The perception mechanism for the strigolactone (SL) class of plant hormones has been a subject of debate because their receptor, DWARF14 (D14), is an α/β-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\textsuperscript{1,2,3}, yet are now known as endogenous plant hormones that control shoot branching and diverse aspects of plant growth\textsuperscript{4–7}. Canonical SLs have a four-ring structure (Fig. 1a, b)\textsuperscript{8–10}.

The SL receptor, DWF14 (D14), was initially characterized from a rice SL-insensitive mutant, d14\textsuperscript{11}.\textsuperscript{12}, and orthologues have since been identified from Arabidopsis (Arabidopsis thaliana D14; AtD14), petunia (DECREASED APICAL DOMINANCE2; DAD2), and pea (RAMOSUS3; RMS3)\textsuperscript{13–14}. In this paper, the rice D14 is referred to as \textit{Oryza sativa} D14 (OsD14) to distinguish from AtD14. The D14 enzymes belong to the \(\alpha/\beta\)-fold hydrolase family and possess the canonical catalytic triad: Ser, His, and Asp. In fact, D14 acts as a hydrolase for some SLs, cleaving them into ABC-ring (ABC-formyltricyclic lactone; ABC-FTL), and D-ring (Hydroxymethylbutenolide; HMB) parts\textsuperscript{8,12,13,15,16}. In rice, signal transduction occurs through a SL-dependent interaction between OsD14 and a negative regulator of SL signaling, D53 (SUPPRESSOR OF MAX2 H1-LIKE (SMXL) 6/7/8 in Arabidopsis), leading to the rapid degradation of D53 through the 26S proteasome pathway in a manner that requires the F-box protein, D3 (MORE AXILLARY GROWTH2 (MAX2) in Arabidopsis)\textsuperscript{17–20} (Fig. 1b). The receptor function of D14 in the SL pathway has been established; however, the role of SL hydrolysis in the signaling mechanism has been a subject of debate. A significant question is when and how the signal is transduced while D14 interacts with SLs: is the signal transduced before, during, or after the hydrolysis of SLs by D14?

Recently, two groups proposed a model in which the hormone signaling is mediated by a covalently linked reaction intermediate of SL with D14\textsuperscript{13,14,16}. A covalent modification involving His of D14 catalytic triad with the D-ring part of SL derived molecule was detected by mass spectrometry analysis. Moreover, Yao et al. solved a crystal structure of an AtD14-D3-ASK1 multiprotein signaling complex induced by an SL analog, GR24 (Fig. 1a)\textsuperscript{16}. The authors proposed that in this signaling complex AtD14 possesses a D-ring derived molecule, which was covalently linked with AtD14 bridging the Ser with the His of the catalytic triad. The authors named this sealed molecule to be covalently linked intermediate molecule (CLIM). Moreover, significant conformational changes were observed for AtD14 in the multiprotein complex when compared with its apo structure. Based on these results, a model has been proposed in which the CLIM is necessary for the induction of conformational changes in the AtD14 protein and the SL signal is transduced during its hydrolysis. However, very recently Carlsson et al. reanalyzed this complex structure data and found that the electron density in the active site is too small to accommodate the proposed CLIM\textsuperscript{21}. More likely, the substance bound to the active site is not the CLIM, rather an iodine ion from the crystallization reagents\textsuperscript{22}. In addition to the uncertain structure data, the hydrolysis reaction by D14 was reported to be extremely slow, on the order of hours\textsuperscript{12,15}, whereas degradation of the target protein, D53/SMXLs, was detected within 5–20 min after the SL treatment in planta\textsuperscript{17–20}. Thus, the CLIM model, which requires the hydrolysis reaction of D14 to transmit the signal, is inconsistent with this rapid response. Taken together, although the structural characterization of the D3-bound form of AtD14 was a significant breakthrough in this research field, the signaling mechanism, in particular the chemical signal that induces the active signaling state of D14, remains a matter of debate.

Here, we report that the active signaling state of D14 can be triggered upon intact SL binding, but not by the hydrolysis 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 AD14-catalyzed hydrolysis reaction were measured using the naturally occurring single isomer of 5-deoxystyrogol (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)\textsuperscript{12}, and the \(K_m\), \(V_{max}\), and \(K_{cat}\) were calculated to be 4.9 \(\mu\)M, 4.0 nmol/min/mg protein, and 0.12 min\(^{-1}\), respectively (Supplementary Fig. 1c). 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\textsuperscript{22,23} (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, in which the double bond of the enol ether bridge is replaced by a single bond, 3′,6′-dihydroGR24\textsuperscript{24}, 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/D3dependent manner\textsuperscript{25–27}. 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\textsuperscript{23–25}, 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\textsuperscript{12,13,26}, 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)\textsuperscript{8}. 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\textsuperscript{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\textsuperscript{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 reflects 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.
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 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 His residue before its release as HMB, which is 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, is 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 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 turn over 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 (AtD14S97A, AtD14L97C, AtD14D218A, and AtD14H247A) and found that the hydrolase activities of all of these mutants are drastically reduced when compared with a negative control (Fig. 3a). Although the RMS5396C mutant was reported to have slight hydrolase activity for GC2423, the hydrolase activity for 5DS in the corresponding mutant in Arabidopsis, AtD14S97C, 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 hydrodrolase 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 AtD14D218A, 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 6 × HA tag fusion\textsuperscript{12}; however, we observed that the expression of untagged AtD14S97C partially complemented the atd14 mutant phenotype (Fig. 3b, c and Supplementary Fig. 6b). In addition, we found that AtD14D218A interacted with SMXL7 in an SL-dependent manner in yeast two hybrid (Y2H) experiments, suggesting that AtD14D218A is still capable of signal transduction despite lacking hydrodrolase activity, while AtD14S97C weakly interacts with SMXL7 in the presence of MeCLA (Fig. 3d and Supplementary Fig. 6c). We also found that AtD14D218A 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 AtD14D218A 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 AtD14D218A slightly, but did not change the Tms for AtD14S97A or AtD14H247A, consistent with the observation that AtD14S97C and AtD14D218A 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 AtD14D218A 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 AtD14D218A suggested that this protein was unstable, even in the absence of 5DS (Supplementary Fig. 7a), we generated transgenic plants expressing AtD14D218A 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 branching phenotype, which was rescued by GR24 treatment, demonstrating that AtD14D218A complements the atd14 mutant in an SL-dependent manner (Fig. 3e, Supplementary Fig. 8a). Furthermore, in this background we observed that AtD14D218A expressing plants were more sensitive to GR24 than the AtD14WT expressing plants, possibly because of the defect in the hydrolytic degradation of GR24 by AtD14D218A (Fig. 3e), implying that the hydrolytic degradation of SL by D14 would be a deactivating 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 hydrodrolase 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 35S 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-deoxyorobanchol (4DO), in the rice overexpressors were decreased relative to WT and the empty vector-expressing plants (Fig. 4e). The SL hydrolysis products...
were reported to have quite weak or no biological activities in shoot branching inhibition\(^1\),\(^2\),\(^28\). 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.

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**Fig. 3** In vitro and in vivo functional analysis of catalytic triad mutants of AtD14. **a** Hydrolysis activities of catalytic triad mutants of AtD14 using 1 \(\mu\)M of 5DS as a substrate. Data are the means ± SD (\(n = 3\)). The control reaction (Cont.) is for MBP protein only. **b** No. of axillary shoots (over 5 mm) of Arabidopsis transgenic plants expressing each catalytic triad mutant of AtD14 in the atd14-2 mutant background. Data are the means ± SD (\(n = 5−10\). Different letters indicate significant differences at \(P < 0.05\) with Tukey-kramer multiple comparison test.). **c** Phenotypes of Arabidopsis transgenic plants expressing each catalytic triad mutant of AtD14. Mature 50 days old plants phenotypes (upper panel) and leaf morphology phenotypes of 25 days old plants (lower panel) are shown. Scale bars = 5 cm (upper panel), 1 cm (lower panel). **d** Y2H analysis of the interaction between SMXL7 and each catalytic triad mutant of AtD14. Yeast transformants were spotted onto the control medium (SD−Leu/−Trp (−TL)) and selective medium (SD−Leu/−Trp/−His (−TLH)) in the absence or presence of SLs (10 \(\mu\)M rac-MeCLA or 10 \(\mu\)M 5DS). Control (Cont.) is acetone only. **e** Shoot branching inhibition assays of the Arabidopsis transgenic lines expressing AtD14\(^{WT}\) and AtD14\(^{D218A}\), respectively, in the atd14\(^{max4}\) double mutants background. The bars indicate the No. of axillary shoots (over 5 mm) in the presence (+) or absence (−) of GR24 at 5 \(\mu\)M (left panel) and 0.5 \(\mu\)M (right panel), respectively. Data are the means ± SD (\(n = 3−13\). Different letters indicate significant differences at \(P < 0.05\) with Tukey-kramer multiple comparison test). Source data are provided as a Source Data file.
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.\(^\text{16}\) 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,\(^\text{19}\) 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, D53/SMXLs and D3/MAX2, to transmit the SL signal. As seen in the AtD14-D3-ASK1 complex structure, D3/MAX2 F-box interacts with the activated D14 protein at the surface of the rearranged helical lid domain. In this complex, D53/SMXLs may bind around the Asp loop region. In our model, after degradation of the negative regulator of SL signaling, the loop returns to reconstruct the catalytic triad, which then induces the hydrolytic degradation and deactivation of bioactive SLs (Fig. 5). The slow rate of the first attack from the Ser residue, as suggested by our hydrolysis monitoring, can be explained by this temporary physical disruption of the catalytic triad. The activation of hormone degradation pathways in signaling is exemplified in other plant hormone pathways, such as GA and, is considered to be an important mechanism to maintain homeostatic levels of active hormones in plants.\(^\text{40}\) Similarly, our data suggest that the D14 receptor protein in the SL signaling pathway is responsible for SL deactivation by hydrolytic catalytic activity native to the α/β-fold hydrolase family.

In conclusion, our targeted biochemical and genetic experiments uncover previously undetected, yet critical aspects of the signaling mechanisms of SLs, as they are mediated by a catalytically active protein that is responsible for both the perception and deactivation of bioactive hormone signals.

**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-n) for construction of transgenic plants.\(^\text{15}\) The rice osd14-2 was characterized from Sasanishiki EMS mutant lines. We used Arabidopsis ecotype Col-0 as the WT, maat-8 (SALK_027590)\(^\text{3}\), and atd14-2 mutants.\(^\text{4}\) 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 branching assays, a hydroponic culture system was used; the growth condition is described below. For the rice phenotype observation, the plants were grown under long day conditions (16 h light at 28°C/8 h dark at 25°C). The number of tillers was measured after 42 days of growth. For SL measurements in rice, the hydroponic culture system was used.

**Chemicals.** Br-PMF and CN-PMF were purchased from Chiralix. Other SLs were prepared as part of our previous studies.\(^\text{2,17}\)

**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 ligated into pET15b plasmid and transformed into E.coli BL21 (DE3) at 37°C. The expressed SL-binding sites of the modified pMALcx5 (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 med- 

**Hydrolyase activity tests of D14.** Hydrolyase 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-Na buffer (pH 7.0) containing 2% acetone. The enzyme reaction was stopped by the addition of 100 μL of acetonitrile, and ABC-FTL, HMB, and remaining substrate was analyzed by LC-MS/MS. For the analysis of hydrolyase 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 hydrolyase activity with d8GR24, 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 concentrations of 5DS (0.25, 2.5, 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%, 0.015 μM 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 (pH 7.4), and at each time point (0, 120, 180, 210, 240, and 240 min, for GR24, 0, 120, 240, 300, 360 min for CN-PMF), 30 μL of GR24, or CN-PMF, in PBS (pH 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. 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 (AD14-f-cacc and AD14-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 cDNA of OsD14R233H/AtD14R133H was cloned into the pENTR/D-TOPO vector and subcloned into the entry vector pDONR207 (Invitrogen). For each entry vector, 1000 μL of each sample was used for hydrolysis assays. For hydrolysis assays, the reaction mixture was directly applied to LC-MS/MS analysis. As for the denatured condition, acetonitrile was added at 50% 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.

**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.

**Fig. 4** In vitro and in vivo functional analysis of OsD14R233H/AtD14R133H. a Phenotypes of 2 weeks old seedlings (left) and mature plants (right) of the rice d14-2 mutant. The white arrow in the left picture indicates the outgrowing tiller. Scale bars = 5 cm (left panel), 20 cm (right panel). b Hydrolase activities of OsD14R233H and AtD14R133H mutants using 1 μM SDS as a substrate. 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.

**Fig. 5** A proposed working model of D14 in the SL signaling pathway. A bioactive SL molecule induces the protein conformational changes of D14, which triggers complex formation with the signaling partners. After the degradation of D53/SMXLs and transmission of the SL signal, D14 reconstructs the catalytic triad to hydrolytically decompose the bioactive SL.

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mutant constructs, which were used for protein expression as described above, as templates, and subcloned into the same entry vector. cDNA was shuttled into the pDEST-p40/16 GATEWAY® vector by the LR reaction according to the manufacturer’s protocols (Invitrogen). Arabidopsis WT, atd14-2, and atd14-2 max1-8 plants were transformed with the resulting constructs by the floral dip method using Agrobacterium tumefaciens. The rice Nipponbare and osd14-1N mutant plants were transformed with the resulting constructs by A. tumefaciens. For genetic complementation of the atd14-2 mutant using a native promoter, a 4,507-bp genomic fragment containing the OsD14 (Os03g023200) 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 (OsD14-genome-F and OsD14-genome-R). The PCR product was subcloned into the entry vector pENTR/D-TOPO. The fragment was then shuttled into a binary vector containing no promoter, pGWB116, by an LR clonase reaction according to the manufacturer’s protocols. Rice osd14-2 mutant was transformed with 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 plated on half strength Murashige and Skoog (MS) medium containing 1% sucrose and 1% agar (pH 5.7) at 22 °C under white light (60 μmol m−2 s−1) for 15 days. The solution was renewed every 7 days. Plants were transferred to a glass pot containing 400 mL hydroponic solution and grown under the same environmental conditions for an additional 15 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 hydroponic culture system for growing rice plants. Rice seeds were sterilized in 70% ethanol for 30 s, sterilized in 2.5% sodium hypochlorite solution for 5 min, rinsed with sterile water, and stratified at 4 °C for 3 days. The seeds were placed on a plate containing 400 mL hydroponic solution and grown under the same environmental conditions for an additional 15 days. The solution was renewed every 7 days.

SL-binding assay. The direct binding assay of AtD14 with SDS was performed according to the GA-binding assay method34. MBP-AtD14 was incubated at 30 °C for 30 min in 10 μL of a standard binding buffer containing 50 μM 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 with 100 mM Phosphate-Na buffer (pH 7.0) containing 150 mM NaCl, and the first 100 μL fractions were collected. The eluates were evaporated to dryness and dissolved in 50 μL of water and subjected to LC-MS/MS analysis. Further information on experimental design is available in Supplementary Table 1 (OsD14-QRT-F and OsD14-QRT-R). PCRs were 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 (Supplementary Table 1). The PCR product was cloned into pGWB2-F and pGWB2-R. The sample was subjected to electrophoresis in 1% agarose gel and visualized with ethidium bromide. The total RNA extracted was then shuttled into the modified pGBKT7 vector (Clontech), and DNA were cloned into the entry vector pENTR/D-TOPO. The fragment was then shuttled into a binary vector containing no promoter, pGWB116, by an LR clonase reaction according to the manufacturer’s protocols. Rice osd14-2 mutant was transformed with resulting constructs by A. tumefaciens.

The membrane was washed three times and analyzed by a chemiluminescence-based detection method using Super Signal West Pico Chemiluminescent Substrate (Pierce).

Yeast two (three) hybrid experiments. The WT and each mutant AtD14 were cloned into pGADT7, and SMX7 was cloned into pGBKTT7. Arabidopsis ASKI was amplified by PCR using primers, ASKI-F-cac and ASKI-R-blunt (Supplementary Table 1), and the PCR product was cloned into the entry vector pENTR/D-TOPO. ASKI was then shuttled into the modified pYES-DEST52 (pYES ADHPro) vector by an LR clonase reaction according to the manufacturer’s protocols (Invitrogen). We were not able to clone Arabidopsis MAX2 into pGBKTT7, thus MAX2 was cloned into a modified pGBD, in which the URA3 marker region was replaced with TRP1 marker. To modify pGBD, the TRP1 region including its promoter was amplified with primers, TRP1-Pro-F-Ndel and TRP1-R-Ncol (Supplementary Table 1), using pGBK7 as a template. The PCR product was ligated by Ndel and Ncol, and cloned into the entry vector pENTR/D-TOPO. The fragment was then shuttled into a binary vector containing no promoter, pGWB116, by an LR clonase reaction according to the manufacturer’s protocols. Rice osd14-2 mutant was transformed with resulting constructs by A. tumefaciens.

For genetic complementation of the atd14-2 mutant using a native promoter, a 4,507-bp genomic fragment containing the OsD14 (Os03g023200) 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 (OsD14-genome-F and OsD14-genome-R). The PCR product was subcloned into the entry vector pENTR/D-TOPO. The fragment was then shuttled into a binary vector containing no promoter, pGWB116, by an LR clonase reaction according to the manufacturer’s protocols. Rice osd14-2 mutant was transformed with resulting constructs by A. tumefaciens.

The membrane was washed three times and analyzed by a chemiluminescence-based detection method using Super Signal West Pico Chemiluminescent Substrate (Pierce).

Reanalyzing of the structurally changed AtD14. The PDB data of AtD14-D3-ASK1 complex structure (SH2G) were downloaded from protein data bank (https://www.rcsb.org/), and reanalyzed by using PyMOL. The cavity volume was calculated using CASTp program server (http://bbsrc.ucl.ac.uk/castp/index.php). Docking was performed using SWISS DOCK (http://www.swissdock.ch/).

The membrane was washed three times and analyzed by a chemiluminescence-based detection method using Super Signal West Pico Chemiluminescent Substrate (Pierce).

qRT-PCR analysis of rice transgenic plants. Rice plants were grown in a growth chamber with a 12 h light/12 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 1 using a Light Cycler & 480 System II (Roche Applied Science).

Reanalyzing of the structurally changed AtD14. The PDB data of AtD14-D3-ASK1 complex structure (SH2G) were downloaded from protein data bank (https://www.rcsb.org/), and reanalyzed by using PyMOL. The cavity volume was calculated using CASTp program server (http://bbsrc.ucl.ac.uk/castp/index.php). Docking was performed using SWISS DOCK (http://www.swissdock.ch/).
Data availability

The source data underlying Fig. 2b, d, 3a,b,e and 4b,c,e and Supplementary Figs 1c, 2b-e, 3b-e,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.

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References


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 analyzed the phenotypes of these plants with guidance from J.K. C.M. performed part of western blot analysis. C.M. and E.S. performed part of Y2H experiments. R.H. performed Y3H experiments. A.S. performed part of Arabidopsis transgenic plants preparation and part of hybridization assays. M.T. performed the characterization of rice ossl4-2 mutant with the guidance from 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 ossl4-2 muax-4 double mutant. Y.H. and T.H. 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

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