatively low affinity is expected to cause difficulties in affinity-based target detection.[9] In contrast to ligand-immobilized AC, a molecular-probe approach would be beneficial because a well-designed molecular probe can retain bioactivity and affinity to the target (Scheme 1b). Additionally, formation of a covalent bond between ligand and target will effectively remove the complex from chemical equilibrium, which is advantageous for the formation of an energetically unfavorable complex. We realized that by using a molecular probe for which high bioactivity can be maintained would be advantageous for target exploration of moderately bioactive ligands. Successful target identification by a molecular-probe approach, such as phytosulfokaine,[10] Pep1,[11] and RALF,[12] usually involves the use of a probe with bioactivity maintained as high as the naturally occurring ligand. These results suggested that retention of the original bioactivity should be the top priority when designing such a molecular probe. A well-designed molecular probe is indispensable for successful target exploration of **1**.

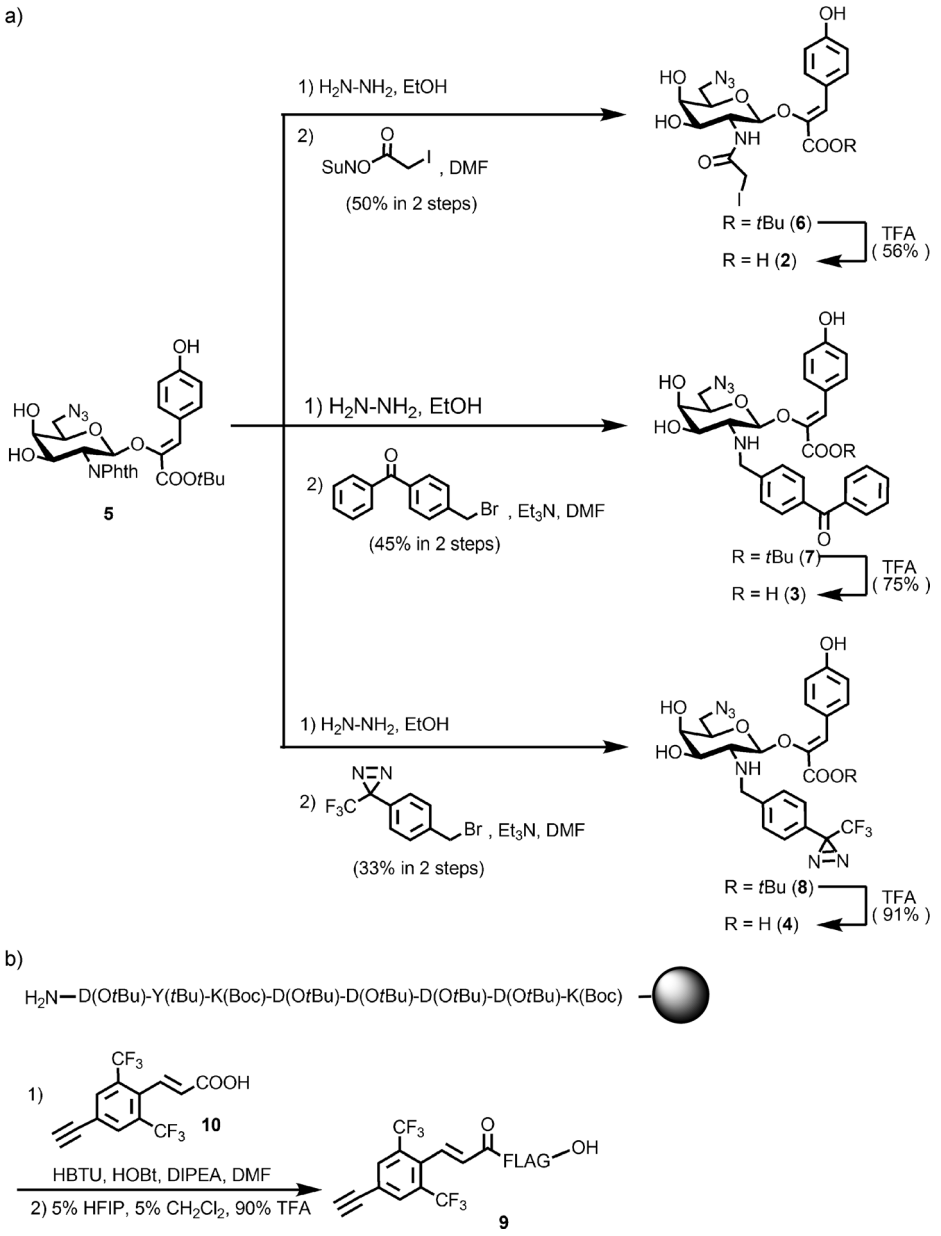
Two major issues must be addressed to successfully achieve a well-designed molecular probe for target detection of **1**. The first is a compact structure of the probe. Introduction of a relatively large functional unit, such as linker-connected biotin, in the compact structure of **1** caused a 100-fold decrease in its bioactivity.[5]

Another serious issue regarding design of molecular probes lies in the occurrence of countless nonspecific interactions between the probe and false targets along with the genuine ligand–receptor interactions.[13] A major cause of an increase in such nonspecific binding can be addressed in the hydrophobic properties of a molecular probe that is brought by the functional units in the probe itself, such as a reactive functional group, molecular tag, and a long linker.[14] Additionally, changes in physical properties, such as solubility and polarity, caused by the introduction of a relatively large functional unit, should be carefully considered. As almost all of these functionalities are hydrophobic, they are prone to nonspecific interactions, and when a biotinyl group is used as a reporter tag, the abundant endogenous biotinylated proteins[15] will interfere with the identification of the desired true signal for the chemically labeled target protein.

Target Identification of Potassium Isolespedezate: Target Identification by a Combination of CMP and the Stepwise Tagging Approach

In comparatively large bioactive molecules, such as oligo- or polypeptidehormones: phytosulfokaine,[10] Pep1,[11] and RALF,[12] it is not so difficult to introduce troublesome functional units without decreasing bioactivity because the pharmacophore and these functional units can be separated spatially because of their large molecular size. However, the same approach cannot be adopted for a small ligand, such as potassium isolespedezate (**1**). Then, the molecular design of the probe is the most important issue to be scrutinized: the more compact the probe is, the better the bioactivity that may be expected. On the other hand, the larger the molecular tag becomes, the less nonspecific binding can occur. This is because the larger tag of many functional groups can be expected to have more sites for molecular recognition, thus leading to highly selective recognition with less nonspecific binding. These conflicting requirements can be made compatible by the exclusion of functional units, such as tags, from the probe. The use of a copper-catalyzed azide alkyne cycloaddition (CuAAC)[13]-based stepwise tagging approach for the target protein[14,15] would be the best way to put this into practice. In this approach, a CMP containing an azide handle can be used for an initial affinity-based labeling of target, and subsequent tagging by CuAAC with a large molecular tag will give a tagged target protein. The use of CMP will enable the retention of moderate affinity between the ligand CMP and its target, in contrast to immobilizing the ligand on affinity beads, to secure selective and efficient binding even for only moderately bioactive ligands.

We designed and synthesized azide-containing lespedezates **2**–**4**[15] as CMPs (Scheme 2a). The arrangement of affinity-labeling functionalities and an azide handle was determined according to previous SAR studies[16] on **1**: both functional units were introduced within the glycone moiety because structural modification of the glycon moiety of **1** little



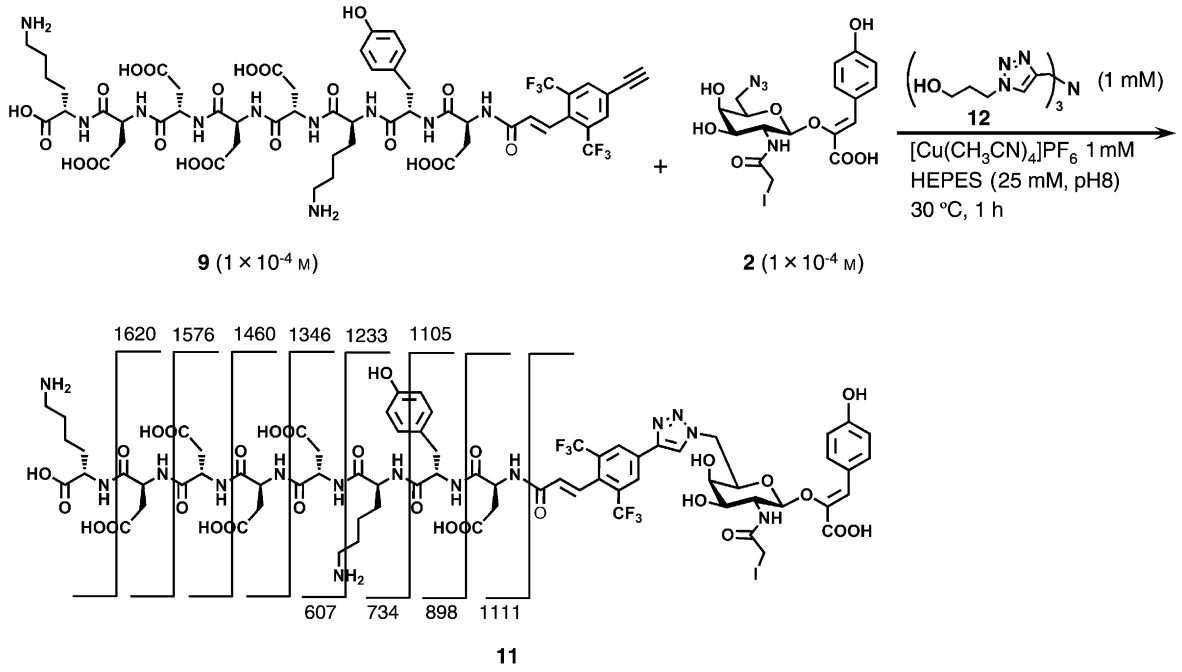
Scheme 2. Syntheses of a) azide-CMPs (**2**–**4**) and b) the FLAG-alkyne unit (**9**). DIPEA = *N*,*N*-diisopropylethylamine; HBTU = [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; HFIP = hexafluoroisopropanol; HOBt = 1-hydroxybenzotriazole; TFA = trifluoroacetic acid.

affects its bioactivity. Probe **2** was effective for leaf opening of *C. obtusifolia* leaves, with 80% of the leaves opening at 1×10^{-5} M, which was as strong as the response to naturally occurring **1** (effective for leaf opening of 87% of leaves at 1×10^{-5} M). Probes **3** and **4** were effective for leaf opening of 40% of leaves at 1×10^{-5} M, which is half as strong as that of **1** (40% at 5×10^{-6} M). A carbon electrophile (iodoacetyl[17] in **2**) and photoreactive groups (benzophenone[18] in **3** and trifluoromethydiazirine[19] in **4**) were selected as reactive functionalities for protein labeling. The remarkably stronger activity of CMPs **2**–**4** than that of a biotin-connected probe (1×10^{-4} M: for structure),[5] used for the detection of 180 and 210 kDa membrane targets, promises successful specific target labeling. This result suggested that avoiding a large molecular tag enables developing a CMP that is expected to retain affinity with its target without a severe decrease in bioactivity.

A FLAG peptide[20] was chosen as a highly potent molecular tag for affinity-based exploration and purification of the target protein of **1**. The FLAG peptide is an artificial octapeptide, DYKDDDDK, and a highly selective anti-FLAG antibody with little nonspecific binding is commercially available. We also developed the alkyne-connected FLAG tag **9**[21] (Scheme 2b), which exerts good reactivity against azide **2** (Scheme 3, Figure 1) under in vitro CuAAC conditions. This large tag is introduced after the azide labeling of target protein in the living cell.

Our previous results suggested that fluorescein-labeled potassium isolespedezate (**13**, see Figure 7) is localized in motor cells of *C. mimosoides* pulvini;[4] thus, we concluded that the target cell for **1** is motor cells. We chose isolated motor cell protoplasts as a material for target exploration because use of isolated protoplasts greatly decreased nonspecific binding.[6] Additionally, we chose *C. obtusifolia* instead of *C. mimosoides* as the plant material. We expected that the larger pulvini of *C. obtusifolia* (1×3 mm) than that of *C. mimosoides* (0.5×0.5 mm) would facilitate the collection of source materials for motor cell protoplasts. Potassium isolespedezate (**1**) was effective for leaf opening of both plants (5×10^{-6} for *C. obtusifolia* and at 1×10^{-6} M for *C. mimosoides*). Motor cell protoplasts were prepared according to the method of Satter and co-workers[21] and were used for the following examination. Next, we moved to target exploration of **1** by using living motor cell protoplasts.

We developed a highly effective stepwise tagging strategy for target exploration of a bioactive metabolite. CMPs **2**–**4** were used for in vivo introduction of an azide handle into the target protein (Figure 2a). Subsequently, the FLAG-tag



Scheme 3. CuAAC between **2** and **9** to give **11** with fragment ions found in MS/MS analysis.

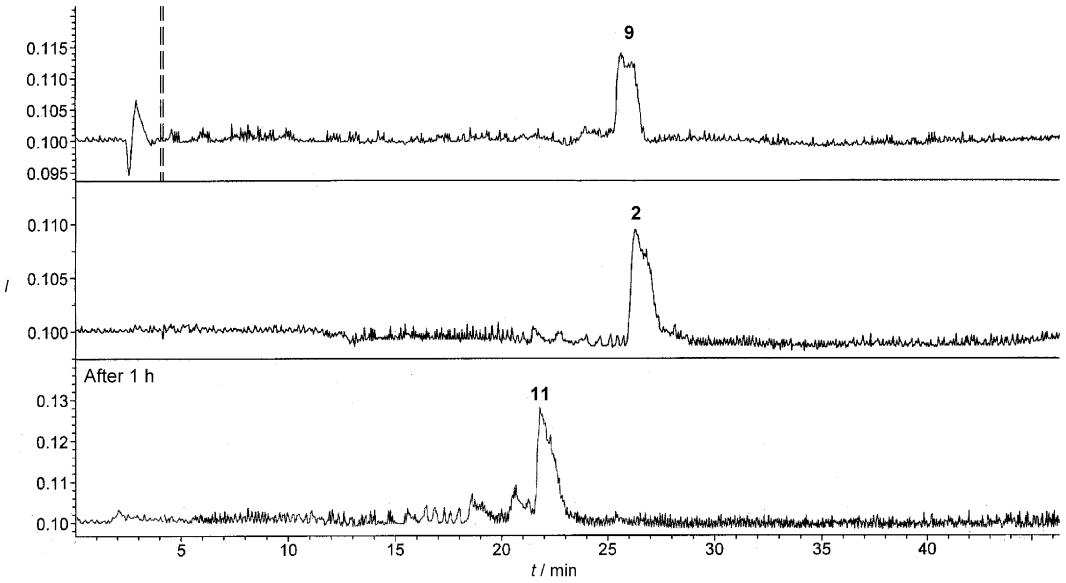


Figure 1. HPLC profile of CuAAC between **2** and **9**: Upper: FLAG unit **9**, middle: CMP **2**, bottom: CuAAC after 1 h (column: Deverosil ODS HG-3, detection: 280 nm, mobile phase: linear gradient in 48 min from 2% CH_3CN aq. containing 0.1% HCO_2H to 98% CH_3CN aq. containing 0.1% HCO_2H).

was coupled with the azide-labeled target protein by a CuAAC reaction. The microsomal fraction yielded no FLAG-tagged protein, whereas the cytosolic fraction gave a FLAG-tagged protein at 83 kDa when the iodoacetyl-type probe 2 was used (Figure 2b, lane 2). This band disappeared in a competitive inhibition experiment in the presence of excess isolespedezate **1** (Figure 2b, lane 3), and thus was confirmed as a putative cytosolic target protein of **1**, named cytosolic target protein for lespedezate (CTPL). Considering the difference found in bioactivity among the three probes (80% at 1×10^{-5} M for **2**, and 40% at 1×10^{-5} M for **3** and **4**), no discriminating difference can be predicted for their affinity with CTPL. Thus, the difference in results in Figure 2 can be attributed to the superiority of the iodoacetyl functionality over a photoaffinity tag. The advantage of iodoacetyl-based protein labeling would lie in its high yield in protein labeling[22] relative to photoaffinity-based protein labeling,

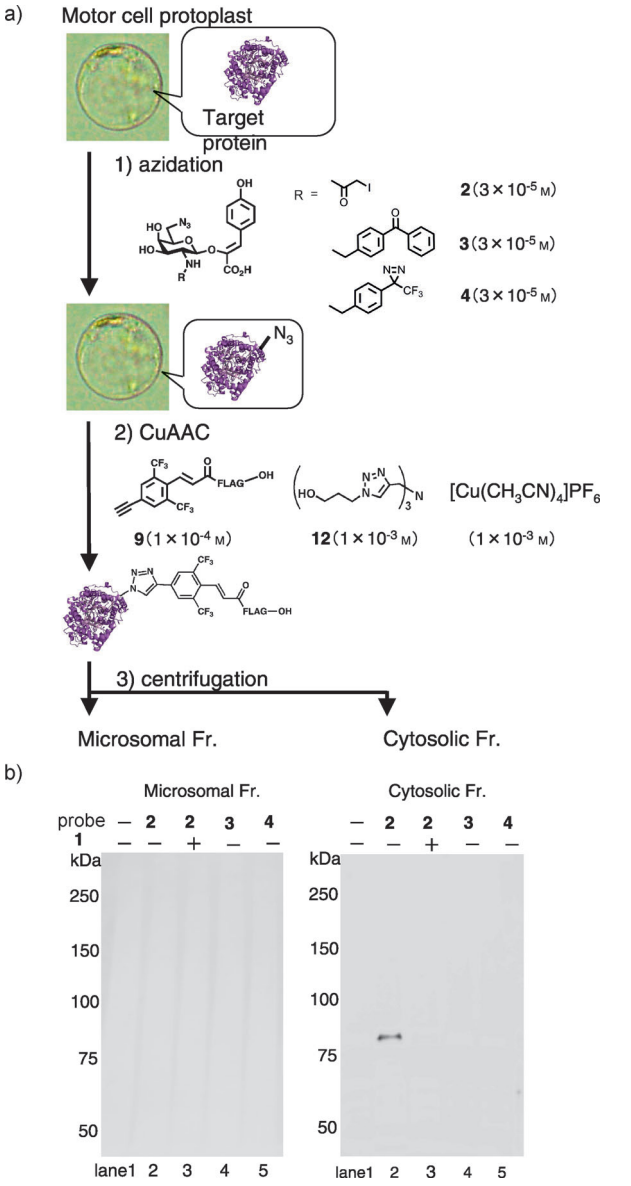


Figure 2. a) Procedures of the CMP approach combined with step-wise FLAG-tagging of target protein. b) SDS-PAGE analyses of separated microsomal and cytosolic fractions.

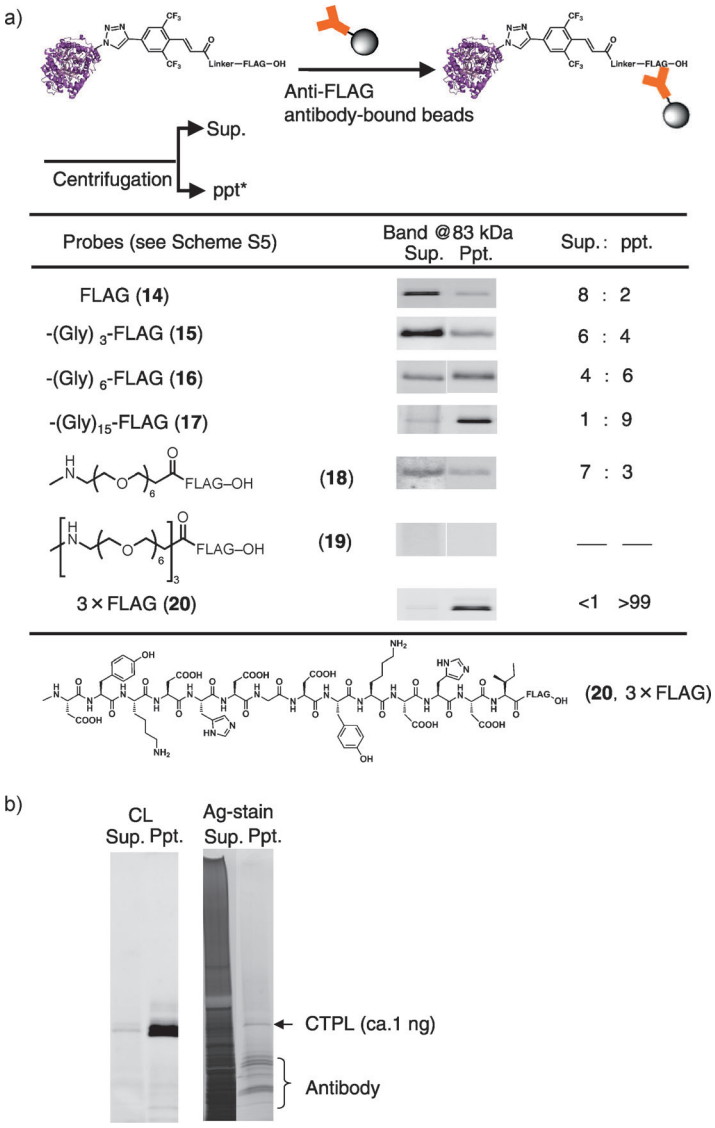


Figure 3. a) Immunoprecipitation (IP) of CTPL by using a panel of probes with linkers of various lengths and structure. b) SDS-PAGE analyses of immunoprecipitated CTPL by using probe **20**; left: chemiluminescence (CL) detection, right: silver-staining (Ag-stain) detection.

which usually results in around a few percent of labeling yield percent.[23]

These results suggested that our CMP approach combined with stepwise FLAG-tagging, first forming a covalent bond between ligand and target by using CMP with bioactivity maintained, followed by CuAAC-based introduction of a FLAG tag, is a potent approach to target exploration of ligands with moderate bioactivity. The resulting tagged CTPL was then immunoprecipitated by using a corresponding anti-FLAG antibody immobilized on beads. Among probes (**14**–**20**, see Schemes S1–S5 in the Supporting Information) of various lengths and structures of linker-connected tags, a 3XFLAG peptide antigen as a molecular tag (**20**)[24] gave the most efficient IP result (Figure 3). The linker structure and length strongly affect IP results.[24] As a result, stepwise tagging of the target by FLAG was successfully applied to the target identification of **1** and led to the discovery of the novel cytosolic target CTPL.

The isolated CTPL was analyzed by de novo sequencing by using LCMS/MS after in-gel digestion by trypsin. The sequences GNASVPAMEMTK, ALGVETVPVLVGPVSYLLLSKPAK, KLNLPILPTTTIGSFPQTLDLR, IQEELDIDVLVHGEPER, GMLTGPVTILNWSFVR, and AGITVIQIDEAALR, YGAGIGPGVYDIHSPR were identified by using a MASCOT search of the NCBInr database to conclude that CTPL is highly similar to MetE (a 5-methyltetrahydropteroyl- triglutamate-homocysteinemethyltransferase) from *Ricinus communis* (Figure 4a).[25] MetE is a cobalamine-independent methyltransferase participating in the final step of methionine biosynthesis, and *Arabidopsis thali-*

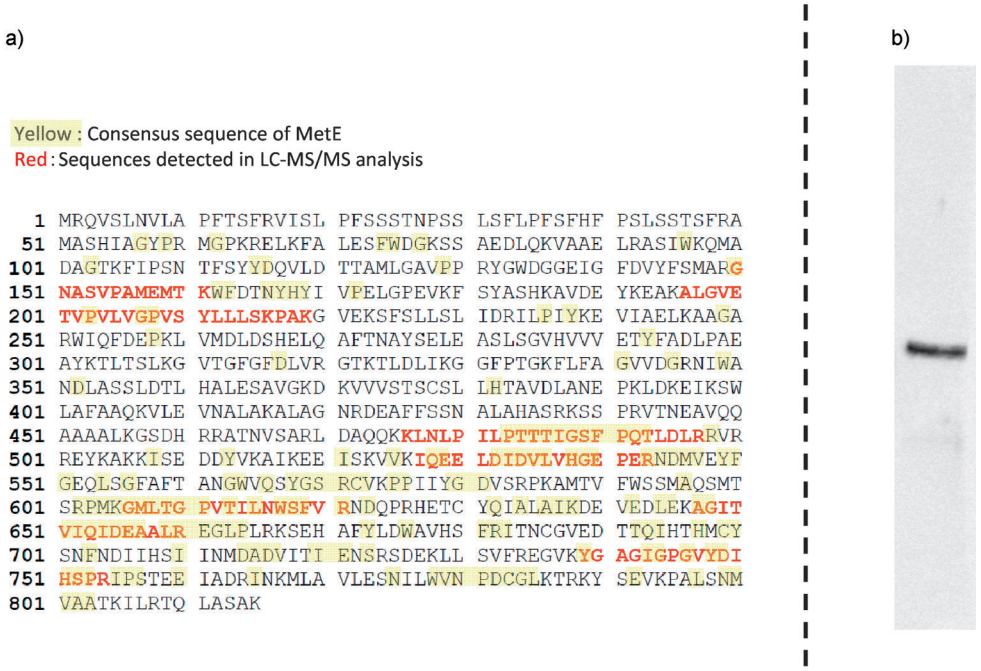


Figure 4. a) Comparison between the partial sequence of CTPL and *Ricinus communis* MetE. b) Chemiluminescence detection of CTPL by using anti-MetE antibody raised against *Lotus japonicus* MetE.

ana has three isoforms with distinct expression profiles.[26] The sequence corresponded well with a consensus sequence for MetE (Figure 4a, colored in yellow).[27] Additionally, an anti-MetE antibody raised against *L. japonicus* MetE cross-reacted with isolated CTPL (Figure 4b). Thus, CTPL was identified as a MetE homologue from *C. obtusifolia* and named CoMetE (*Cassia obtusifolia* MetE).

FLAG-tagged CTPL (=CoMetE) was detected only in the precipitate obtained by CMP-IP (Figure 5a). This indicates that all of FLAG-tagged CTPL was recovered by IP. However, non-FLAG-tagged CTPL, which can be detected by using anti-MetE antibody, was also found in supernatant after CMP-IP (Figure 5b, right). Overall isolation yield of CTPL (=CoMetE) by the CMP-IP strategy was estimated to be 20% by comparing the content of CoMetE in each supernatant without CuAAC (motor cell lyzate as a control), precipitate obtained by CMP-IP, and supernatant after CMP-IP (Figure 5a,b).

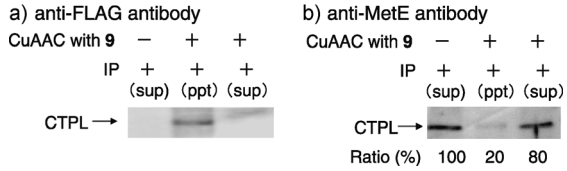


Figure 5. Chemiluminescence detection of IP results by using a) anti-FLAG antibody or b) anti-MetE antibody: from the left, supernatant without CuAAC (cell lyzate as a control), precipitate obtained by CMP-IP, and supernatant after CMP-IP.

Membrane Targets of 180 and 210 kDa

In the current study, previously observed membrane targets (180 and 210 kDa)[5] were not found in the microsomal fraction. This may be attributed to the different quantities of microsomal fractions used in these experiments: in the previous study, the microsomal fraction, which was directly prepared from chopped pulvini, contained as much as 134 μg protein (membrane ATPase activity:[28] 0.67 mmol mg^{-1} protein/min).[5] On the other hand, a microsomal fraction prepared from isolated motor cell protoplasts contained as little as 2.84 μg protein (membrane ATPase activity: 2.81 mmol mg^{-1} protein/min) because the azide labeling using motor cells was carried out with few protoplasts due to the low yield during protoplast preparation. A large amount of motor cells was lost and disrupted during enzyme digestion during protoplast preparation. The use of protoplasts was effective for decreasing nonspecific binding by avoiding incorporation of other plant materials, such as vascular bundles or epidermis, but the overwhelming difference in the quantity of the microsomal fraction would make it difficult to find trace amounts of a membrane target of 180 and 210 kDa.

CoMetE

Target identification of a bioactive metabolite requires appropriate validation. In general, the most reliable approach would be to use a knock-out or -down strain impaired in the response to the bioactive metabolite. However, no genetic mutant has been obtained in *Cassia* plants, and it is impossible to use model legumes, such as *Lotus japonicus*, instead

of *Cassia* because potassium isolespedezate (**1**) is not effective for leaf movement of *L. japonicus*, even at 3×10^{-4} M. Additionally, to the best of our knowledge, no inhibitor is known for MetE. We thus carried out validation by using unique features in the bioactivity of **1**.

Fortunately, potassium isolespedezate (**1**) has two unique features in its bioactivity. One is a unique SAR pattern:[16] modification of the aglycone moiety severely lowers its leaf-opening activity, whereas modification of the glycone moiety does not affect it at all. The other is its unique genus-specific leaf-opening activity:[2,29] potassium isolespedezate (**1**) is effective only for plants belonging to the genus *Cassia*. The correspondence between these unique features of **1** and its affinity with CoMetE was examined to validate MetE as a target.

Binding affinity between CoMetE and biologically active (**2**)/inactive (**21**, **22**) CMPs showed a good correspondence

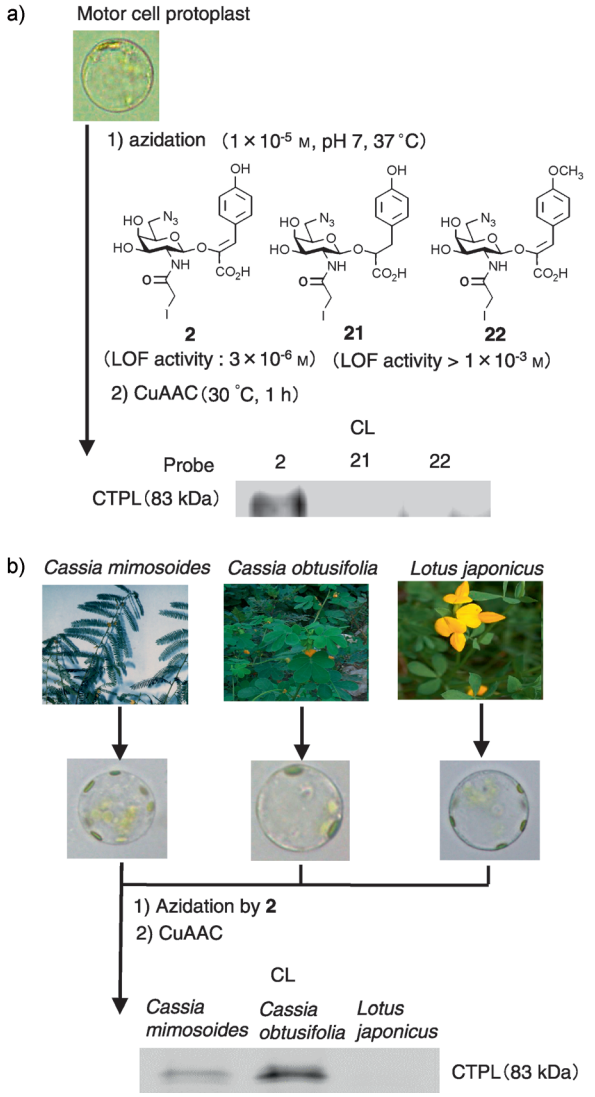
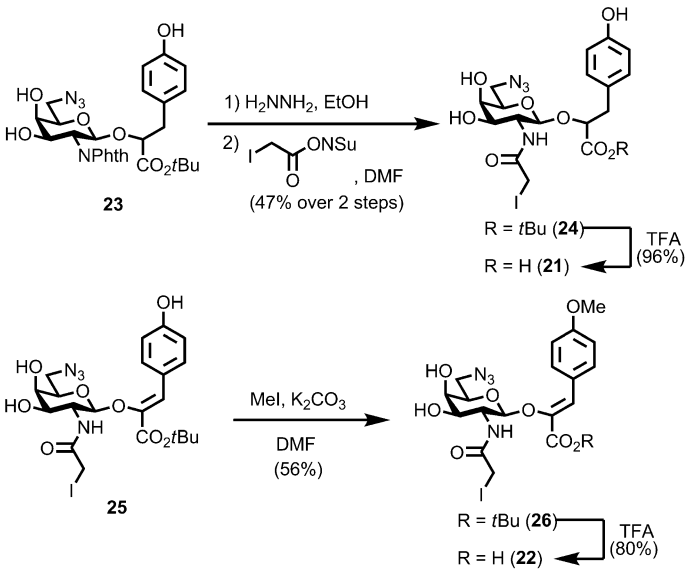


Figure 6. Experiments for the varidation of CTPL as a target protein of **1**: a) A clear correlation was observed between the leaf-opening activity of CMPs (**2**, **21**, and **22**) and affinity with CTPL. b) Genus-specific labeling of CTPL was observed by using CMP **2**.



Scheme 4. Syntheses of biologically inactive CMPs (**21** and **22**).

with this unique SAR pattern of **1** in cell-based binding studies (Figure 6a). We synthesized biologically inactive CMPs **21** and **22** (Scheme 4), in which the aglycone moiety was modified to exert no leaf-opening activity even at 1×10^{-3} M. CoMetE in *Cassia* motor cells was not labeled by either of these probes, and a clear relationship between leaf-opening activity and affinity with CoMetE was demonstrated. Additionally, CMP **2** gave a FLAG-labeled 83 kDa protein in the motor cells of both *C. mimosoides* and *C. obtusifolia*, whereas no labeled protein was found in *L. japonicus* (Figure 6b). This result also reflects the other unique feature of **1**, its genus-specific leaf-opening activity. Thus, these two validation experiments gave results in accordance with the unique features of bioactivity in **1**.

CoMetE and Cytosolic Localization

Two of the three isoforms of *A. thaliana* MetE (AtMetE) were revealed to be localized in the cytosol.[26] CoMetE was also isolated from the cytosol fraction. This means that highly polar potassium isolespedezate (**1**) can be transported into the cytosol through the plasma membrane of the motor cell to bind CoMetE.

Waring[30] reported that log*D* and molecular weight are the most important factors in determining the membrane permeability of small molecules based on a study by using a panel of over 9500 compounds. According to the study, log*D* > 1.1 for MW: 300–350, log*D* > 1.7 for MW: 350–400, log*D* > 3.1 for MW: 400–450, log*D* > 3.4 for MW: 450–500, log*D* > 4.5 for MW: > 500, are expected criteria for successful membrane permeability. The calculated log*D*[36] of **1** (MW: 342, log*D* = −4.5 at pH 7, −2.5 at pH 5) and of CMP **2** (MW: 534, log*D* = −3.7 at pH 7, −1.7 at pH 5) are lower than the criteria necessary for passive (diffusion-driven) transport. Additionally, the localization of fluorescein-la-

beled **1** (**13**)[4] in motor cell protoplasts showed that **13** can be transported into the cytosol through the plasma membrane (Figure 7). This may be due to transport by a carrier-mediated facilitated diffusion process, perhaps involving a transporter protein, because **13** at a pH below 7 lost its membrane permeability due to dissociation of the carboxylate to form a salt.

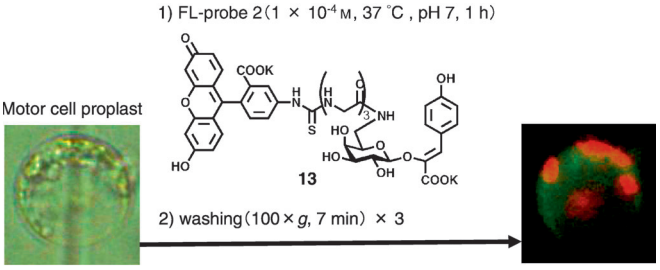


Figure 7. Cytosolic localization of fluorescein-labeled **1** (**13**) in motor cell protoplasts.

The previously identified 180 and 210 kDa membrane proteins were reported to be able to recognize the structure related to the leaf-opening activity of **1**,[5] and thus, can be considered potential transporters with the appropriate selectivity. Additionally, the detection of cytosolic CoMetE by using **2** implies that CMP **2** can be transported into the cytosol with putative carrier protein, as naturally occurring **1**. This means that our CMP strategy was successful in maintaining transport ability and bioactivity of naturally occurring **1**.

Conclusions

Our CMP strategy was proven to be effective for the detection of a moderate affinity target for which AC cannot be applied successfully. Then, our CMP approach can be considered as a powerful alternative of AC when the ligand has moderate bioactivity. Additionally, it is also emphasized that our CMP approach enables the tracing of the fate of bioactive metabolites to detect cytosolic targets because CMP can maintain transport ability and bioactivity equivalent to naturally occurring **1**.

MetE is a ubiquitous enzyme participating in the biosynthesis of methionine. However, in *Cassia* motor cells, the putative membrane transporter recognizes potassium isolespedezate (**1**) and transports it into the cytosol by a carrier-mediated diffusion process. Recently, it was revealed that a leaf-closing substance of *Samanea saman* worked as a trigger for motor cell shrinkage by activation of ion channels.[31] On the other hand, the leaf-opening factor **1** can be involved in a different mechanism and induce leaf opening in *Cassia* plants.

Experimental Section

Compound ***6***

Compound **5**[5] (56.5 mg, 0.103 μmol) was dissolved in ethanol (2 mL) and hydrazine monohydrate (40 μL, 0.8 mmol) was added to this solution. After overnight stirring at RT under an Ar atmosphere, the reaction mixture was evaporated to dryness. The residue was dissolved in EtOAc, washed with H_2O, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated to give the crude amine (49.5 mg). Iodoacetic acid *N*-hydroxysuccinimide ester[35] (5.1 mg, 17.9 μmol) was added to a *N*,*N*-dimethylformamide (DMF; 200 μL) solution of the crude amine (6.3 mg) and the mixture was stirred for 6 h at RT under an Ar atmosphere. The reaction was quenched by the addition of AcOH (3 drops). After drying, the residue was purified by pTLC ($CHCl_3$/MeOH 10:1) to give **6** (4.0 mg, 6.5 μmol 50%) in two steps. ^{1}H NMR (500 MHz, CD_3OD): δ=7.68 (d, J=8.5 Hz, 2H), 6.82 (s, 1H), 6.76 (d, J=8.5 Hz, 2H), 5.32 (d, J=8.5 Hz, 1H), 4.21 (dd, J=11.0, 8.5 Hz, 1H), 3.77 (s, 2H), 3.74 (dd, J=3.5, 1.0 Hz, 1H), 3.67 (dd, J=11.0, 3.5 Hz, 1H), 3.59 (ddd, J=8.5, 4.0, 1.0 Hz, 1H), 3.50 (dd, J=12.5, 8.5 Hz, 1H), 3.23 (dd, J=12.5, 4.0 Hz, 1H), 1.55 ppm (s, 9H); ^{13}C NMR (125 MHz, CD_3OD): δ=172.0, 165.0, 159.5, 140.4, 133.7, 126.2, 125.8, 116.2, 100.5, 82.8, 76.2, 73.0, 70.0, 54.9, 52.4, 28.5, −1.58 ppm; IR (film): $\tilde{\nu}$=3295, 2978, 2927, 2102, 1698, 1606, 1584, 1556, 1512, 1369, 1316, 1276, 1254, 1158, 1115, 1072, 837, 755 cm^{-1}; $[\alpha]_D^{27}$=−52.7 (c=0.10 in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{21}H_{28}IN_4NaO_8$: 613.0771 $[M+Na]^+$; found: 613.0800.

Compound ***2***

TFA (1 mL) was added to compound **6** (12.5 mg, 21.2 μmol). After stirring for 5 min at RT under an Ar atmosphere, the reaction mixture was dried in vacuo. The residue was purified by HPLC (25% CH_3CN aq. containing 0.1% TFA) to give **2** (6.3 mg, 11.3 μmol, 56%). ^{1}H NMR (500 MHz, CD_3OD): δ=7.71 (d, J=8.5 Hz, 2H), 7.00 (s, 1H), 6.76 (d, J=8.5 Hz, 2H), 5.26 (d, J=8.5 Hz, 1H), 4.21 (dd, J=10.5, 8.5 Hz, 1H), 3.77 (s, 2H), 3.74 (dd, J=3.0, 0.5 Hz, 1H), 3.66 (dd, J=10.5, 3.0 Hz, 1H), 3.60 (ddd, J=8.0, 4.5, 0.5 Hz, 1H), 3.49 (dd, J=13.0, 8.0 Hz, 1H), 3.23 ppm (dd, J=13.0, 4.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ= 172.2, 167.6, 159.7, 139.8, 133.9, 127.2, 126.2, 116.1, 101.2, 76.1, 73.3, 70.0, 55.0, 52.3, −1.71 ppm; $[\alpha]_D^{23}$=−24.6 (c=0.10 in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{17}H_{20}IN_4NaO_8$: 557.0145 $[M+Na]^+$; found: 557.0157.

Compound ***7***

Compound **5**[5] (32.8 mg, 59.4 μmol) was dissolved in ethanol (2 mL) and hydrazine monohydrate (40 μL, 0.8 mmol) was added to this solution. After overnight stirring at RT under an Ar atmosphere, the reaction mixture was evaporated to dryness. The residue was purified by RP-pTLC (water/acetonitrile 1:1, containing 1% TFA) to give the crude amine (33.0 mg, crude). 4-Bromomethylbenzophenone (32.5 mg, 0.119 mmol) and TEA (41.3 mL, 0.297 mmol) were added to the DMF solution (1 mL) of the crude amine (33.0 mg, crude). After stirring for 2.5 h at RT under an Ar atmosphere, the reaction was quenched with AcOH. After being dried, the residue was purified by pTLC ($CHCl_3$/MeOH 10:1) to give **7** (16.3 mg, 26.5 μmol 45% in two steps). ^{1}H NMR (500 MHz, CD_3OD): δ=7.75–7.67 (m, 6H), 7.61 (t, J=6.5 Hz, 1H), 7.55 (d, J= 8.5 Hz, 2H), 7.49 (t, J=7.8 Hz, 2H), 6.92 (s, 1H), 6.70 (d, J=8.5 Hz, 2H), 5.13 (d, J=8.0 Hz, 1H), 4.22 (d, J=13.5 Hz, 1H), 4.19 (d, J= 13.5 Hz, 1H), 3.70 (d, J=3.0 Hz, 1H), 3.61 (dd, J=10.0, 3.5 Hz, 1H), 3.58 (m, 1H), 3.44 (dd, J=13.0, 8.3, 1H), 3.17 (dd, J=13.0, 4.3, 1H), 3.07 (dd, J=10.0, 8.3, 1H), 1.55 ppm (s, 9H); ^{13}C NMR (125 MHz, CD_3OD): δ=198.5, 165.2, 159.7, 147.0, 140.6, 139.0, 137.4, 133.7, 131.3, 130.9, 129.8, 129.5, 126.9, 126.1, 116.1, 103.8, 82.9, 76.1, 74.3, 70.0, 60.5, 53.7, 52.4, 28.5 ppm; IR (film): $\tilde{\nu}$=3381, 2978, 2931, 2102, 1698, 1651, 1606, 1512, 1369, 1317, 1279, 1159 cm^{-1}; $[\alpha]_D^{22}$=−43.8 (c=0.10 in CH_3OH); HRMS (ESI, positive): m/z: calcd for $C_{33}H_{37}N_4O_8$: 617.2606 $[M+H]^+$; found: 617.2602.

*Compound **3***

TFA (1 mL) was added to compound **7** (8.2 mg, 13.2 μmol). After stirring for 1 h at RT under an Ar atmosphere, the reaction mixture was dried in vacuo. The residue was purified by RP-pTLC (water/acetonitrile 1:1, containing 1% TFA) to give **3** (5.0 mg, 9.9 μmol, 75%). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.74–7.30 (m, 11H), 6.89 (s, 1H), 6.62 (d, J = 8.5 Hz, 2H), 4.96 (d, J = 8.5 Hz, 1H), 4.36 (d, J = 13.0 Hz, 1H), 4.28 (d, J = 13.0 Hz, 1H), 3.96 (dd, J = 11.0, 3.0 Hz, 1H), 3.75 (d, J = 3.0 Hz, 1H), 3.63 (m, 1H), 3.42 (dd, J = 12.5, 7.8, 1H), 3.35 (dd, J = 11.0, 8.5, 1H), 3.26 ppm (dd, J = 12.5, 4.0, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 197.7, 169.3, 160.7, 139.8, 138.4, 137.1, 134.4, 134.0, 131.5, 131.4, 130.9, 130.1, 129.6, 126.5, 125.1, 116.2, 101.0, 76.2, 70.7, 69.6, 61.3, 52.3, 52.0 ppm; IR (film): $\tilde{\nu}$ = 3304, 2924, 2102, 1658, 1607, 1512, 1366, 1279, 1064, 703 cm^{-1}; $[\alpha]_D^{22}$ = −38.3 (c = 0.10 in CH_3OH); HRMS (ESI, positive): m/z: calcd for $C_{29}H_{28}N_4NaO_8$: 583.1799 $[M+Na]^+$; found: 583.1801.

*Compound **8***

Compound **5**[5] (28.0 mg, 50.7 μmol) was dissolved in ethanol (1 mL) and hydrazine monohydrate (20 μL, 0.8 mmol) was added to this solution. After overnight stirring at RT under an Ar atmosphere, the reaction mixture was evaporated to dryness. The residue was dissolved in EtOAc, washed with H_2O, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated to give crude amine (26.1 mg). 3-[4-(Bromomethyl)phenyl]-3-(trifluoromethyl)diazirine[34] (28.2 mg, 0.101 mmol) and TEA (21.2 mL, 0.152 mmol) were added to a solution of the crude amine (26.1 mg, crude) in DMF (0.5 mL) and the mixture was stirred for 1 h at 0°C under an Ar atmosphere. Then, the mixture was slowly allowed to stand to RT. After overnight stirring, the reaction was quenched with AcOH. After being dried, the residue was purified by pTLC ($CHCl_3$/MeOH 10:1) to give **8** (12.5 mg, 16.7 μmol 33%) in two steps. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.67 (d, J = 8.5 Hz, 2H), 7.48 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.5 Hz, 2H), 6.91 (s, 1H), 6.71 (d, J = 8.5 Hz, 2H), 5.11 (d, J = 8.5 Hz, 1H), 4.15 (d, J = 13.5 Hz, 1H), 4.09 (d, J = 13.5 Hz, 1H), 3.68 (d, J = 3.5 Hz, 1H), 3.56 (m, 2H), 3.44 (dd, J = 13.0, 8.5 Hz, 1H), 3.16 (dd, J = 13.0, 4.0 Hz, 1H), 3.01 (d, J = 8.5 Hz, 1H), 1.54 ppm (s, 9H); ^{13}C NMR (125 MHz, CD_3OD): δ = 165.2, 159.7, 143.9, 140.5, 133.7, 130.4, 128.5, 127.5, 127.0, 126.1, 123.5 (q, J = 261.2 Hz), 116.1, 103.8, 82.9, 76.1, 74.2, 69.9, 60.5, 53.4, 52.4, 29.5 (q, J = 40.2 Hz), 28.5 ppm; IR (film): $\tilde{\nu}$ = 3312, 2980, 2932, 2103, 1697, 1606, 1585, 1513, 1455, 1394, 1370, 1346, 1316, 1277, 1254, 1233, 1158, 1114, 1067, 759 cm^{-1}; $[\alpha]_D^{27}$ = −11.1 (c = 0.10 in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{28}H_{32}F_3N_6O_7$: 621.2285 $[M+H]^+$; found: 621.2259.

*Compound **4***

Compound **8** (8.2 mg, 13.2 μmol) was dissolved in TFA (1 mL). After stirring for 1 h at RT under an Ar atmosphere, this solution was dried in vacuo. The residue was purified by HPLC (40% CH_3CN aq. containing 0.1% TFA) to give **4** (6.8 mg, 12.0 μmol, 91%). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.75 (d, J = 8.5 Hz, 2H), 7.65 (d, J = 8.5 Hz, 2H), 7.31 (d, J = 8.5 Hz, 2H), 7.24 (s, 1H), 6.74 (d, J = 8.5 Hz, 2H), 5.26 (d, J = 8.5 Hz, 1H), 4.68 (d, J = 13.0 Hz, 1H), 4.48 (d, J = 13.0 Hz, 1H), 4.03 (dd, J = 11.0, 3.0 Hz, 1H), 3.83 (dd, J = 3.0, 0.5 Hz, 1H), 3.69 (ddd, J = 8.0, 5.0, 0.5 Hz, 1H), 3.52 (dd, J = 11.0, 8.5 Hz, 1H), 3.46 (dd, J = 13.0, 8.0 Hz, 1H), 3.26 ppm (dd, J = 13.0, 5.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 169.2, 160.7, 134.8, 134.3, 132.8, 132.1, 131.3, 129.8, 128.2, 125.1, 123.4 (q, J = 273.1 Hz), 116.1, 101.0, 76.5, 76.2, 70.6, 69.6, 61.4, 52.0, 29.3 ppm (q, J = 40.3 Hz); IR (film): $\tilde{\nu}$ = 3311, 2926, 2855, 2358, 2106, 1672, 1606, 1513, 1439, 1374, 1261, 1232, 1175, 1156, 1079, 940, 838, 801, 763, 722, 505, 458, 444, 418 cm^{-1}; $[\alpha]_D^{27}$ = +20.1 (c = 0.10 in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{24}H_{24}F_3N_6O_7$: 565.1659 $[M+H]^+$; found: 565.1651.

*Compound **9***

Protected-FLAG (12.5 mg on beads, 5 μmol) was suspended in DMF (200 μL). Compound **10**[36] (15.4 mg, 50 μmol), HOBt (6.8 mg, 50 μmol), iPr$_2$NEt (8.7 μL, 50 μmol), and HBTU (19.0 mg, 50 μmol) were added to this suspension. After shaking for 8 h with a shaker at RT under an Ar atmosphere, the reaction mixture was diluted with MeOH (2 mL) and the supernatant was removed. The collected precipitate was re-suspended in MeOH (2 mL) and the supernatant was decanted. This sequence was repeated twice and the resin was dried by flushing with N_2. The resultant resin was suspended in CH_2Cl_2/HFIP/TFA 5:5:90 (1 mL) and stirred for 1 h at RT under an Ar atmosphere. After the reaction mixture was concentrated in vacuo, purification by HPLC (linear gradient over 40 min from 20% CH_3CN aq. containing 0.1% TFA to 40% CH_3CN aq. containing 0.1% TFA) gave **9** (3.3 mg, 2.5 μmol, 50%); HRMS (ESI, positive): m/z: calcd for $C_{54}H_{66}F_6N_{10}O_{21}$: 652.2149 $[M+2H]^{2+}$; found: 652.2149.

*Compound **11** (in vitro CuAAC)*

Azide **2** (2×10^{-8} mol) was dissolved in ligation buffer (90 μL, 25 mM HEPES (pH 8.0)) and then FLAG unit **9** (2×10^{-8} mol in 90 μL ligation buffer) with $[Cu(CH_3CN)_4]PF_6$ (2×10^{-8} mol) and **12** (2×10^{-8} mol) in ligation buffer (10 μL) containing 10% DMSO were added. This solution was incubated with gentle agitation of 50 rpm for 1 h at 30°C. The CuAAC reaction was monitored by LCMS/MS analysis (linear gradient over 48 min from 2% CH_3CN aq. containing 0.1% HCOOH to 98% CH_3CN aq. containing 0.1% HCOOH, flow rate: 0.2 mL min^{-1}, detection 280 nm coupled with positive mode-ESI by using an esquire 4000, Bruker Daltonics). HRMS (ESI, positive): m/z: calcd for $C_{71}H_{84}F_6I_1N_{14}O_{29}$: 1837.4477 $[M+H]^+$; found: 1837.4490.

*Compound **24***

Compound **23**[5] (22.1 mg, 39.8 μmol) was dissolved in ethanol (2 mL) and hydrazine monohydrate (40 μL, 0.8 mmol) was added to this solution. After overnight stirring at RT under an Ar atmosphere, the reaction mixture was evaporated to dryness. The residue was dissolved in EtOAc, washed with H_2O, and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated to give crude amine (22.3 mg). Iodoacetic acid *N*-hydroxysuccinimide ester[34] (22.6 mg, 79.8 μmol) was added to a DMF (1 mL) solution of the crude amine (22.3 mg) and the mixture was stirred for 45 min at RT under an Ar atmosphere. The reaction was quenched with AcOH (3 drops). After being dried, the residue was purified by pTLC ($CHCl_3$/MeOH 10:1) to give **24** (11.1 mg, 18.0 μmol 47% in two steps). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.04 (d, J = 8.5 Hz, 2H), 6.65 (d, J = 8.5 Hz, 2H), 4.53 (d, J = 8.5 Hz, 1H), 4.51 (t, J = 6.0 Hz, 1H), 3.92 (dd, J = 10.5, 8.5 Hz, 1H), 3.75 (s, 2H), 3.72 (d, J = 3.0 Hz, 1H), 3.67–3.63 (m, 3H), 3.22 (q, J = 8.5 Hz, 1H), 2.97 (dd, J = 14.0, 6.0 Hz, 1H), 2.90 (dd, J = 14.0, 6.0 Hz, 1H), 1.33 ppm (s, 9H); ^{13}C NMR (125 MHz, CD_3OD): δ = 172.3, 171.8, 157.1, 132.0, 128.4, 115.8, 101.0, 82.9, 77.7, 76.2, 72.7, 70.2, 54.8, 52.5, 39.3, 28.3, −1.21 ppm; IR (film): $\tilde{\nu}$ = 3319, 2979, 2931, 2100, 1720, 1656, 1517, 1369, 1250, 1154, 1114, 1073, 757 cm^{-1}; $[\alpha]_D^{27}$ = −24.4 (c = 0.10 in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{21}H_{29}IN_4NaO_8$: 615.0928 $[M+Na]^+$; found: 615.0935.

*Compound **21***

TFA (1 mL) was added to compound **24** (5.3 mg, 8.95 μmol). After stirring for 5 min at RT under an Ar atmosphere, the reaction mixture was dried in vacuo. The residue was purified by HPLC (25% CH_3CN aq. containing 0.1% TFA) to give **21** (4.6 mg, 8.2 μmol 96%). ^{1}H NMR (500 MHz, CD_3OD): δ = 7.05 (d, J = 8.5 Hz, 2H), 6.64 (d, J = 8.5 Hz, 2H), 4.58 (t, J = 6.0 Hz, 1H), 4.54 (d, J = 8.0 Hz, 1H), 3.91 (dd, J = 11.0, 8.0 Hz, 1H), 3.71 (d, J = 3.0 Hz, 1H), 3.70 (s, 2H), 3.64 (m, 3H), 3.23 (q, J = 8.5 Hz, 1H), 2.99 (dd, J = 14.5, 6.0 Hz, 1H), 2.96 ppm (dd, J = 14.5, 6.0 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): δ = 181.2, 174.9, 157.1, 131.9, 128.6, 115.9, 101.2, 77.8, 76.1, 72.9, 70.2, 54.9, 52.5, 39.1, −1.41 ppm; IR (film): $\tilde{\nu}$ = 3290, 2924, 2358, 2342, 2105, 1733, 1653, 1558, 1541, 1517, 1457, 1122, 1069, 470, 456, 426 cm^{-1}; $[\alpha]_D^{27}$ = −12.7 (c = 0.10 in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{17}H_{21}IN_4NaO_8$: 559.0302 $[M+Na]^+$; found: 559.0317.

*Compound **26***

K_2CO_3 (0.8 mg, 5.93 μmol) and MeI (3.7 mL, 59.3 μmol) were added to a DMF (0.6 mL) solution of **25**[15] (3.5 mg, 5.93 μmol). After the mixture was stirred for 4 h at RT under an Ar atmosphere, the reaction was quenched by the addition of water (10 mL). After adding EtOAc

(10 mL), the organic layer was extracted and dried over anhydrous Na_2SO_4. After filtration, the filtrate was evaporated. The residue was purified by pTLC ($CHCl_3$/MeOH 10:1) to give **26** (2.0 mg, 3.2 μmol, 56%). ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.68$ (d, $J = 9.0$ Hz, 2H), 6.81 (d, $J = 9.0$ Hz, 2H), 6.74 (s, 1H), 5.26 (d, $J = 8.5$ Hz, 1H), 4.13 (dd, $J = 11.0$, 8.5 Hz, 1H), 3.72 (s, 3H), 3.69 (s, 2H), 3.65 (dd, $J = 3.5$, 1.0 Hz, 1H), 3.58 (dd, $J = 11.0$, 3.5 Hz, 1H), 3.51 (ddd, $J = 8.5$, 4.0, 1.0 Hz, 1H), 3.42 (dd, $J = 13.0$, 8.5 Hz, 1H), 3.14 (dd, $J = 13.0$, 4.0 Hz, 1H), 1.47 ppm (s, 9H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 172.0$, 164.8, 161.6, 141.0, 133.5, 127.4, 125.2, 114.8, 100.5, 82.9, 76.3, 73.0, 70.1, 55.7, 54.8, 52.5, 28.5, −1.67 ppm; IR (film): $\tilde{\nu} = 3309$, 3084, 2931, 2102, 1704, 1604, 1551, 1510, 1369, 1320, 1303, 1254, 1159, 1119, 1074, 1034, 757 cm^{-1}; $[\alpha]_D^{27} = -60.7$ ($c = 0.10$ in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{22}H_{29}IN_4NaO_8$: 627.0928 $[M+Na]^+$; found: 627.0951.

*Compound **22***

TFA (1 mL) was added to compound **26** (2.5 mg, 4.1 μmol). After stirring for 5 min at RT under an Ar atmosphere, the reaction mixture was dried in vacuo. The residue was purified by HPLC (35% CH_3CN aq. containing 0.1% TFA) to give **25** (1.8 mg, 3.1 μmol, 80%). ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.81$ (d, $J = 9.0$ Hz, 2H), 7.00 (s, 1H), 6.90 ($J = 9.0$ Hz, 2H), 5.30 (d, $J = 8.5$ Hz, 1H), 4.22 (dd, $J = 10.5$, 8.5 Hz, 1H), 3.81 (s, 3H), 3.77 (s, 2H), 3.74 (dd, $J = 3.5$, 1.0 Hz, 1H), 3.66 (dd, $J = 10.5$, 3.5 Hz, 1H), 3.61 (ddd, $J = 8.0$, 4.5, 1.0 Hz, 1H), 3.49 (dd, $J = 13.0$, 8.0 Hz, 1H), 3.23 ppm (dd, $J = 13.0$, 4.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD): $\delta = 172.1$, 167.4, 161.8, 140.5, 133.7, 127.3, 126.6, 114.7, 101.1, 76.2, 73.3, 70.0, 55.7, 55.0, 52.3, −1.70 ppm; IR (film): $\tilde{\nu} = 3309$, 2925, 2854, 2104, 1700, 1604, 1558, 1511, 1458, 1424, 1303, 1254, 1175, 1121, 1069, 1030, 883, 831 cm^{-1}; $[\alpha]_D^{27} = -41.4$ ($c = 0.10$ in MeOH); HRMS (ESI, positive): m/z: calcd for $C_{18}H_{21}IN_4NaO_8$: 571.0302 $[M+Na]^+$; found: 571.0327.

Plant Materials and Bioassay

C. obtusifolia and *C. mimosoides* were grown in a Biotron LPH-1000S chamber (Nippon Medical & Chemical Instruments) under a condition of 16 h light from 5:00 to 21:00 at 32±2°C with 80% humidity and 150 μmol m^{-2}s PAR/8 h dark from 21:00 to 5:00 at 22±2°C with 80% humidity.

Eight- to 12-week-old *C. obtusifolia* leaves were used for the bioassay. All of the procedures were carried out in a Biotron chamber because the results were strongly affected by changes in temperature, climate, and other environmental factors. The leaves were detached from the stem with a sharp razor blade in water around 16:00 h. One leaf was placed in a 5 mL glass tube with H_2O and allowed to stand overnight. The leaves, which opened again in the morning (around 9:30 h), were then used for the bioassay. Each test solution was transferred into test tubes with a micropipette around 10:00 h. The LOF activity was determined as the concentration at which over 40% of sample leaves had opened at 21:00 h.

On the other hand, leaves of *L. japonicus* were bioassayed according to the procedures in references [33]. The bioactivity of a fraction was assessed by the status of leaves at 21:00 h. Potassium isolespedezate (**1**; Table 1) was not effective in this assay even at 1×10^{-4} (0/4) or 3×10^{-4} mol L^{-1} (0/4), whereas potassium (*R*)-eucomate,[33] a LOF of *L. japonicus*, was effective at 1×10^{-5} mol L^{-1} (5/10).

Table 1. Leaf-opening activities of naturally occurring **1** and CMPs (**2**–**4**, **21**, and **22**).

Compounds	Number of opened leaves at 21:00/sample leaves	Percentage of LOF activity
blank	0/24	0
1 (1×10^{-5} mol L^{-1})	13/15	87
1 (5×10^{-6} mol L^{-1})	4/10	40
2 (1×10^{-5} mol L^{-1})	8/10	80
3 (1×10^{-5} mol L^{-1})	4/10	40
4 (1×10^{-5} mol L^{-1})	5/10	50
21 (1×10^{-3} mol L^{-1})	0/5	0
22 (1×10^{-3} mol L^{-1})	0/5	0

Preparation of Protoplasts

Protoplasts were isolated from primary pulvini of eight- to 12-week-old *C. obtusifolia* seedlings according to the method of Gorton and Satter[15] with modifications. About 30 pulvini were chopped finely with a sharp razor blade and soaked in a predigestion solution (1 mL, 50 mM MES (pH 5.5), 0.3 M sorbitol, 0.2% bovine serum albumin (BSA), 8 mM $CaCl_2$, Gamborg's B-5) for 10 min. The osmotic pressure of the predigestion solution was then raised to the desired level (0.6 M sorbitol) for plasmolysis in three steps over 30 min with osmotic adjustment solution (50 mM MES (pH 5.5), 4.0 M sorbitol, 0.2% BSA, 8 mM $CaCl_2$, Gamborg's B-5). The solution was replaced with an enzyme solution (2 mL, 50 mM MES (pH 5.5) buffer containing 0.6 M sorbitol, 0.3% (w/v) Macerozyme R-10, and 1% (w/v) cellulase Onozuka RS). This suspension containing the protoplasts was placed under reduced atmospheric pressure for 2 min. Protoplasts were released by incubation of the tissue in the solution with gentle incubation at 50 rpm for 3 h at 30°C. Debris was removed by filtration of the protoplast suspension through a 50 μm nylon mesh. Protoplasts in the filtrate were sedimented at $110\times g$ for 7 min and the supernatant was discarded. The protoplasts were re-suspended in HEPES buffer (4 mL, 25 mM HEPES-KOH (pH 7.0), 0.6 M sorbitol, 1 tablet/500 mL complete protease inhibitor cocktail (Roche, 1 tablet/500 mL) was added and the mixture was sedimented again at $100\times g$ for 7 min. This washing procedure was repeated three times. The yield of protoplasts was 4×10^5.

FLAG-Tagging of CTPL by using Protoplasts of C. occidentalis Motor Cells

Each sample (6×10^{-10} mol of **2**, 6×10^{-10} mol of **2** with 6×10^{-8} mol of **1**, 6×10^{-10} mol of **3**, 6×10^{-10} mol of **4**) was added to a suspension of protoplasts (about 5×10^4 protoplasts in 20 μL of wash solution (25 mM HEPES-KOH (pH 7), complete protease inhibitor cocktail (1 tablet/500 mL), 0.6 M sorbitol)). Biologically inactive analogues (**21**, **22**: 6×10^{-10} mol) were also used instead of **2**, **3**, and **4** in control experiments with a suspension of protoplasts (about 3×10^4). Cross-linking with CTPL was conducted at 4°C as follows: cross-linking by using iodoacetoamide-type probes (**2**) was achieved by incubation for 5 min, whereas photo-cross-linking by using photoaffinity-type probes (**3** and **4**) was achieved by UV irradiation (365 nm, 1820 μW cm^{-2}) at a distance of 5 cm from UV lamp for 30 min with ice cooling. After cross-linking, the protoplasts were sedimented three times by centrifugation ($100\times g$, 7 min, 4°C) with wash solution (100 μL) to remove excess unreacted probe. The sediment was resuspended in 25 mM HEPES buffer (pH 7) (6 μL); FLAG unit **9** (1×10^{-9} mol) in 25 mM HEPES buffer (2 μL, pH 8) was added to this suspension. The CuAAC reaction was started by the addition of 25 mM HEPES buffer (2 μL, pH 8), 5% DMSO solution containing ligand **12** (1×10^{-8} mol), and $[Cu(CH_3CN)_4]PF_6$ (1×10^{-8} mol, Aldrich). After incubation for 1 h at 30°C, extraction buffer (40 μL, 25 mM Tris-MES buffer (pH 7.2) containing 0.25 M sucrose, 3 mM EDTA-2K, 2.5 mM DTT, and complete protease inhibitor cocktail (1 tablet/μL)) was added to this solution and the protoplasts were crushed by ultrasonification (DU-200 with UR-20P, TOMY Industry). Centrifugation of the lysate twice (1st: $3{,}000\times g$, 15 min, 4°C; 2nd: $100\,000\times g$, 1 h, 4°C) gave a crude cytosolic homogenate as the supernatant (this fraction was designated crude CTPL) with a crude membrane fraction as the pellet. After lyophilization, electrophoresis buffer (15 μL, 0.3 M Tris-HCl buffer (pH 6.8) containing 10% sodium dodecyl sulfate (SDS), 30% glycerol, and 9.3% dithiothreitol (DTT) was added to each fraction and each solution was heated at 95°C for 5 min. The reaction mixtures were analyzed by SDS-PAGE on Ready Gel J 5–10% polyacrylamide gradient gels (Bio-Rad Laboratories) with a molecular weight marker. After western blotting by using Hybond-P PVDF membrane (GE Healthcare), the membrane was exposed to a rabbit anti-FLAG antibody (Delta Biolabs) or an anti-AtMetE polyclonal antibody raised against bacterially expressed *L. japonicus* MetE. Subsequently, the membrane was exposed to goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology). These procedures were carried out by using a BenchPro TM4100 (Invitrogen). The protein bands were detected by chemiluminescence by using an ECL Advance west-

ern blotting detection kit (GE Healthcare UK) with an LAS-4000 IR multicolor Image Analyzer (Fujifilm Corp.).

Immunoprecipitation of CTPL by using Protoplasts or Cytosolic Fractions

FLAG tagging by using protoplasts and subsequent immunoprecipitation were carried out by probe **2**. After FLAG-tagging by **2** and subsequent ultracentrifugation (10000×*g*, 15 min, 4°C), the cytosolic fraction (50 μL) obtained as a supernatant was mixed with four volumes of acetone and allowed to stand for 1 h at −80°C. After centrifugation (10000×*g*, 15 min, 4°C), the pellet was dissolved in lysis buffer (100 μL, 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100). The solution was incubated with anti-FLAG antibody-connected beads (FLAG M Purification Kit, Sigma–Aldrich) for 12 h at 4°C. After centrifugation (5,000×*g*, 4°C, 30 sec), the supernatant was lyophilized, whereas the precipitated affinity beads were resuspended in wash buffer (100 μL, 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl) and centrifuged (5,000×*g*, 4°C, 30 s). This washing process was repeated five times. The precipitated affinity beads were suspended in wash buffer, and FLAG-tagged CTPL was eluted from the affinity beads by incubation with 10 μg of 3×FLAG (Sigma) at RT for 10 min. After centrifugation (5,000×*g*, 4°C, 30 sec), the supernatant was lyophilized to give purified CTPL. The purified CTPL was analyzed by using an ECL Advance western blotting detection kit (GE Healthcare UK) or a silver staining kit (Wako Pure Chemical Industries). The amount of purified CTPL and the ratio of supernatant to precipitate in Figure 4 were obtained by using an LAS-4000 Bioimager (Fujifilm). The amount of purified CTPL was estimated from a comparison of the CTPL band with a standard protein on silver staining.

Immunoprecipitation by probes **14**–**20** was carried out by using the cytosolic fraction instead of living protoplasts. Protoplasts (1×10^4) in extraction buffer (50 μL, 25 mM Tris-MES (pH 7.2), 0.25 M sucrose, 3 mM EDTA-2K, 2.5 mM DTT, 1 complete tablet/50 mL) were crushed by ultrasonification (DU-200 with UR-20P, TOMY Industry). The solution was centrifuged twice (1st: 3,000×*g*, 15 min, 4°C, 2nd: 100000×*g*, 1 h, 4°C), and each probe (**14**–**20**, 5×10^{-11} mol each) was added to the cytosolic fraction obtained as a supernatant. After cross-linking by incubation for 1 h at 30°C, the FLAG-tagged cytosolic fraction was mixed with four volumes of acetone and allowed to stand for 1 h at −80°C, then treated according to the above procedure. The yield of immunoprecipitated CTPL was calculated by comparing the density of bands detected at 83 kDa: the cytosolic fraction containing CMP-labeled CTPL was applied to IP procedure without CuAAC reaction and the density of 83 kDa band detected in supernatant was defined as control (100%). In this case, no 83 kDa band was detected in the IP fraction. On the other hand, the cytosolic fraction containing CMP-labeled CTPL was applied to the IP after the CuAAC reaction by using **9**, and the density of 83 kDa bands in both of the IP fractions and supernatant were compared with the control.

Microsequencing of CTPL

Immunoprecipitation by using probe **20** was run at a 10-fold scale by using 1×10^5 protoplasts. The resulting supernatant and eluent from the affinity beads were lyophilized and dissolved in 20 μL of sample buffer each. An aliquot of 2 μL each was separated by SDS-PAGE followed by chemiluminescence detection and the remaining 18 μL was separated by SDS-PAGE with silver-staining detection (1 ng×5 lanes). Protein bands detected by silver staining with a Silver Stain MS Kit (Wako Pure Chemical Industries) were excised from the gel, destained, and in-gel digested with trypsin at 35°C for 20 h. The peptide fragments were analyzed on a Q-Tof2 positive mode nanoflow-LC ESIMS (Waters Micromass, UK, capillary voltage: 1.8 kV, collision energy: 20–56 eV) equipped with an L-column ODS (ϕ 0.1×50 mm). The linear gradient conditions were: 95% A:5% B to 45% A:55% B over 0–35 min (solvent A: 2% CH_3CN aq. containing 0.1% HCOOH, solvent B: 90% CH_3CN aq. containing 0.1% HCOOH).

Preparation of Protoplasts from C. mimosoides and L. japonicus

Protoplasts were isolated from primary pulvini of eight- to 12-week-old *C. mimosoides* and *L. japonicus* seedlings according to the method of Gorton and Satter[21] with modifications.

About 120 of *C. mimosoides* pulvini or 200 of *L. japonicus* pulvini were chopped finely with a sharp razor blade and soaked in a predigestion solution (1 mL, 50 mM MES (pH 5.5), 0.3 M sorbitol, 0.2% BSA, 8 mM $CaCl_2$, Gamborg's B-5) for 10 min. The osmotic pressure of the predigestion solution was then raised to the desired level (0.6 M sorbitol) for plasmolysis in three steps over 30 min with osmotic adjustment solution (50 mM MES (pH 5.5), 4.0 M sorbitol, 0.2% BSA, 8 mM $CaCl_2$, Gamborg's B-5). The solution was replaced by an enzyme solution (2 mL, 50 mM MES (pH 5.5) buffer containing 0.6 M sorbitol, 0.3% (w/v) Macerozyme R-10, and 1% (w/v) cellulase Onozuka RS). This solution was placed under reduced atmospheric pressure for 2 min. Protoplasts were released by incubation of the tissue in the solution with gentle incubation at 50 rpm for 3 h at 30°C. Debris was removed by filtration of the protoplast suspension through a 50 μm nylon mesh. Protoplasts in the filtrate were sedimented at 100×*g* for 7 min and the supernatant was discarded. The protoplasts were resuspended in HEPES buffer (4 mL, 25 mM HEPES-KOH (pH 7.0), 0.6 M sorbitol, 1 complete tablet/500 mL), and sedimented again at 100×*g* for 7 min. This washing procedure was repeated three times. The yield of protoplasts was 4×10^4 from 120 *C. mimosoides* pulvini or 200 *L. japonicus* pulvini.

FLAG tagging of CTPL by using Motor Cells of C. Mimosoides or L. Japonicus

Iodoacetamide probe **2** (6×10^{-10} mol) was added to a suspension of *C. obtusifolia*, *C. mimosoides*, or *L. japonicus* protoplasts (about 1×10^4) in wash solution (20 μL, 25 mM HEPES-KOH (pH 7), complete protease inhibitor cocktail (1 tablet/500 mL), 0.6 M sorbitol). The remainder of the procedure was carried out as for *C. obtusifolia* cells.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from MEXT (no. 20310128). We also thank the Experimental Station for Medicinal Plant Studies, Tohoku University, for cultivation of plants, and Mr. S. Park for carrying out bioassays by using *L. japonicus*.

[1] a) N. Jessani, B. F. Cravatt, *Curr. Opin. Chem. Biol.* **2004**, *8*, 54; b) M. J. Evans, B. F. Cravatt, *Chem. Rev.* **2006**, *106*, 3279–3301; c) U. Hillaert, M. Verdoes, B. I. Florea, A. Saragliadis, K. L. L. Habets, J. Kuiper, S. V. Calenbergh, F. Ossendrop, G. A. van der Marel, C. Driessen, H. S. Overkleeft, *Angew. Chem.* **2009**, *121*, 1657–1660; *Angew. Chem. Int. Ed.* **2009**, *48*, 1629–1632.

[2] H. Shigemori, E. Miyoshi, Y. Shizuri, S. Yamamura, *Tetrahedron Lett.* **1989**, *30*, 6389–6392.

[3] T. Ohnuki, M. Ueda, S. Yamamura, *Tetrahedron* **1998**, *54*, 12173–12184.

[4] T. Sugimoto, Y. Wada, S. Yamamura, M. Ueda, *Tetrahedron* **2001**, *57*, 9817–9825.

[5] T. Fujii, Y. Manabe, T. Sugimoto, M. Ueda, *Tetrahedron* **2005**, *61*, 7874–7893.

[6] B. J. Leslie, P. J. Hergenrother, *Chem. Soc. Rev.* **2008**, *37*, 1347–1360.

[7] a) M. W. Harding, A. Galat, D. E. Uehling, S. L. Schreiber, *Nature* **1989**, *341*, 758–760; b) J. Taunton, C. A. Hassig, S. L. Schreiber, *Science* **1996**, *272*, 408–411.

[8] a) L. Burdine, T. Kodadek, *Chem. Biol.* **2004**, *11*, 593–597; b) S. Sato, A. Murata, T. Shirakawa, M. Uesugi, *Chem. Biol.* **2010**, *17*, 616–623.

[9] a) J. L. Erion, J. E. Fox, *Plant Physiol.* **1981**, *67*, 156–162; b) S. Sakai, T. Hanagata, *Plant Cell Physiol.* **1983**, *24*, 685–693; c) S. Sakai, *Plant Cell Physiol.* **1985**, *26*, 185–192.
[10] Y. Matsubayashi, Y. Sakagami, *J. Biol. Chem.* **2000**, *275*, 15520–15525.
[11] Y. Yamaguchi, G. Pearce, C. A. Ryan, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 10104–10109.
[12] J. M. Scheer, G. Pearce, C. A. Ryan, *Planta* **2005**, *221*, 667–674.
[13] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.
[14] L. Ballell, K. J. Alink, M. Slijper, C. Versluis, R. M. Liskamp, R. J. Pieters, *ChemBioChem* **2005**, *6*, 291–295.
[15] Y. Manabe, M. Mukai, S. Ito, N. Kato, M. Ueda, *Chem. Commun.* **2010**, *46*, 469–471; all of the physical data on **3**–**5** and **6** were presented in the Supporting Information of this paper.
[16] a) H. Shigemori, N. Sakai, E. Miyoshi, Y. Shizuri, S. Yamamura, *Tetrahedron* **1990**, *46*, 383–394; b) M. Ueda, Y. Sawai, S. Yamamura, *Tetrahedron* **1999**, *55*, 10925–10936.
[17] C. Huggins, E. V. Jensen, *J. Biol. Chem.* **1949**, *179*, 645–654.
[18] a) R. E. Galardy, L. C. Craig, J. D. Jamieson, M. P. Printz, *J. Biol. Chem.* **1974**, *249*, 3510–3518; b) G. Dorman, G. D. Prestwich, *Biochemistry* **1994**, *33*, 5661–5673.
[19] Y. Hatanaka, M. Hashimoto, H. Kurihara, H. Nakayama, Y. Kanaoka, *J. Org. Chem.* **1994**, *59*, 383–395.
[20] A. Einhauer, A. Jungbauer, *J. Biochem. Biophys. Methods* **2001**, *49*, 455–465.
[21] a) H. Gorton, R. L. Satter, *Plant Physiol.* **1984**, *76*, 680–684; b) N. Moran, D. Fox, R. L. Satter, *Plant Physiol.* **1990**, *94*, 424–431.
[22] A. Saghatelian, B. F. Cravatt, *Nat. Chem. Biol.* **2005**, *1*, 130–142.
[23] M. Hashimoto, Y. Hatanaka, *Eur. J. Org. Chem.* **2008**, 2513–2523.
[24] M. Ueda, Y. Manabe, M. Mukai, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 1359–1362.
[25] a) S. Ravanel, B. Gakiere, D. Job, R. Douce, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7805–7812; b) http://ca.expasy.org/cgi-bin/niceprot.pl?B9RQ33.
[26] S. Ravanel, M. A. Block, P. Rippert, S. Jabrin, G. Curien, F. Rebeille, R. Dounce, *J. Biol. Chem.* **2004**, *279*, 22548–22557.
[27] R. Pejchal, M. L. Ludwig, *PLoS Biol.* **2005**, *2*, 254–265.
[28] R. P. Sanderson, A. H. de Boer, T. L. Lomax, R. E. Cleland, *Plant Physiol.* **1987**, *85*, 693.
[29] M. Ueda, H. Shigemori, N. Sata, S. Yamamura, *Phytochemistry* **2000**, *53*, 39–44.
[30] M. J. Waring, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2844–2851.
[31] a) I. V. Tetko, G. I. Poda, *J. Med. Chem.* **2004**, *47*, 5601–5604; b) I. V. Tetko, P. Bruneau, *J. Pharm. Sci.* **2004**, *93*, 3103–3110.
[32] a) Y. Nakamura, A. Mithöfer, E. Kombrink, W. Boland, S. Hamamoto, N. Uozumi, K. Tohma, M. Ueda, *Plant Physiol.* **2011**, *155*, in press; b) Y. Nakamura, R. Miyatake, M. Ueda, *Angew. Chem.* **2008**, *120*, 7399–7402; *Angew. Chem. Int. Ed.* **2008**, *47*, 7289–7292.
[33] M. Okada, S. Park, T. Koshizawa, M. Ueda, *Tetrahedron* **2009**, *65*, 2136–2141.
[34] H. Nakashima, M. Hashimoto, Y. Sadakane, T. Tomohiro, Y. Hatanaka, *J. Am. Chem. Soc.* **2006**, *128*, 15092–15093.
[35] I. Schechter, E. Clerrici, E. Zazepitzki, *Eur. J. Biochem.* **1971**, *18*, 561–572.
[36] Y. Nakamura, S. Inomata, M. Ebine, Y. Manabe, I. Iwakura, M. Ueda, *Org. Biomol. Chem.* **2011**, *9*, 83–85.

Received: April 18, 2011
Published online: August 3, 2011

