Supplementary information, Data S1 Methods

Protein Preparation

Wild type OsD14 (residues 51–318) was expressed as an N-terminal His6-Sumo fusion protein from pSUMO (LifeSensors). BL21 (DE3) cells transformed with the expression plasmid were grown in LB broth at 16°C to an OD600 of ~1.0 and induced with 0.1 mM IPTG for 16 hrs. Cells were harvested, resuspended in extract buffer (20 mM Tris, pH 8.0, 200 mM NaCl, and 10% glycerol), and lysed using a French press. The lysate was centrifuged at 16,000 rpm in a Sorvall SS34 rotor for 30 min, and the supernatant was loaded on a 50 ml Nickel HP column. The column was washed with 600 ml of 10% buffer B (20 mM Tris, pH 8.0, 200 mM NaCl, 500 mM imidazole, and 10% glycerol) and eluted in two steps with 200 ml of 50% buffer B followed by 100 ml of 100% buffer B. The eluted H6SUMO-D14 protein was dialyzed against extract buffer and cleaved overnight with SUMO protease at a protease/protein ratio of 1:1000 at 4ºC. The cleaved H6SUMO tag was removed by passing through a Nickel HP column, and the protein was further purified by chromatography through a HiLoad 26/60 Superdex 200 gel filtration column in 20 mM Tris, pH 8.0, 200 mM ammonium acetate, 1 mM dithiotreitol and 1 mM EDTA. Apo-D14 eluted as a sharp single peak with an estimated molecular weight of 30 kD and a purity of >95% as judged by 10% SDS/PAGE, suggesting that apo-D14 is a monomer in solution (predicted MW of 29.2 kD).

Wild type KA12 (AT4g37470) (residues 1–267) from *Arabidopsis thaliana* was also expressed as a His6-Sumo fusion protein from the expression vector pSUMO (LifeSensors). The expression and purification of KA12 followed the same method as for D14. Apo-KA12 eluted as a sharp single peak with an estimated molecular weight of 30 kD and a purity of >95% as judged by 10% SDS/PAGE, suggesting that apo-KA12 is a monomer in solution (predicted MW of 29.79 kD).

Wild type AtD14 (At3g03990) (residues 1–270) from *Arabidopsis thaliana* was also expressed and purified as His6-Sumo fusion protein following the same expression and purification conditions as for OsD14. AtD14 eluted as a sharp single peak with an estimated molecular weight of 30 kD and purity of >95% as judged by 10% SDS/PAGE, suggesting that apo-AtD14 is a monomer in solution (predicted MW of 29.6 kD).

SABP2 (salicylic acid-binding protein; residues 1–260) was expressed as a His6-Thioredoxin (H6Trx) fusion protein from the expression vector pETDuet1 (Novagen). The expression and purification of SABP2 followed the same method as for D14 except that the H6Trx tag was cleaved overnight with 3C protease at a protease/protein ratio of 1:500 at 4ºC. Biotin-SABP2 eluted from the gel filtration column as a broad peak that ranged from an estimated MW of 32 kD, suggesting the Biotin-SABP2 may exhibit a monomer-dimer equilibrium in solution.

To generate biotinylated SABP2 for hydrolase activity assays, SABP2 was expressed as H6-Trx-3C-avitag-fusion protein in *E. coli* BL21 (DE3) cells from a pETDuet (Novagen) derived vector. The first T7 RNA polymerase-driven expression unit of this vector contains SABP2 as fusion with a H6-thioredoxin-3C protease cleavage site-avitag tag, the second site the *E. coli* biotin-ligase gene *BirA*. The 14 amino acid avitag functions as a defined *in vivo* biotinylation site in *E. coli*. Cells grown in the presence of 40 μM biotin were lysed and fusion proteins purified over Nickel HiTrap columns as above. Following 3C protease release of the H6-Trx tag, SABP2 proteins with biotinylated avitags were purified over 5 ml Nickel HP column followed by gel filtration chromatography. Residual biotin was removed by extensive dialysis prior to their use in hydrolase assays.

Crystallization

Apo-KA12 crystals were grown at room temperature in hanging drops containing 1.0 µl of the purified KA12 protein at a concentration of 11.75 mg ml^{-1} and 1.0 µl of well solution containing 0.1 M HEPES, pH 7.5, 2% w/v polyethylene glycol 400, 2.0 M ammonium sulfate. Crystals of about 150-200 µm in length appeared within 1–2 days. Crystals were flash frozen in liquid nitrogen before data collection.

Apo AtD14 crystals were grown at room temperature in hanging drops containing 1.0 µl of the purified AtD14 protein at a concentration of 10.2 mg ml^{-1} and 1.0 µl of well solution containing 0.2 M $MgCl_2$, 0.1 M Tris, pH 8.5, 30% w/v polyethylene glycol 4000, and 1.1 mM C12E8I. Crystals appeared within 1–2 days and grew to a dimension of 50 µm in length over a period of 4 days. Crystals were serially transferred to well buffer with increasing glycerol concentration (20% v/v final) before flash freezing in liquid nitrogen.

OsD14-GR24 hydrolysis product crystals were obtained by adding GR24 at a 20-30fold molar excess and concentrated to 5.4 mg/ml. Crystals were grown at room temperature in hanging drops containing 1.0 µl D14/GR24 and 1.0 µl of well solutions in two conditions. One contained 0.1 M HEPES, pH 7.5, 10% w/v polyethylene glycol 6,000, 5% v/v (+/-)-2-Methyl-2,4-pentanediol, and the other contained 0.1 M BICINE, pH 9.0, 2% v/v 1,4-Dioxane, 10% w/v polyethylene glycol 20,000. Crystals appeared within 1–2 days and grew to a dimension of 30–40 µm in length over a period of 4 days. Crystals were serially transferred to well buffer with increasing glycerol concentration (20% v/v final) prior to flash freezing in liquid nitrogen for data collection.

Data Collection and Structure Determination

The X-ray diffraction data sets for apo-D14 crystals were collected at SSRF(Shanghai Synchrotron Radiation Facility) beam line BL17U and KA12 and AtD14 crystals were collected using MAR300 and MAR225 CCD detectors (MAR Research) at the ID-D and ID-F lines of

sector-21 (LS-CAT) at the Advanced Photon Source at Argonne National Laboratory. The observed reflections were reduced, merged, and scaled with DENZO and SCALEPACK in the HKL2000 package. Crystals of apo-OsD14, apo-AtD14, apo-AtKA12 and the AtD14-GR24 hydrolysis product complex diffracted to resolutions of 1.55 Å, 3.5 Å, 1.55 Å, and 1.55 Å, respectively. Molecular replacement was performed by using the Collaborative Computational Project 4 (CCP4) program Phaser using the RbsQ structure (PDB code 1WOM) as the initial model. Programs O and Coot were used to manually fit the protein model. Model refinement was performed with CNS and the CCP4 program Refmac5. The volumes of the ligand binding pockets were calculated with the program Voidoo by using program default parameters and a probe with a radius of 1.4 Å. All structure figures were prepared by using PyMOL (DeLano Scientific). The statistics of data collection and the model refinement are summarized in Supplementary Table 1.

Enzymatic hydrolysis of GR24 by OsD14, KA12, AtD14, and SABP2

The hydrolysis of GR24 by D14, KAI2, AtD14, and SABP2 was performed by incubating 10 μg of GR24 with 40 μg of D14 in 220 μl of reaction buffer (20 mM HEPES, pH 7.0, 200 mM NaCl, 10% glycerol, 10% DMSO) at 37° for 4 hours. 10 μl of the reaction mixtures were separated by a reverse-phase, ultra-performance liquid chromatography (UPLC, Waters) C18 column (Agilent Eclipse Plus, 100 mm × 2.1 mm, 3.5 μm) at a flow rate of 0.4 ml/min. The mobile phase was 10 mM ammonium acetate including 0.05% formic acid and acetonitrile. GR24 and the ABC-ring eluted at 11 and 8 minutes, respectively. The UPLC eluent was subjected to electrospray ionization quadrupole time-of-flight mass spectrometric analysis (ESI-Q-TOF, Waters). The data were acquired with Masslynx V4.1 software (Waters).

4-Nitrophenyl butyrate hydrolysis assay

Enzymatic reactions were performed in 96 well plates. Reactions contained 2 μM SABP2/

OsKAI2/ AtKAI2/ OsD14/ AtD14, 1 mM 4-Nitrophenyl butyrate with or without 1 mM PMSF in total volumes of 200 µl 20 mM HEPES, pH 7.0, 200 mM NaCl, 10% glycerol. Light absorbance at 405 nm was measured every half hour using a Perkin Elmer 2104 multilabel reader, and converted into corresponding percentage of substrate hydrolysis and turnover rate. The data shown is normalized by setting SABP2 activity as 100% and subtracting non-enzymatic substrate hydrolysis determined in a reaction buffer control.