Strigolactone perception and deactivation by a hydrolase receptor DWARF14

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The perception mechanism for the strigolactone (SL) class of plant hormones has been a subject of debate because their receptor, DWARF14 (D14), is an $\alpha/\beta$-hydrolase that can cleave SLs. Here we show via time-course analyses of SL binding and hydrolysis by Arabidopsis thaliana D14, that the level of uncleaved SL strongly correlates with the induction of the active signaling state. In addition, we show that an AtD14D218A catalytic mutant that lacks enzymatic activity is still able to complement the atd14 mutant phenotype in an SL-dependent manner. We conclude that the intact SL molecules trigger the D14 active signaling state, and we also describe that D14 deactivates bioactive SLs by the hydrolytic degradation after signal transmission. Together, these results reveal that D14 is a dual-functional receptor, responsible for both the perception and deactivation of bioactive SLs.
Trigolactones (SLs) were originally characterized as root-derived signals for parasitic and symbiotic interactions, yet are now known as endogenous plant hormones that control shoot branching and diverse aspects of plant growth. Canonical SLs have a four-ring structure (Fig. 1a, b). The SL receptor, DWARF14 (D14), was initially characterized from a rice SL-insensitive mutant, d14, and orthologues have since been identified from Arabidopsis, Oryza sativa, and others. The signaling mechanism, in particular the chemical signal that induces the active signaling intermediate. We also demonstrate that D14 can deactivate SLs by hydrolytic degradation after signal transmission.

**Results**

**Structural requirements for the D14-SL interaction**. In order to understand the relationship between the hydrolysis reaction and the signal transducing role of D14, we first comprehensively examined the structural requirements for the D14-SL interaction using various naturally occurring SLs and synthetic analogs by hydrolysis assays and differential scanning fluorimetry (DSF) experiments, which can evaluate protein-chemicals interactions by monitoring protein melting temperature (Tm) shifts induced upon exposure to chemicals. The hydrolysis kinetics of the AtD14-catalyzed hydrolysis reaction were measured using the naturally occurring single isomer of 5-deoxyxystrogol (5DS) (Fig. 1a). We detected two previously reported hydrolysis products, ABC-FTL and HMB, generated in a catalytic triad Ser-dependent manner (Supplementary Fig. 1a, b). Among the stereoisomers of SLs, (2′R)-isomers have a demonstrably greater effect on the inhibition of shoot branching than the (2′S)-isomers in both Arabidopsis and rice (Supplementary Fig. 2a). We observed that AtD14 hydrolyzes the (2′R)-isomers more efficiently than the (2′S)-isomers across all tested SLs (Supplementary Fig. 2b). OsD14 also preferred (2′R)-isomers of 5DS (Supplementary Fig. 2c). A biologically inactive analog, 3,6′-dihydroGR24, was not hydrolyzed by AtD14/OsD14 (Supplementary Fig. 2d). Debranones, such as Br-PMF and CN-PMF, are a class of non-enol ether-type SL analogs (Supplementary Fig. 2e). They were reported to inhibit shoot branching in an AtD14/OsD14- and MAX2/D3-dependent manner. We quantitatively examined the hydrolyzability of Br-PMF and CN-PMF by AtD14, which demonstrated that both of them are significantly poorer substrates when compared with GR24 (racemic mixture, Supplementary Fig. 2e). The low catalytic activity of AtD14 for debranones, coupled with the observations that debranones induce a similar signaling response to GR24 in planta, raises the question of whether SL hydrolysis is required for D14-mediated SL signaling.

We next performed DSF experiments using various SLs and analogs. Previously, bioactive SLs were found to lower the Tm of D14, including GR24 and a newly found endogenous SL-like molecule called methyl carlactonoate (MeCLA), whereas the SL biosynthetic precursors such as carlactone (CL) or its carboxylated derivative, carlactonoic acid (CLA), were not able to induce clear temperature shift of AtD14 (Fig. 1a)\(^6\). An AB-ring truncated analog, GR5 (Supplementary Fig. 2a), was reported to inhibit shoot branching, and the (2′R)-isomer, (+)-GR5, showed much stronger activity than the (2′S)-isomer in shoot branching inhibition\(^22\). Here we found that only (+)-GR5, but not (−)-GR5, induces a clear melting temperature shift similar to 5DS (Fig. 2a, Supplementary Fig. 2f). By comparison, the biologically inactive analogs, such as ABC-FTL, HMB, and 3,6′-dihydroGR24, were insufficient to induce the same Tm shifts (Fig. 2a). Debranones were also reported to induce a Tm shift for OsD14/DAD2\(^26\), and here we show that CN-PMF clearly induces a Tm shift for AtD14/OsD14 similar to 5DS, despite the fact that CN-PMF is less hydrolyzable (Fig. 2a and Supplementary Fig. 2f). Taken together with the hydrolysis assays, these data conclusively demonstrate that the Tm shift of D14 directly correlates with biological activity of the SL-related compounds, suggesting that the temperature shifts reflect the induction of an active signaling state of D14, possibly related to the conformational changes seen in the...
AtD14-D3-ASK1 complex structure. In addition, a previous report demonstrated that the biologically active SL-related compounds could specifically promote OsD14-D3 interaction. Given that the bioactive SL-dependent Tm shift observed in our DSF experiments, thermal destabilization of OsD14 is likely correlated with D3 binding.

**Fig. 1** Chemical structures of SL-related compounds and a scheme for their biosynthesis and signaling pathways. a Structures of SL-related compounds. b The scheme for the SL biosynthesis and signaling pathways. Red, blue, orange, and green characters indicate genes of Arabidopsis, rice, petunia, and pea, respectively. Black arrows indicate the biosynthetic steps, and a white arrow indicates the signaling step. (CCD; carotenoid cleavage dioxygenase)

**Fig. 2** Evaluation of the AtD14-SL interaction using hydrolysis and DSF assays. a Melting temperature curves of AtD14 in the presence of various SLs and analogs. The names in red and blue denote biologically active and inactive (or weakly active) compounds, respectively. b and d, Monitoring by LC-MS/MS of the AtD14 hydrolysis reaction of GR24 (b) and CN-PMF (d). HBN; Hydroxybenzenenitrile. Data are the means ± SD (n = 3). c and e Melting temperature curves of AtD14 pre-incubated with GR24 (c) and CN-PMF (e) for indicated time period. Source data are provided as a Source Data file

Time course analysis of D14 activation and SL hydrolysis. In order to determine when the active state of D14 is triggered relative to the timing of SL hydrolysis, we designed a time-course DSF experiment coupled with quantitative detection of GR24 and its hydrolysis products. RMS3 was proposed to be a single turnover enzyme with a pro-fluorescent analog, GC24. The authors

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-018-08124-7 | www.nature.com/naturecommunications
suggested the hydrolyzed D-ring was trapped through a covalent bond with the catalytic triad His, and this might be the reason for the single turnover reaction. However, we observed that all of the GR24 was consumed when the reaction was performed at a 1:6 molar ratio of AtD14:GR24 (Fig. 2b). This clearly demonstrates that AtD14 is not a single turnover enzyme with GR24. Interestingly, we further observed that ABC-FTL and HMB were released at almost the same rate that GR24 was consumed (Fig. 2b). Generally, the hydrolysis reaction catalyzed by this protein family is a two-step process. First, the activated hydroxyl group of the catalytic triad Ser acts as a nucleophile attacking to possibly the D-ring part of SLs to release the first product, ABC-FTL. At the same time, the protein forms a covalently-linked reaction intermediate with the D-ring part through the Ser residue of D14. Then, if we apply a conventional reaction model of this protein family, the activated water molecule triggers the second attack to the D14/D-ring complex to cleave the covalent bond, and HMB is released as the second product. On the other hand, according to two recent reports, the D-ring part is likely transferred to the catalytic triad residue before its release as HMB, which was speculated to be significantly slower. However, our observation makes it unlikely that the D-ring derived product gets covalently trapped within the protein for a significant amount of time because the HMB release was detected at almost same turnover as the ABC-FTL release, and suggests that the second attack from a water molecule must occur immediately after the first attack from the catalytic Ser. Even if the covalently linked modification of His takes place, it must be released quickly as a product. Thus, it is likely that the first attack is rate limiting, which may cause slow catalysis.

We then performed the DSF experiments simultaneously with hydrolysis monitoring in order to observe the correlation between the degree of hydrolysis and the Tm shift. Strikingly, the maximum Tm shift was detected upon initial incubation with SL (0 min), before gradually returning to the control Tm over the course of the hydrolysis reaction (Fig. 2c), such that the GR24 consumption curve was highly correlated with the degree of the Tm shift (Supplementary Fig. 3a, b). These results strongly suggest that the signal that induces the Tm shift of AtD14 is GR24, not the hydrolysis intermediate or products, suggesting that the active state of AtD14 is triggered upon GR24 binding, prior to its hydrolysis.

When GR24 was used as a substrate, we found that the background hydrolysis reaction was ongoing during DSF detection (Supplementary Fig. 3c). To observe the correlation between the hydrolysis reaction and the temperature shift more precisely, we used a less-hydrolyzable analog, CN-PMF. As expected, CN-PMF was much more stable than GR24 during DSF detection (Supplementary Fig. 3d). Although, the effect of CN-PMF on the Tm shift of AtD14 was smaller when compared with GR24, the slight shift persisted for a longer time because the rate of CN-PMF hydrolysis was much slower than GR24 (Supplementary Fig. 3e, f). To monitor the Tm shift more closely, the concentration of CN-PMF was increased and we were able to observe a clear correlation between substrate consumption and the Tm shift that is similar to GR24 (Fig. 2d, e and Supplementary Fig. 3g-i). Taken together, our data suggest that the uncleaved substrate itself, not the hydrolysis intermediate or the products, induces the Tm shift of AtD14. In contrast to reports regarding the role of CLIM in inducing conformational changes of AtD14, our results strongly suggest that the active state of AtD14 is triggered by the intact SL molecule prior to its hydrolytic degradation. Given that the two products were released at almost the same turnover rate, the lifetime of the covalently attached intermediate must be exceedingly short. It is unlikely that such a short-lived intermediate induces the drastic conformational changes necessary to recruit other partner proteins.

To address this question more directly, we analyzed the D-ring modification of AtD14 using LC-MS/MS during hydrolysis. Consistent with the reported data, we also detected a peak whose molecular mass increased by 98 ± 5, suggesting that AtD14 forms a covalently attached intermediate with the D-ring part of SL during the hydrolysis reaction (Supplementary Fig. 4a). This conjugate peak was detected in both the native and denatured samples (Supplementary Fig. 4a). We performed a time-course analysis of this modified AtD14, and found that the intensity of the conjugate peak initially increased from 0 to 15 min, then gradually decreased over-time until 4 h when most of the AtD14 protein existed as unbound form. These results suggest that the D-ring part, which is once bound to AtD14, is released rapidly without being trapped tightly (Supplementary Fig. 4b). Moreover, it is noteworthy that the level of the covalently-linked AtD14 does not correlate with the degree of the Tm shift of AtD14, suggesting that the reaction intermediate is not a chemical that induces the transition of the AtD14 state. Because a small peak for modified AtD14 was still detectable after 4 h when all the substrate, GR24, was consumed, we individually incubated AtD14 with a reaction product, HMB. We found that even HMB alone could induce the formation of modified AtD14 at 400 μM (Supplementary Fig. 4c). Although at a lower concentration (40 μM), the modified AtD14 peak was almost undetectable, HMB produced in the ligand-binding pocket by the hydrolysis reaction possibly forms the conjugate more effectively than the exogenously applied HMB, because HMB was reported to be trapped in the pocket after its release as a product. Thus it would be difficult to distinguish whether this modified protein is a hydrolysis intermediate or a conjugate formed with HMB after the completion of hydrolysis. We analyzed the covalently modified AtD14 formation using CN-PMF as a substrate, and found a tendency similar to the case with GR24 (Supplementary Fig. 4d and e). Notably, a gradual increment of the modified AtD14 peak was more clearly observed in the case of CN-PMF than GR24, which does not correlate with the melting temperature shift. These results again support the idea that the substrate, but not the reaction intermediate, induced the melting temperature shift.

Our time-course DSF experiments demonstrate that the Tm of AtD14 is initially lowered by SL binding before it returns to the unbound state temperature. This result indicates that the SL-inducible dynamics of AtD14 is reversible. To further examine this, we spiked in fresh GR24 to the 4 h pre-incubated sample in which all the initially added substrate was consumed. As a result, the enzyme was still capable of hydrolyzing freshly added GR24, and fresh GR24 could induce the melting temperature shift of pre-incubated AtD14. These results again demonstrate that AtD14 is not a single turnover enzyme with GR24, and that the SL-inducible transition of AtD14 state is reversible depending on the presence of intact SL (Supplementary Fig. 5). Moreover, our results clearly reveal that debranones, which are much less hydrolyzable than GR24, cause the Tm shift to last for a longer time, thus providing an explanation for the potent biological activities of these analogs.

Functional analysis of D14 catalytic triad mutants. To evaluate the necessity of the hydrolytic function of D14, we next prepared four catalytic triad mutants (AtD14597A, AtD14597C, AtD14D218A, and AtD14H247A) and found that the hydrolytic activities of all of these mutants are drastically reduced when compared with a negative control (Fig. 3a). Although the RMS3596C mutant was reported to have slight hydrolysis activity for GC24, the hydrolysis activity for 5DS in the corresponding mutant in Arabidopsis, AtD14597C, was reduced to the same level as other catalytic triad mutants (Fig. 3a). For each
mutant we also observed that all of the substrate stayed intact after the incubation with enzyme, suggesting that the hydrolase reaction gets stuck before the initial nucleophilic attack step (Supplementary Fig. 6a). To investigate the signal transducing function of these mutants, complementation tests were performed by expressing each mutant in the Arabidopsis atd14-2 mutant background under the control of the cauliflower mosaic virus (CaMV) 35 S promoter. The expression of AtD14S97A and AtD14H247A did not complement the atd14 mutant, which corroborates previous reports (Fig. 3b, c, and Supplementary Fig. 6b)\textsuperscript{12,13}. Very interestingly, we found that AtD14=D218A, which has not previously been tested, completely complemented the atd14 mutant phenotype (Fig. 3b, c and Supplementary Fig. 6b). In a previous report, AtD14S97C was unable to complement the atd14 mutant when it was expressed as 6xHis-tag fusion\textsuperscript{12}; however, we observed that the expression of untagged AtD14H247C, partially complemented the atd14 mutant phenotype (Fig. 3b, c and Supplementary Fig. 6b). In addition, we found that AtD14=D218A interacted with SMXL7 in an SL-dependent manner in yeast two hybrid (Y2H) experiments, suggesting that AtD14(D218A) is still capable of signal transduction despite lacking hydrolase activity, while AtD14S97C weakly interacts with SMXL7 in the presence of MeC1A (Fig. 3d and Supplementary Fig. 6c). We also found that AtD14=D218A interacted with another signaling partner, MAX2, as examined by Y3H experiments, in which an SCF complex component, ASK1, was co-expressed without any tag as a third protein (Supplementary Fig. 6d). Interestingly, the SL-dependent interaction between AtD14=D218A and MAX2 was observed only in the presence of ASK1, possibly because ASK1 stabilizes MAX2. The DSF experiments using the catalytic triad mutant proteins revealed that 5DS lowered Tms for AtD14S97C and AtD14=D218A slightly, but did not change the Tms for AtD14S97A or AtD14H247A, consistent with the observation that AtD14S97C and AtD14=D218A retain the capacity for SL signaling in planta (Supplementary Fig. 7a). According to the D14 apo structures, the catalytic triad Ser and His are present at the surface of the active site pocket, whereas the Asp residue does not form part of the pocket surface\textsuperscript{12,15,28,29} (Supplementary Fig. 7b), suggesting that these two residues might be important not only for the catalytic triad formation but also for the direct interaction with the ligand/substrate molecules. Therefore, the mutation to Ser and His possibly affected the initial interaction with SLs. Consistent with this idea, we found that AtD14=D218A and AtD14S97C mutant proteins, which were capable of signal transduction, exhibit higher binding activity with 5DS than other two mutants, AtD14S97A and AtD14H247A (Supplementary Fig. 7c). Because the Tm curve of AtD14=D218A suggested that this protein was unstable, even in the absence of 5DS (Supplementary Fig. 7a), we generated transgenic plants expressing AtD14=D218A in the max4 atd14 double knockout mutant background (max4; CCD8 knockout defective for SL biosynthesis) to examine the SL dependency of this mutant protein function. These transgenic lines exhibited the severe phenotype due to the reduction in bioactive hormone levels as were the cases with other plant hormones deactivating enzymes\textsuperscript{30}. Based on this speculation, we overexpressed OsD14R233H/AtD14R183H in each WT background (Nipponbare/Col-0) under the control of the CaMV 35 S promoter, which resulted in increased branching phenotype (Fig. 4c, d and Supplementary Fig. 11e-g). We also found that the levels of an endogenous SL, 4-deoxoxyroboanchol (4DO), in the rice overexpressors were decreased relative to WT and the empty vector-expressing plants (Fig. 4e). The SL hydrolysis products

Reanalysis of the conformationally altered AtD14 structure. Conformational changes in AtD14, as shown in the complex with D3, were observed around the helical lid domain, through the rearrangement of a-helix structures, and as an open-to-close transition of the V-shaped lid domain\textsuperscript{16}. In addition, the geometry of the catalytic triad is disrupted in this structure due to the reordering of the loop containing the catalytic Asp (D218) residue (Asp loop)\textsuperscript{16}. The observed changes were reported to cause a significant reduction in the size of the active site pocket due to closure of the V-shaped lid (Supplementary Fig. 9a), and thus the authors suggested that the structural changes were triggered by a small molecule such as CLIM. On the other hand, our aforementioned data strongly suggest that the D14 active site is triggered by intact SL molecules. We downloaded the structural data of AtD14-D3-ASK1 complex (PDB code; 5HZG), and reanalyzed the structural changes in AtD14. Intriguingly, we found that the conformational change of the D218-containing loop enlarges the cavity adjacent to the catalytic Ser. The calculated volume of this new pocket, 944 Å\textsuperscript{3}, is larger than that of the original pocket in the apo structure, 863 Å\textsuperscript{3} (Supplementary Fig. 9a). Molecular docking experiments using the structurally altered AtD14 structure showed favorable binding of GR24 and CN-PMF within the newly formed pocket, suggesting that AtD14 is able to accommodate an entire SL molecule after its conformational changes (Supplementary Fig. 9b). These results support the idea that the intact SL molecules, but not the hydrolysis intermediate, induce the conformational changes to D14.

Functional analysis of a new d14 allele. Our data suggests that the hydrolysis function of D14 is not necessary for its role in signal transduction. What then is the physiological role of SL hydrolysis by D14? To address this question, we used a mutant version of D14 characterized from a phenotypic screen for shoot branching in rice. We characterize a new d14 allele, d14-2 (osd14-2 in this paper to distinguish from atd14-2), containing a missense mutation of a highly conserved amino acid, R233H (Fig. 4a, Supplementary Fig. 10). Interestingly, both OsD14R233H and the corresponding mutant in Arabidopsis, AtD14R183H, showed hydrolyase activity similar to each wild type (Fig. 4b). However, AtD14R183H neither complemented the atd14 mutant phenotype (Supplementary Fig. 11a-c), nor interacted with SMXL7 in the presence of SLs (Supplementary Fig. 11d). Thus this mutation significantly influenced the signal transducing role of D14 without affecting its hydrolyase ability, suggesting that these mutant proteins would be a good tool to further investigate the physiological role of SL hydrolysis by D14. As mentioned above, the results with AtD14=D218A mutant implied that the hydrolysis function of D14 is a deactivateing step of bioactive hormone compounds because the transgenic plants expressing this enzymatically inactive mutant in the atd14 max4 double mutant was highly sensitive to exogenously applied SL. If we hypothesize correctly, overexpression of the osd14-2 type mutant protein, which has only the hydrolyase function, should cause the SL-deficient phenotype due to the reduction in bioactive hormone levels as were the cases with other plant hormones deactivating enzymes\textsuperscript{30}. Based on this speculation, we overexpressed OsD14R233H/AtD14R183H in each WT background (Nipponbare/Col-0) under the control of the CaMV 35 S promoter, which resulted in increased branching phenotype (Fig. 4c, d and Supplementary Fig. 11e-g). We also found that the levels of an endogenous SL, 4-deoxyroboanchol (4DO), in the rice overexpressors were decreased relative to WT and the empty vector-expressing plants (Fig. 4e).
were reported to have quite weak or no biological activities in shoot branching inhibition\textsuperscript{12,28}. Moreover, our time-course analysis revealed that the signal is transduced prior to SL hydrolysis. Considering all together, our data support the hypothesis that the hydrolase reaction catalyzed by D14 would be a deactivating step of SLs after transducing the signal.

**Discussion**

We conclude that intact SL molecules induce the active signaling state of D14, and that D14 deactivates bioactive SLs by the hydrolytic degradation after signal transmission. Therefore, our data demonstrate that D14 is a dually functional protein (Fig. 5). Notably, we could successfully separate two functions of D14...
biochemically and genetically just by introduction of single amino acid substitution.

Our data enable us to speculate more detailed mechanisms of signal transduction as illustrated in Fig. 5. When a bioactive SL binds the active site pocket of D14, it induces conformational changes to D14\textsuperscript{16}. Considering the presence of the enlarged pocket observed in the AtD14-D3 protein complex, it seems likely that SL initially induces a conformational change of the loop containing the catalytic triad Asp. After the formation of the enlarged pocket, SL may translocate within this pocket, triggering the open-to-close transition of the helical lid domain. Although there has been no evidence of such detailed events, our time-course DSF experiments together with the catalytic triad mutant analysis demonstrate that the induction of the D14 active state is triggered by an intact SL molecule, not by the hydrolysis intermediate or products. This means that the signaling process does not require the hydrolytic function of D14. In the AtD14-D3-ASK1 complex structure, an intact SL molecule was not observed in this newly formed pocket. It is likely that SL in the AtD14-D3-ASK1 complex crystal was partly hydrolyzed during crystallographic experiments due to a relatively high pH condition\textsuperscript{15}, resulting in poor electron density for the ligand molecules. Or it is also possible that the disordering of the Asp loop induced the detachment of the SL molecule during crystallization or other experimental procedures.

Upon binding of the intact SL, D14 initially adopts a destabilized conformation characteristic of a catalytically inactive state that is due to the disruption of the catalytic triad formation. In this state, the conformationally altered D14 protein interacts with its signaling partners, D3/SMXLs and D3/MAX2, to transmit the signal. As seen in the AtD14-D3-ASK1 complex structure, D3/MAX2 F-box interacts with the protein interacts with its signaling partners, D53/SMXLs and D3/MAX2 F-box, in this newly formed pocket. It is likely that SL in the AtD14-D3-MAX2 F-box pocket observed in the AtD14-D3 protein complex, it seems likely that SL initially binds around the ASK1 complex structure, D3/MAX2 F-box interacts with the protein interacts with its signaling partners, D53/SMXLs and D3/MAX2 F-box, in this newly formed pocket. It is likely that SL in the AtD14-D3-MAX2 F-box pocket, SL may translocate within this pocket, triggering the open-to-close transition of the helical lid domain. Although there has been no evidence of such detailed events, our time-course DSF experiments together with the catalytic triad mutant analysis demonstrate that the induction of the D14 active state is triggered by an intact SL molecule, not by the hydrolysis intermediate or products. This means that the signaling process does not require the hydrolytic function of D14.

Methods

Plant materials and growth conditions. We used rice cultivar (Oryza sativa L. cv. Nipponbare) as the WT. The rice d14-1 mutant was used after backcrossing with Nipponbare 3 times (d14-1 N) for construction of transgenic plants\textsuperscript{19}. The rice osd14-2 was characterized from Sasanishki EMS mutant lines. We used Arabidopsis ecotype Col-0 as the WT, mash-8 (SALK_072750)\textsuperscript{3}, and atd14-2 mutants\textsuperscript{9}. For Arabidopsis phenotype observation, the seeds were directly sown on soil and grown under long day conditions (16 h light/8 h dark) at 22 °C. For shoot growth, the untagged AtD14 recombinant protein was adjusted to be 5 μM (or 10 μM) of substrates in 50 mM Phosphate buffer (pH 7.1) containing 2% acetone. The enzyme reaction was stopped by the addition of 100 μl of acetonitrile, and ABC-FITC, HMB, and remaining substrate was analyzed by LC-MS/MS. For the analysis of hydrolysis activity with 5DS, GR24, orobanchol, and GR7 each corresponding formylactone part was analyzed by using each deuterium labeled standard as an internal standard, and the exact amount of reaction product was calculated. For the analysis of hydrolysis activity with dhGR24, GR5, Br-PMF, and CN-PMF, a common reaction product, HMB was analyzed by LC-MS/MS and its peak area was used for the calculation of relative activities. The kinetic parameters were calculated from Lineweaver-Burk plots. The effect of substrate concentration on reaction velocity was examined at various concentration of SDS (1.25, 2.5, 5, 10, 20 μM). Detailed conditions for the LC-MS/MS analysis are described in Supplementary Table 3.

Chemicals. Br-PMF and CN-PMF were purchased from Chiralix. Other SLs were prepared as part of our previous studies\textsuperscript{22,13}.

Functional expression of AtD14 and OsD14 proteins. The coding sequences for OsD14 and AtD14 were amplified by PCR from cDNA synthesized from total mRNA of the rice and Arabidopsis seedlings, respectively, using the primers described in Supplementary Table 1 (AtD14-F-blunt and AtD14-R EcorI for AtD14, OsD14-F-blunt and OsD14-R EcorI for OsD14). For OsD14, the conserved esterase domain lacking the N-terminal region (residues 1–54) was used. The PCR products were digested by EcoRI and cloned into pET49b (Novagen) and pMALc5x (New England Biology) vector, containing a polyhistidine tag and HRV 3C protease site from PET49b (Novagen), to yield OsD14-pMALHis and AtD14-pMALHis. E.coli Rosetta-gami 2 (Novagen) was used for recombinant protein expression. Overnight cultures (10 ml) were inoculated to fresh LB medium containing 50 μg/ml ampicillin, 30 μg/ml streptomycin, 25 μg/ml tetracyclin, and 34 μg/ml chloramphenicol. After OD\textsubscript{600} reached 0.6–1.0, 0.1 mM IPTG was added and the cells were further incubated at 16 °C for 20 h. The cells were pelleted by centrifugation, then resuspended and sonicated in a lysis buffer (20 mM Tris-HCl (pH 8.0), containing 150 mM NaCl). The supernatants from the resuspending lysates were subjected to MBTTrap column chromatography (5 ml, GE Healthcare). After washing with the lysis buffer, bound proteins were eluted using elution buffer (Lysis buffer containing 10 mM Maltose). To obtain MBP-OsD14 or MBP-AtD14, the eluate was further purified by TALON column chromatography (GE Healthcare). After applying a wash buffer (20 mM Tris (pH 8.0)) containing 300 mM NaCl and 2 mM Imidazole, bound proteins were eluted using an elution buffer (20 mM Tris (pH 8.0) containing 300 mM NaCl and 50 mM Imidazole). The eluate was concentrated using an Amicon Ultra-4 10 K (Millipore) and the concentration of purified protein was adjusted to 5 μg/μL. The recombinant protein solution was divided into aliquots, immediately frozen in liquid nitrogen, and stored at −80 °C until use. To prepare the untagged AtD14 recombinant protein from the MBP Trap column (MBP-AtD14), was treated with HRV 3C protease at 4 °C for 3 h. The resulting sample was diluted with 20 mM Tris (pH 8.0) buffer and subjected to Hit trap Q HP anion exchange column chromatography (GE Healthcare). Absorbed proteins were eluted across a linear gradient of 0–700 mM NaCl (20 mM Tris (pH 8.0)). The fractions containing AtD14 were collected and concentrated using an Amicon Ultra-4 10 K (Millipore). The concentration of recombinant protein was adjusted to be 5 μg/μL and proteins were divided into aliquots, immediately frozen in liquid nitrogen, and stored at −80 °C until use. For AtD14, we used untagged protein in our initial experiments (Supplementary Fig. 1), although we later used MBP fusion protein expression (Supplementary Fig. 2a, and b) for the mutants was carried out as described above.

Hydrolase activity tests of D14. Hydrolase activity tests of AtD14 and OsD14 were carried out at 30 °C for 15 min in 100 μL of a standard reaction buffer that containing 10 μg of recombinant protein, 1 μM (or 10 μM) of substrates in 50 mM Phosphate buffer (pH 7.1) containing 2% acetone. The enzyme reaction was stopped by the addition of 100 μl of acetonitrile, and ABC-FITC, HMB, and remaining substrate was analyzed by LC-MS/MS. For the analysis of hydrolysis activity with 5DS, GR24, orobanchol, and GR7 each corresponding formylactone part was analyzed by using each deuterium labeled standard as an internal standard, and the exact amount of reaction product was calculated. For the analysis of hydrolysis activity with dhGR24, GR5, Br-PMF, and CN-PMF, a common reaction product, HMB was analyzed by LC-MS/MS and its peak area was used for the calculation of relative activities. The kinetic parameters were calculated from Lineweaver-Burk plots. The effect of substrate concentration on reaction velocity was examined at various concentration of SDS (1.25, 2.5, 5, 10, 20 μM). Detailed conditions for the LC-MS/MS analysis are described in Supplementary Table 3.

Differential scanning fluorimetry experiments. DSF experiments were carried out using Mx3000P (Agilent). Sypro Orange (Ex/Em: 490/610 nm, Invitrogen) was used as the reporter dye. Reaction mixtures were prepared in 96-well plates, and each reaction was carried out on a 20 μL scale in PBS buffer containing 10 μg protein. SLs in acetone so that the final acetone concentration was 5%, and 0.015 μL Sypro Orange. In the control reaction acetone was added instead of the chemical solution. Samples were heated from 25 °C to 95 °C after incubation at 25 °C for 10 min in the absence of light. The denaturation curve was obtained using MxPro software.

Time-course DSF experiments and hydrolysis monitoring. MBP-AtD14 (24 μg) was incubated in 15 μL of PBS (pH7.4), and at each time point (0, 120, 180, 210, 230, and 240 min, for GR24; 0, 120, 240, 300, 330, 360 min for CN-PMF), 30 μL of GR24, or CN-PMF, in PBS (7.5% acetone) was added to the protein solution to initiate the reaction. The final concentration of the chemicals was 40 or 200 μM for...
both chemicals. After 4 h (for GR24) or 6 h (for CN-PMF) incubation at 30 °C, the reaction was terminated and 15 μL of each sample were used for DSF experiments and hydrolysis analysis, respectively. For DSF experiments, 5 μL of Sypro Orange solution, diluted 333X with PBS, was added to each sample. DSF analysis condition for each chemical is described in Supplementary Table 3. BioAnalyst software was used for acquisition and data processing including deconvolution of multiply charged ions.

**Analysis of the AtD14 protein modification during hydrolysis.** MBP-AtD14 (24 μg) was incubated in 45 μL of PBS (pH 7.4) containing a chemical (GR24 (40 μM), CN-PMF (40 μM or 200 μM), or HMB (40 μM or 400 μM)) and 5% acetone. For the native condition analysis, the reaction mixture was directly applied to LC-MS/MS analysis. As for the denatured condition, acetonitrile was added at 50% final concentration to each sample to terminate the reaction. LC-MS/MS analysis of MBP-AtD14 was carried out using a system consisting of a quadruple/time-of-flight tandem mass spectrometer (TripleTOF 5600, AB SCIEX) and an Ultra high performance liquid chromatograph (Nexera, Shimadzu). The detailed analysis condition is described in Supplementary Table 3. BioAnalyst software was used for acquisition and data processing including deconvolution of multiply charged ions.

**Generation of transgenic plants.** The cDNA of OsD14 and AtD14 was obtained by PCR amplification using primers as described in Supplementary Table 1 (AtD14-F-cacc and AtD14 R-blunt for AtD14, OsD14-F-cacc and OsD14-R-blunt for OsD14), and each PCR product was subcloned into the entry vector pENTR/D-TOPO (Invitrogen). For OsD14, the conserved esterase domain was used, as described above. Each point mutation construct was generated by PCR using primers as described in Supplementary Table 1. The white arrow in the left picture indicates the outgrowing tiller. Scale bars = 5 cm (left panel), 20 cm (right panel).

**Phenotypes of 42 days old transgenic plants overexpressing OsD14R233H in the WT (Nipponbare) background. Empty vector expressing plants are indicated as EV. Data are the means ± SD (n = 3-4).** c. No. of tillers of rice transgenic plants overexpressing OsD14R233H in the WT (Nipponbare) background. Empty vector expressing plants are indicated as EV. Data are the means ± SD (n = 3-5). d. Phenotypes of 42 days old transgenic plants overexpressing OsD14R233H (OsD14R233HOE). Scale bars = 10 cm. e. Quantitative analysis of 4DO in the root exudates (left panel) and extracts (right panel) of OsD14R233H overexpressing (OsD14R233HOE) plants. Data are the means ± SD (n = 3-4). Different letters in c and e indicate significant differences at P < 0.05 with Tukey-kramer multiple comparison test. Source data are provided as a Source Data file.
mutant constructs, which were used for protein expression as described above, as templates, and subcloned into the same entry vector. Each cDNA was shuttled into the pGBKT7, pGADT7, and pGBT9 vectors by an LR clonal reaction according to the manufacturer’s protocols (Invitrogen). Arabidopsis WT, athy1-2, and athy1-4 mutant plants were transformed with the resulting constructs by the floral dip method using Agrobacterium tumefaciens. The rice Nipponbare and Os03g0203200 mutant plants were transformed with the resulting constructs by A. tumefaciens. For genetic complementation of the athy1-2 mutant using a native promoter, a 4,507-bp genomic fragment containing the OsATD14 (Os03g0203200) gene, as well as regions 2 kb upstream and 1 kb downstream of the transcribed region, was amplified by PCR using primers described in Supplementary Table 1 (OsATD14-forward/BamHI and OsATD14-reverse/PstI). The PCR product was subcloned into the entry vector pENTR/D-TOPO. The fragment was then shuttled into a binary vector containing no promoter, pGWB115, by an LR clonal reaction according to the manufacturer’s protocols. Rice athy1-2 mutant was transformed using resulting constructs by A. tumefaciens.

Arabidopsis shoot branching assay. Arabidopsis seeds were sterilized in a 1% sodium hypochlorite solution for 5 min, rinsed with sterile water, and stratified for one day at 4 °C. The seeds were placed on half strength Murashige and Skoog (MS) medium containing 50 μM 2,4-D (pH 5.7) and incubated in a 150 rpm shaker at 22°C for 10 days. The solution was renewed every 7 days. The solution was renewed every 7 days. The solution was renewed every 7 days.

Quantitative analysis of endogenous SLs in rice plants. In order to quantitatively analyze the endogenous SLs, we used a hydropod system culture for growing rice. Rice plants were cultured in 70% ethanol for 30 s, sterilized in 2.5% sodium hypochlorite solution for 5 min, rinsed with sterile water, and then incubated in sterile water at 28 °C under fluctuating white light (150 μmol m−2 s−1) with a 16 h light/8 h dark photoperiod for 15 days. Plants were transferred to a glass pot containing 400 mL hydropod solution and grown under the same environmental conditions for an additional 15 days. The solution was renewed every 7 days.

Western blot analysis of AtD14 protein in Arabidopsis. Rice plants were grown in a growth cabinet with a 15 h light/9 h dark photoperiod for 6 weeks. Genotyping was carried out to select the transgenic plants, using primers described in Supplementary Table 1 (pGWB2-F and pGWB2-R). Protein samples (each 1 μg) were separated by 15% SDS-PAGE and the blots were transferred to a nitrocellulose membrane. For the detection of the AtD14 protein fused with the GAL4 activation domain, the blots were treated with 5% skim milk in TBST (0.1% Tween20 in 2 mM NaCl) for 2 h and subsequently incubated with GAL4 AD Monoclonal Antibody (Clontech, 630402, 4000 times dilution) at 25 °C overnight. After washing three times with TBST for 10 min each wash, the membrane was incubated with goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (abcam, ab97023, 2000 times dilution) at 25°C for 2 h. The membrane was washed three times, and analyzed by a chemiluminescence-based detection method using Super Signal West Pico Chemiluminescence Substrate (Pierce).

SL-binding assay. The direct binding assay of AtD14 with SDS was performed according to the GA-binding assay method1. MBP-AtD14 was incubated at 30°C for 30 min in 10 μL of a standard binding buffer that containing 50 μg recombinant protein and 20 μM of SDS in 50 mM Phosphate-Na buffer (pH 7.0) containing 150 mM NaCl and 2% acetone. After 30 min incubation, the sample was loaded onto the NAP-5 column chromatography (GE Healthcare). The column was eluted by 100 mM Phosphate-Na buffer (pH 7.0) containing 150 mM NaCl and the first 100 mL of the SDS-Rich fraction was discarded. The remaining 200 mL of the eluents was collected. 1 mL of the eluates was then loaded onto the NAP-5 column chromatography (GE Healthcare). The column was eluted by 100 mM Phosphate-Na buffer (pH 7.0) containing 150 mM NaCl and the first 100 mL of the SDS-Rich fraction was discarded. The remaining 200 mL of the eluents was collected. To determine the activity of the fusion, the 10 mL of the eluent was precipitated by 100 μL of 20% trichloroacetic acid, and the precipitate was dissolved in 1 M NaOH and incubated for 2 h at 30°C. The 150 μL of the sample was subjected to Western blot analysis using an antibody against MBP-AtD14. To determine the activity of the fusion, the 10 mL of the eluent was precipitated by 100 μL of 20% trichloroacetic acid, and the precipitate was dissolved in 1 M NaOH and incubated for 2 h at 30°C. The 150 μL of the sample was subjected to Western blot analysis using an antibody against MBP-AtD14. The membrane was washed three times and analyzed by a chemiluminescence-based detection method using Super Signal West Pico Chemiluminescence Substrate (Pierce).

Yeast two (three) hybrid experiments. The WT and each mutant AtD14 were cloned into pGADT7, and SMX7 was cloned into pGBKKT7. Arabidopsis ASKI was amplified by PCR using primers, ASKI-F-cac and ASKI-R-blunt (Supplementary Table 1), and the PCR product was subcloned into the entry vector pENTR/D-TOPO. ASKI was then shuttled into the modified pYES-DEST52 (pYES ADHPro) vector by an LR clonal reaction according to the manufacturer’s protocols (Invitrogen). We were not able to clone Arabidopsis MAX2 into pGBKKT7, thus MAX2 was cloned into a modified pGBD-U, in which the URA3 marker region was replaced with TRP1 marker. To modify pGBD-U, the TRP1 region including its promoter was amplified with primers TRP1-Pro-F-Ndel and TRP1-R-Ncol (Supplementary Table 1), using pGBK7T as a template. The PCR product was digested by Ndel and Ncol, and cloned into yeast expression enzyme sites of pGBD-U. We named the resulted vector pGBDT. MAX2 was amplified by PCR using primers MAX2-F-BamHI and MAX2-R-PstI (Supplementary Table 1), and the PCR product was cloned into the corresponding enzyme sites of pGBD-U. Resulting constructs were co-transformed with the yeast strain Pichia and the transformants were grown on SD-Trp/-Leu or SD-Trp/-Leu/-Ura plates for 3 days at 30°C. Interactions between the two proteins were examined on selective media (SD-Trp/-Leu/-His or SD-Trp/-Leu/-Ura/-His) containing a 1:1000 dilution of the tested SL from an acetone-dissolved stock solution (0.1% acetone was used as a control). The plates were kept for 5 days at 30°C. For western blot analysis, each transformant was pre-incubated in SD-Trp/-Leu overnight at 30°C, transferred to fresh YPDA medium, and incubated until the OD600 reached 0.5–0.6. The cells were collected by centrifugation then resuspended with an Urea/SDS protein extraction buffer (40 mM Tris (pH 6.8) containing 8 M Urea, 5% SDS, 0.1 mM EDTA, 0.4 mg/mL Bromophenol blue, and 1 mM PMSF). Each sample was filtered twice through a 0.2 μm filter via a Millipore filter and then centrifuged to remove debris. The supernatants were separated by SDS-PAGE and the blots were treated with 5% skim milk in TBST (0.1% Tween20 in 2 mM NaCl) for 2 h and subsequently incubated with anti-MAX2 rabbit IgG antibody overnight at 4°C. After washing three times with TBST for 10 min each wash, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Sigma, A4914, 100 times dilution) at 25°C for 2 h. The membrane was washed three times, and analyzed by a chemiluminescence-based detection method using Super Signal West Pico Chemiluminescence Substrate (Pierce).

Reanalyzing of the structurally changed AtD14. The PDB data of AtD14-D3-ASK1 complex structure (SHZG) were downloaded from protein data bank (https://wws.rcsb.org/), and reanalyzed by using PyMOL. The cavity volume was calculated using CASTp program server (http://cbsi.bio.uc.ie/castp/index.php). Docking was performed using SWISS DOCK (http://www.swisdock.ch/).

qRT-PCR analysis of rice transgenic plants. Rice plants were grown in a growth cabinet with a 15 h light/9 h dark photoperiod for 6 weeks. Genotyping was carried out to select the transgenic plants, using primers described in Supplementary Table 1 (pGWB2-F and pGWB2-R). Total RNA was extracted from leaf blades of the plants using a Plant RNA Isolation mini kit (Agilent). After DNase I treatment, first-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). The primer sets used to amplify the transcripts were described in Supplementary Table 1 (OsD14-QRT-F and OsD14-QRT-R). PCRs were performed with SYBR green I using a Light Cycler 480 System II (Roche Applied Science).

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.
Data availability

The source data underlying Figs 2b, d, 3a,b,e, and 4b,c,e and Supplementary Figs 1c, 2b–c, 3b–g,i, 4a–e, 5a, 6a–c, 7c, 8b–d, and 11b–g are provided by a Source Data file. A reporting summary for this Article is available as a Supplementary Information File. All other data are available from the corresponding authors upon a reasonable request.

Received: 27 March 2018 Accepted: 17 December 2018
Published online: 14 January 2019

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Acknowledgements

This work was supported by JSPS/MEXT KAKENHI (Grant Nos, 24780117, 24114010, and 17H06474), the Program for Promotion of Basic and Applied Research for Innovations in Bio-Oriented Industry, and the Core Research for Evolutional Science and Technology (CREST) by JST (Grant No, JPMJCR13B1). Y.S. was a JSPS Postdoctoral Fellow for Research Abroad. We thank Dr. Mitsunori Seo for providing the yeast expression vector, pYES ADHpro.

Author contributions

Y.S. and S.Y. designed the research. Y.S. and R.Y. performed the majority of experiments with the guidance from K.M. and S.Y. H.K. generated the rice transgenic plants and Arabidopsis transgenic plants and analyzed the phenotypes of these plants with guidance from J.K.C. M.U. technically supported rice and Arabidopsis plant growth. A.I. performed part of D14H experiments. R.T. M.U. technically supported rice and Arabidopsis plant growth. A.H. and T.K. technically supported the LC-MS/MS analysis. K.A. prepared SI chemicals. N.T.-K. and W.L. generated Arabidopsis osd14-2 mutant with the guidance from R.T. M.U. technically supported rice and Arabidopsis plant growth. A.H. and T.K. supported the protein expression work. Y.S. and J.B. analyzed the reported structural data with guidance from J.P.N., Y.S., R.Y., J.B., and S.Y. wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-08124-7.

Competing interests: The authors declare no competing interests.

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Journal peer review information: Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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