Structural plasticity of D3–D14 ubiquitin ligase in strigolactone signalling

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The strigolactones, a class of plant hormones, regulate many aspects of plant physiology. In the inhibition of shoot branching, the α/β hydrolase D14—which metabolizes strigolactone—interacts with the F-box protein D3 to ubiquitinate and degrade the transcription repressor D53. Despite the fact that multiple modes of interaction between D14 and strigolactone have recently been determined, how the hydrolase functions with D3 to mediate hormone–dependent D53 ubiquitination remains unknown. Here we show that D3 has a C-terminal α-helix that can switch between two conformational states. The engaged form of this α-helix facilitates the binding of D3 and D14 with a hydrolysed strigolactone intermediate, whereas the dislodged form can recognize unmodified D14 in an open conformation and inhibits its enzymatic activity. The D3 C-terminal α-helix enables D14 to recruit D53 in a strigolactone-dependent manner, which in turn activates the hydrolase. By revealing the structural plasticity of the SCFΔD3–ΔD14 ubiquitin ligase, our results suggest a mechanism by which the E3 coordinates strigolactone signalling and metabolism.

Strigolactones represent a class of plant hormones that regulate a variety of plant growth and developmental processes, such as shoot branching, root development, leaf senescence and flower size1–3. Strigolactones are also exuded by plant roots for stimulating interactions with symbiotic fungi4 and exploited by parasitic plants to time their seed germination5–8. As a group of terpenoid lactones, strigolactones typically comprise a butenolide ring (D ring) connected to a variable tricyclic lactone (the ABC rings) via an enol-ether bridge9,10. Functional dissection of both natural and synthetic strigolactone molecules has indicated that the C and D rings and their linkage are essential for strigolactone activity, whereas separated ABC or D rings are inactive in plants10–13. The perception and signal propagation of strigolactones are coordinated by three highly conserved components: DWARF3 (D3) in rice, or the Arabidopsis thaliana orthologue MAX2 (also known as AT2G42620, D14 (AT3G03990) and D33 (LOC4349543) in rice, or the Arabidopsis homologues SMXL6 (AT1G07200), SMXL7 (AT2G29970) and SMXL8 (AT2G40130)12,14–22. As a member of the α/β serine hydrolase superfamily, D14 not only serves as the strigolactone receptor but also metabolizes strigolactones into tricyclic ABC- and D-ring products, albeit at a rate that is much slower than most known α/β hydrolases12,13,23. D3 in rice (or MAX2 in Arabidopsis) encodes an F-box protein and binds Arabidopsis SKP1-like protein (ASK1) to function as a substrate receptor of an SKP1–CUL1–F-box (SCF) ubiquitin ligase complex20,24. Recent studies have shown that D3 or MAX2, when bound to D14, mediates the inhibition of shoot branching by sensing strigolactones and ubiquitinating D53 (or the Arabidopsis homologues SMXL6, SMXL7 and SMXL8), which is a key nuclear repressor that regulates distinct developmental processes and target genes of strigolactone signalling15,17,22,25–28.

Early structural studies of strigolactone perception focused on the binding of the hormone to isolated D14 orthologues12,13,23,29. Crystal structures of several D14 orthologues—either in their apo or ligand-bound forms—revealed a common α/β fold with a large, solvent-exposed ligand-binding pocket6,12–14,23,30. Thus, strigolactones have previously been thought to be perceived by D14 orthologues in this open conformation, although possible conformational changes have also been suggested. A recent study of the pea (Pisum sativum) D14 orthologue RAMOUSUS3 (RMS3) suggested that the α/β-fold hydrolase is a single-turnover enzyme, which produces a covalent D-ring-enzyme complex via the catalytic histidine after substrate hydrolysis and the rapid release of the ABC ring23. The crystal structure of rice ASK1–D3 in complex with Arabidopsis D14 (AtD14; all uses of D14 without a species prefix refer to D14 from Oryza sativa) further uncovered a closed conformation of D3-bound AtD14, which sequesters the covalently linked intermediate molecule (CLIM) of strigolactone inside a small enclosed pocket23. These results raised the possibility that CLIM might represent the active form of the hormone. However, this proposition is complicated by the identification of multiple non-hydrolysable strigolactone agonists6,33–35.

To better delineate the signalling-competent form of D14 in the context of substrate recognition by the SCF E3 and its relationship with hormone hydrolysis, we have performed structure–function studies of the homogeneous rice D14–D3–D53 system. Our analyses have revealed not only structural plasticity in D3 but also functional states of SCFΔD3–ΔD14 that are switchable by D53 for strigolactone hydrolysis.

Structural plasticity of C-terminal α-helix of D3

We first independently determined the crystal structure of D3 in complex with ASK1. D3 contains an N-terminal F-box motif that forms a canonical interface with ASK1. The C-terminal domain of D3 consists of 20 leucine-rich repeats (LRRs) and adopts a fully circularized solenoid fold; the last LRR (LRR20) of D3 makes direct contact with the three N-terminal LRRs and the C-terminal α-helix (CTH) of ASK1 (Fig. 1a). Distinct from most F-box proteins that contain LRRs, the extreme C-terminal residue of D3 (Asp720) is strictly conserved among D3 orthologues in diverse plant species (Extended Data Fig. 1a). The backbone and side-chain carboxyl groups of this Asp720 are simultaneously anchored to a positively charged pocket constructed by ASK1, the F-box motif and the LRR domain of D3 (Extended Data Fig. 1b, c).

We subsequently identified a second crystal form of the D3–ASK1 complex, which was crystallized in a different space group with two

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D3–CTH binds and inhibits D14

This unusual structural feature of D3–CTH prompted us to investigate its role in the D3–D14 interaction. We first established a quantitative method for measuring the GR24-dependent interaction between D3 and D14 in an AlphaScreen assay (Extended Data Fig. 4a). In a dose-dependent manner, a 28-amino-acid peptide of D3-CTH was able to compete with D3 for binding D14 at a saturating concentration of GR24 (Fig. 2a). When fused to glutathione S-transferase (GST), D3-CTH robustly pulled down D14 in a GR24-dependent manner (Fig. 2b), indicating that it can directly interact with the α/β hydrolase. In the previously reported crystal structure of the AtdD14–D3–ASK1 complex, D3-CTH is fully engaged with the LRR domain32. D3-CTH uses its N-terminal tip and preceding loop to assist the recognition of the CLIM-bound α/β hydrolase by three other D3 C-terminal LRRs (LRR17–LRR19) (Extended Data Fig. 4b). Without the rest of the F-box protein, it is unlikely that our D3-CTH peptide interacts with CLIM-bound D14 in a similar manner.

D14 hydrolyses the fluorogenic striaglactone agonist Yoshimulacon Green (YLG) with a biphasic time course that is characterized by a rapid initial phase followed by slow linear hydrolysis (Fig. 2c). Such a two-stage reaction has been reported for RMS3, which becomes completely inhibited by CLIM after hydrolysing a substrate molecule33. Instead of being a single-turnover enzyme, however, D14 slowly released the D-ring under our experimental conditions and continued to hydrolyse additional substrate, as evidenced by the slow linear phase of its enzyme kinetics. Consistent with the recognition and stabilization of CLIM-bound D14 by D3–ASK1, the addition of recombinant D3–ASK1 to D14 reduced the substrate hydrolysis rate of D14 in the slow linear phase without compromising the rapid initial reaction (Fig. 2d). An increasing amount of the isolated D3 C-terminal peptide not only blocked the slow linear hydrolysis but also inhibited the enzymatic activity of D14 (Fig. 2e). Moreover, the half maximal inhibitory concentration of the D3 C-terminal peptide in inhibiting D14 and its affinity to the C-terminal region of D3—when dislodged from the LRR domain—can interact with and block the enzymatic activity of D14 in a manner that is different from the engaged form of this C-terminal region.

Structure of a D3–CTH–D14–GR24 complex

To map the binding mode of a D3 C-terminal peptide to D14 bound to GR24, we crystallized and determined the structure of D14 that is bound to D3-CTH (Extended Data Fig. 5a). In a two-stage reaction has been reported for RMS3, which becomes completely inhibited by CLIM after hydrolysing a substrate molecule33. Instead of being a single-turnover enzyme, however, D14 slowly released the D-ring under our experimental conditions and continued to hydrolyse additional substrate, as evidenced by the slow linear phase of its enzyme kinetics. Consistent with the recognition and stabilization of CLIM-bound D14 by D3–ASK1, the addition of recombinant D3–ASK1 to D14 reduced the substrate hydrolysis rate of D14 in the slow linear phase without compromising the rapid initial reaction (Fig. 2d). An increasing amount of the isolated D3 C-terminal peptide not only blocked the slow linear hydrolysis but also inhibited the enzymatic activity of D14 (Fig. 2e). Moreover, the half maximal inhibitory concentration of the D3 C-terminal peptide in inhibiting D14 and its affinity to the C-terminal region of D3—when dislodged from the LRR domain—can interact with and block the enzymatic activity of D14 in a manner that is different from the engaged form of this C-terminal region.
Second, the electron density is extended beyond the D ring, and points towards the exit of the hormone-binding pocket. Third, the location of the D ring predicts that the tricyclic ABC rings are largely solvent-exposed, which could explain their missing electron density. In comparison to the previously reported GR24–D14 structure\(^2\), the hormone is markedly removed from the catalytic centre instead of being poised for hydrolysis (Fig. 3b). The relative position of the hormone, and its orientation to the active site, suggest that it is bound to the enzyme in a non-reactive configuration.

Upon binding to D14, the D3-CTH sequence adopts the same \(\alpha\)-helical conformation as seen in its engaged form (Fig. 3a, d). D3-CTH docks to a surface site on D14 that is opposite to where D3 binds in the structure of the \(\alpha\)D14–D3–ASK1 complex (Extended Data Fig. 5e). At one end of this interface, Glu700 clamps the D3 \(\alpha\)-helix to the hydrolyase by making polar interactions with Ser224 and His133 on the D14 \(\alpha\)E helix and \(\beta6\)–\(\alpha\)T1 loop, respectively. At the other end of the interface, D3-CTH inserts Leu707 into a hydrophobic cleft formed between the \(\alpha\)E helix and \(\beta8\) strand of D14 (Fig. 3c). As a whole, the helical portion of the D3 C-terminal sequence buries a total of 800 Å\(^2\) surface area on D14. If the acidic C terminus of D3-CTH were not fused to the N terminus of the neighbouring D14 molecule, it might be able to interact with a nearby basic D14 surface (Extended Data Fig. 5f).

Superposition analysis of free D14 and D14 bound to D3-CTH reveals a slight rotation of the cap domain around the hormone-binding pocket, which could couple the docking of D3-CTH to the binding of the unhydrolysed hormone (Extended Data Fig. 5g). A closer comparison of all D14 structures also reveals a potential allosteric pathway that links D3-CTH binding to the D14 catalytic triad (Extended Data Fig. 5h). Importantly, D3-CTH uses several common residues to either bind D14 in its open conformation or engage with the rest of the LRR domain (Fig. 3d). The incompatibility of the two structures strongly suggests that D3-CTH binds D14 when dislodged from the LRR domain. Overall, the binding mode of the D3 C-terminal peptide to D14 reflects a functional state of SCF\(^{D3–D14}\) that is different from D14 bound to CLIM.

Reactivation of D3–bound D14 by D53

In an in vitro protein degradation system, we next reconstituted proteasome-mediated degradation of recombinant D53 with cell-free extracts prepared from \(Arabidopsis\) Col-0 seedlings (Fig. 4a). Consistent with the essential role of the MAX2 in strigolactone signalling, max2-1 extracts lack D53-degrading activity but can be rescued by the addition of recombinant D3 and D14. On the basis of its sequence homology with proteins of the class I Clp ATPase family, D53 is predicted to contain an N-terminal domain and two putative ATPase domains (D1 and D2). We purified each of these D53 domains fused to GST and found that the D2 domain of D53 (D53-D2) is solely responsible for binding D14 in a GR24-dependent manner\(^16\) (Extended Data Fig. 6a). Both full-length D53 and the isolated D2 domain can form a stable complex with D14–D3–ASK1 in the presence of GR24 as detected by size-exclusion chromatography (Fig. 4b, Extended Data Fig. 6b). Although previous studies have suggested that D14 and D3 can individually interact with D53\(^3\),\(^5\)–\(^17\), we found that the D2 domain of D53 becomes stably associated with D14 only in the presence of D3 and GR24. The three binding partners, therefore, assemble cooperatively into a ternary complex, which explains the degradation of the D2 domain of recombinant D53 by the proteasome in a MAX2-dependent manner (Fig. 4a).

We next used the D2 domain of D53 to probe the role of the C-terminal region of D3 in D14-mediated substrate binding. Consistent with the ability of D3 to flip out its CTH without compromising its structural integrity, truncating the 28-amino-acid C-terminal region had no detectable effect on the folding and solution behaviour of D3 (Extended Data Fig. 6c). However, the C-terminally truncated D53 mutant protein could neither form a ternary complex with D14 and D53-D2 on a sizing column, nor restore the D53 degradation activity of the max2-1 extracts (Fig. 4a, Extended Data Fig. 4c, d), which indicates a critical role of the C-terminal region of D53 in substrate recruitment by SCF\(^{D3–D14}\). The isolated C-terminal peptide of D3 was able to stimulate D14 and D53-D2 to pull down one another in the presence of GR24 (Fig. 4c, Extended Data Fig. 7a). In the more-quantitative AlphaScreen assay, the D3 peptide—but not two shorter versions—elicited the same effect in a dose-dependent manner (Extended Data Fig. 7b). Mutation of a single D14 residue (S224E) at the interface revealed in our D3–CTH–D14 structure compromised D3–CTH–D14 binding and was sufficient to prevent GST–D53–D2 from pulling down D3 (Extended Data Fig. 7c, d). These data strongly suggest that the C-terminal region of D3 helps recruit D53 when D3-CTH is liberated from the LRR domain of D3 and becomes compatible for binding D14 with the canonical open conformation. This notion is further corroborated by the impaired D53 degradation observed with either the isolated D3-CTH peptide or the D14(S224E) mutation in the cell-free extracts (Extended Data Fig. 7e, f). D53 was originally identified through the gain-of-function rice mutant \(d53\), the gene product of which becomes resistant to strigolactone-induced degradation owing to the loss of four amino acids in the D2 domain\(^5,\(^16\). Accordingly, the D2 domain of the recombinant D53 mutant protein was unable to pull down D14 in the presence of D3-CTH and GR24, and remained stable in Col-0 cell extracts (Extended Data Fig. 7a, g). These results further support the functional relevance of the D3-CTH-mediated recruitment of D53 to SCF\(^{D3–D14}\).

Given the structural flexibility of D14, we next investigated the effect of substrate binding on the hydrolase activity of D14. Similar to GR24, YLG can induce complex assembly among D14, D3–ASK1 and the D2 domain of D53 (Extended Data Fig. 7h). By monitoring YLG hydrolysis, we detected little change in the enzymatic kinetics of D3–ASK1-arrested D14 when the D2 domain of D53 was present (Extended Data Fig. 7i). By contrast, the addition of D53 robustly blocked the inhibition of the enzymatic activity of D14 by the D3 C-terminal peptide, both in the rapid initial reaction and the slow linear phase (Fig. 4d). Together, these results suggest that the enzymatic activity of D14 within the SCF\(^{D3–D14}\) ubiquitin ligase complex is susceptible to modulation...
Fig. 4 | Interactions among D3-CTH, D53 and D14. a, Time-dependent degradation of GST–D35 and GST–D35–D2 in Arabidopsis seedlings of Col-0 and max2-1 mutant extracts, with and without recombinant D14 and either D3 or D3(ΔCTH). MG132, proteasome inhibitor. b, Size-exclusion chromatography analysis of the interaction between the D2 domain of D35 and D14–GR24 ± ASK1–D3, with sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the elution fractions. c, GST pull-down assay using recombinant GST–D14 and non-tagged D35–D2 ± D3-CTH and/or GR24. d, Kinetics of YLG hydrolysis by D14 in the presence of D3-CTH at increasing concentrations. All experiments repeated three times.

Fig. 5 | SMXL7–YFP stability in response to GR24 and D3-CTH expression. a–d, Representative images of levels of SMXL7 in response to GR24 application in tobacco epidermal cells. Levels of SMXL7–YFP (yellow) at 0 and 120 min post-treatment are shown for single nuclei that co-express empty vector (a), D14–mCherry (b), D14-CTH–mCherry (c) or CTH-NLS–mCherry (d), all of which are displayed in magenta. Scale bars, 10 μm. e, Relative SMXL7 abundance at 2 h post-treatment, comparing GR24 and mock treatment in cells that either express SMXL7–YFP alone or co-express SMXL7 and D14–mCherry, D14-CTH–mCherry or CTH-NLS–mCherry, respectively. Yellow dots and bars are mean ± s.e.m., ***P < 0.001, n = 7 (nuclei), two-tailed Student’s t-test. Black dots, data more than 3 s.d. from mean. Coloured boxes represent the central 50% of the distribution, with the median shown as a horizontal bar. Top and bottom vertical bars represent 75–100% and 0–25% of the distribution of data points, respectively. P values for empty vector, D14, D14-CTH and CTH-N7 (N7 is a nuclear localization sequence) are 1.29 × 10−5, 7.188 × 10−4, 4.401 × 10−2 and 3.86 × 10−1, respectively.

SMXL7 levels are compromised by D3–CTH
To further validate the role of D3-CTH in vivo, we expressed SMXL7–YFP alone or in combination with AtD14, AtD14 fused to MAX2-CTH (AtD14-CTH), or MAX2-CTH (CTH) alone in tobacco epidermal cells. Despite the cross-species reactions, SMXL7 was markedly destabilized upon GR24 treatment (23% reduction, Fig. 5a, e), which indicates that the strigolactone perception machinery that is endogenous to tobacco epidermal cells is sufficient to induce GR24-dependent degradation of SMXL7. In support of the functionality of AtD14 in tobacco epidermal cells, the above response was further accentuated in nuclei that co-express AtD14–CTH–mCherry—reaching a nearly 50% reduction in the level of SMXL7 by the end of the incubation time (Fig. 5b, e). However, this enhancement effect was completely eliminated and reversed to 11.8% and 6% reduction when SMXL7–YFP was co-expressed with D14–CTH–mCherry or CTH-NLS (nuclear localization sequence)–mCherry, respectively (Fig. 5c–e). The MAX2-CTH, therefore, not only prevented AtD14 from accelerating the degradation of SMXL7 but also impaired the destabilization of SMXL7 by endogenous strigolactone-signalling components, in response to GR24 treatment.

A model of the functional states of SCF D3–D14
Our studies have uncovered a structural plasticity in the D3 F-box protein, which can adopt two distinct structural states by altering the topology of its CTH. With an engaged CTH, the F-box protein is structurally compatible for binding the inactive closed conformation of CLIM-bound D14. When the D3-CTH is unleased from the LRR domain, D3 uses the helical structural element to capture hormone-bound D14 via a different interface. In this binding mode, the hydrolase maintains its open conformation, which allows its enzymatic activity to be tunable by D35, the substrate of the SCF E3. We postulate that the plant SCFD3–D14 complex has evolved these unusual features to orchestrate strigolactone sensing, substrate polyubiquitination and hormone metabolism in a highly coordinated manner. To explain the activity of non-hydrolysable strigolactone agonists5,33–35, we propose that D14 perceives and transduces the hormonal signal in its open conformation, which is recognized by the D3-CTH and is competent for D35 binding (Extended Data Fig. 8, Supplementary Discussion). Before loading the SCF substrate, D3 arrests strigolactone-bound D14 to prevent premature hydrolysis of the hormone. Hormone-dependent association of D35 with SCFD3–D14 not only triggers D35 polyubiquitination but also licenses D14 to catalyse strigolactone metabolism, which takes place while D35 is being fully modified (or after D35 has been fully modified) by a ubiquitin chain. A more-detailed quantitative understanding of the timing of strigolactone hydrolysis and polyubiquitin chain assembly on a substrate awaits further studies.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0743-5.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

Protein preparation and purification. The full-length rice D3 (O. sativa) and A. thaliana ASK1 were co-expressed as a 6 × His–2 × Msb (mxyB)36 fusion protein and an untagged protein, respectively, in Hi5 suspension insect cells. The ASK1–D3 complex was isolated from the soluble cell lysate by Q Sepharose High Performance resin (GE Healthcare). NaCl eluates (500 mM) were subjected to Nickel Sepharose Fast Flow (GE Healthcare) and were eluted with 250 mM imidazole. To remove the 6 × His–2 × Msb fusion tag, the clarified complex was cleaved at 4 °C for 16 h by tobacco etch virus (TEV) protease, and was further purified by anion exchange and gel-filtration chromatography. For crystallization and biochemical analysis purposes, the D3-expressing construct was designed to eliminate a non-conserved 40-residue disordered loop between amino acid 476 and amino acid 514 after affinity purification. The resulting D3 fusion protein contains an 6 × His–2 × Msb tag at the N terminus and three TEV protease sites: between the Msb tag and D3, after T476 and before L514, yielding a purified split form of D3 with D3 N-terminal domain (1–476) and C-terminal domain (514–720) stably associated (Extended Data Figs. 2, 3). D3(∆CTH) (O. sativa, residues 1–693) was co-expressed with ASK1 and purified in the same manner as full-length D3. Purified ASK1–D3 and ASK1–D3(∆CTH) complexes were independently eluted as a single monodisperse peak off a Superdex-200 gel-filtration column (GE Healthcare) with an estimated molecular weight of 93 kDa or 90 kD, respectively. The D3(∆CTH) construct, which lacks the C-terminal 10 amino acids, was also purified using a similar procedure. Rice D14 protein (O. sativa, residues 52–318) was expressed as a 6 × His–SUMO fusion protein from the expression vector pSUMO (LifeSensors, and a gift from E. Xu). 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 h. Cells were collected, re-suspended and lysed in extract buffer (20 mM Tris–HCl, pH 8.0, 200 mM NaCl). His–SUMO–D14 was isolated from soluble cell lysate by Ni-NTA resin. The eluted His–SUMO–D14 was subjected to anion exchange and the eluted His–SUMO–D14 was cleaved overnight with SUMO protease (ULP1, LifeSensors) at a protease-to-protein ratio of 1:1,000 at 4 °C. The clarified His–SUMO tag was removed by passing through a Nickel Sepharose column, and the protein was further purified by chromatography through a Superdex-200 gel-filtration column in 20 mM Tris, pH 8.0, 200 mM NaCl, 2 mM DTT. Full-length D53 (O. sativa) was expressed as a GST fusion protein in Hi5 suspension insect cells. D14 (O. sativa, residues 52–318), D3-CTH (O. sativa, residues 693–720), D53 N domain (D53-N, residues 1–181), D53 D1 domain (D53-D1, residues 182–406), D53 D2 domain (D53-D2, residues 718–1,131), and the D2 domain of the D53 mutant (F811T followed by deletion of residues 812–818, as previously described13,17) were expressed as GST fusion proteins in BL21 (DE3) cells. The recombinant protein containing the tagged protein (isolated by Ni-NTA resin) was incubated in the presence of 5 μM ASK1–D3 complex proteins and PHENIX and the final model was built, refined and rebuilt with COOT39 and PHENIX. The small final model was built and refined with a native dataset. The crystals of ASK1–D3 form 2 and ASK1–D3 form 3 complexes were determined by molecular replacement using ASK1–D3 form 1 structure as the search model. The D14–D3-CTH structure was determined by molecular replacement using rice D14 structure (PDB 4I9H)38 as the search model. All structural models were manually built, refined, and rebuilt with PHENIX and COOT.

AlphaScreen luminescence proximity assay. AlphaScreen assays for determining the proximity of protein interactions were performed using Enzyme Immuno assay plates (PerkinElmer). GST-tagged D53 or D14 was attached to glutathione AlphaScreen donor beads. His-tagged D14 or D3 was attached to anti-6 × His conjugated AlphaScreen acceptor beads. The donor and acceptor beads were brought into proximity by the interactions between D14, D3 and ASK1–D3 complex, which were measured with and without GR24 and/or non-tagged proteins at indicated concentrations. When excited with a laser beam of 680 nm, the donor beads emit singlet oxygen that activates thioxene derivatives in the acceptor beads, which then release photons of 520–620 nm as the binding signal. The experiments were conducted with 100–500 nM of D14 or D3 and 1 μM ASK1–D3 complex proteins in the presence of 5 μg/ml donor and acceptor beads in a buffer of 50 mM MES, pH 6.5, 150 mM NaCl, 1 mM DTT and 0.1 mg/ml bovine serum albumin. The results were based on an average of three experiments with standard errors typically <10% of the measurements. Half maximal inhibitory concentration values were determined using nonlinear curve-fitting of graphs generated with Prism 6 (GraphPad).

YLG hydrolysis assay. YLG (TCL America) hydrolysis assays were performed using 1–2 μg of recombinant proteins in a reaction buffer (50 mM MES pH 6.5, 150 mM NaCl and 1 mM DTT) at 100-μl volume on a 96-well black plate (Greiner). The fluorescence intensity was measured by EnSpire 2300 multilabel plate reader (PerkinElmer) at excitation by 480 nm and detection by 520 nm. Ninety-six-well black half-area plates were covered with Viewdrop III UV plate seals to prevent evaporation. Time-course experiments were performed in 10–s intervals over 50–60 min. Fluorescence data were converted directly to fluorescein concentration using a standard curve. Data generated in Excel were transferred to Prism 6 for graphical analysis and curve-fitting. In all cases in which synthesized peptides (Genscript and Biomatik) were analysed, dimethylsulfoxide (DMSO) was added in equivalent concentration into the reaction.

Size-exclusion chromatography. Purified proteins (20–50 μM) were incubated with 100–200 μM GR24 (Chiralx), or equal amount of acetone as the solvent control, at 4 °C for one hour in 20 mM HEPES, pH 7.0, 150 mM NaCl and 2 mM DTT. The reaction was injected onto a Superdex-200 Increase 10/300 column (GE Healthcare) for analysis at a flow rate of 0.5 ml min−1. The elution fractions (0.5 ml per fraction) were resolved by SDS–PAGE and analysed by Coomassie blue G-250 stain.

Limited proteolytic digestion. One milligram per millilitre of purified ASK1–D3 (or ASK1–D3(∆CTH)) was incubated at 4°C for 12 h with increasing amounts of trypsin (2.5 μg, 5 μg, 10 μg, 15 μg and 20 μg), 4°C for 10 min. To monitor protein degradation in the cell-free system, 0.5 μg of purified GST-tagged proteins (either full-length D53, D53-D3 or D53-D2, as indicated) was incubated at 22°C
in a reaction mixture that contained, at a final volume of 12.5 μl, 1–2 μl of plant extract supplemented with 10 μM GR24, 25 mM Tris–HCl, pH 7.4, 0.625 mM ATP, 5 mM MgCl₂ and 0.5 mM DTT. Where indicated, the proteasome inhibitor MG132 (Calbiochem) was added at a concentration of 100 μM. Reactions were terminated at the indicated times by the addition of fivefold-concentrated sample buffer. Boiled samples were resolved via SDS–PAGE, and proteins were visualized using western blot and polyclonal anti-GST antibodies (Sigma).

Plant growth conditions. Nicotiana benthamiana plants were grown on F2 compost (Levington Horticulture) pre-treated with 0.2 g l⁻¹ Intercept (Everris). Glasshouse conditions as follows: 16 h light:8 h darkness, minimum irradiance 88 W per m², shading implemented at 500 W per m² and cooling implemented at 31 °C. Humidity and temperature were determined by the ambient conditions.

Cloning and plant transformation. All constructs were cloned using Multi-site Gateway (Invitrogen). A previously generated CaMV 35S promoter and SMXL7–YFP expression vector were used²⁵. D14, D14-CTH and CTH-NLS sequences were synthesized and cloned into pDONR221. The mCherry and YFP fluorescent tags were cloned into the pDONR P2-P3R and all ENTRY vectors were then recombined in the relevant combinations in pH7m24GW (https://gateway.psb.ugent.be/). Agrobacterium tumefaciens strain GV3101 was transformed using standard electroporation procedure.

Assays for transient gene expression mediated by A. tumefaciens in N. benthamiana. A. tumefaciens (strain GV3101) carrying the desired transfer DNA construct was grown overnight at 28 °C with the appropriate antibiotics. Cells were collected by centrifugation at 8,000g and resuspended in agro-infiltration medium with 5 mM MES, 10 mM MgCl₂, pH 5.6, before syringe infiltration into leaves of 3–4-week-old N. benthamiana plants. Bacteria carrying each construct were infiltrated at a final OD₆₀₀ₙₐₙ of 0.4. Leaves were detached 48 h post-infiltration for confocal imaging.

Confocal microscopy. All confocal images were captured on a Leica SP8 laser scanning confocal with a W Plan-Apochromat 20× 1.0 numerical aperture objective (Zeiss). Detection wavelengths: 520–540 for SMXL–YFP, and 600–620 for mCherry-tagged proteins. The pinhole was set to one airy unit for all nuclei. Detection gain and laser power were kept constant between t₁ and t₂ for intensity comparison and the same settings were used for all nuclei of the same construct combination. Laser power was adjusted across construct combinations as necessary to account for differences in expression level and avoid signal saturation.

SMXL7 quantification. Two days after infiltration, leaves were infiltrated with A. thaliana salt (ATS) with 0.1% v/v acetone for the mock or 10 μM GR24, 0.1% v/v acetone for the treated samples. Between 7 and 13 nuclei expressing 35S:SMXL7–YFP alone or in combination with either 35S:D14–mCherry, 35S:D14-CTH–mCherry or 35S:CTH-NLS–mCherry were located and imaged at time 0. The same nuclei were then imaged again using identical settings after 120 min for each construct combination and treatment. Areas of interest were drawn around each nucleus in ImageJ version 2.0.0 and the mean signal intensity was recorded. The ratio of the means at 0 min and 120 min post-treatment was used to compute relative fluorescence at t₂, which is expressed as the percentage of change in the level of SMXL7. Distributions for each combination and treatment condition were compared using a two-tailed Student’s t-test with Bonferroni correction.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Structural coordinates and structural factors have been deposited in the RCSB Protein Data Bank under accession numbers 6BRO, 6BRP, 6BRQ and 6BRT. Uncropped gels and blots are available in the Supplementary Information. All other data are available from the corresponding author upon reasonable request.

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Extended Data Fig. 1 | Conservation and conformation of D3 C-terminal α-helix. a, Sequence alignment of the C-terminal regions of 14 orthologues of MAX2 or D3. Highly conserved residues are coloured in blue. b, Electrostatic-potential surface map of D3 with CTH shown in cartoon representation (orange). The C terminus aspartic acid residue (Asp720) is anchored to a positively charged pocket. c, Close-up view of the D3 extreme C-terminal residue (Asp720) and its interacting residues in D3 and ASK1. d, Electron densities of the D3-CTH region in two different crystal forms, adopting either a regular helical conformation (left) or an extended conformation (right).
Extended Data Fig. 2 | Sequence alignment and analysis of selected orthologs of D3 or MAX2. Orthologs of D3 or MAX2 are selected and aligned from rice (O. sativa) (accession XP_015643693), A. thaliana (NP_5655979), castor (Ricinus communis) (XP_002582851), poplar (Populus trichocarpa) (XP_002320412), grapevine (Vitis vinifera) (XP_010657042), cucumber (Cucumis sativus) (XP_001437031), monkey flower (Erythranthe guttata) (XP_012832933), tobacco (Nicotiana sylvestris) (XP_009757168), medicago (Medicago truncatula) (XP_030630792), pea (Pisum sativum) (ABD67495), soybean (Glycine max) (XP_003540983), and sorghum (Sorghum bicolor) (XP_002436499) and moss (Physcomitrella patens) (XP_024400746). The non-conserved region designed to be truncated by TEV cleavage during recombinant D3 purification is underlined in green.
Extended Data Fig. 3 | Comparison of D3–ASK1 structures. a, Top view of ASK1–D3 crystal structure (orange) based on PDB 5HYW. Red arrows indicate a gap in the polypeptide model. Note that PDB 5HYW has a polypeptide register error ranging from amino acid 373 to 473 before the gap. b, Superposition of ASK1–D3 determined in this study (light blue) with PDB 5HYW. The region truncated by design ranges from N474 to L516, which are indicated by red arrows. c, Superposition of all three crystal forms of ASK1–D3 determined in the current study. d, Limited trypsin digestion assay of ASK1–D3 and ASK1–D3(ΔCTH). Proteins were resolved by SDS–PAGE followed by Coomassie blue stain, focusing on D3 C-terminal domain. The experiment was repeated three times.
Extended Data Fig. 4 | Established binding between D3 and D14.

**a**, AlphaScreen assay measuring direct interaction between GST–D14 and His–D3 in response to increasing amounts of GR24 (mean ± s.d. of biological triplicates).

**b**, The binding interface between CLIM-bound D14 (magenta) and the LRR domain of D3 (blue) (PDB 5HZG). The last four LRRs are labelled, and D3-CTH in LRR20 is coloured in orange.
Extended Data Fig. 5 | Structural analysis of D3-CTH–D14–GR24 complex. a, Packing of two D14 molecules that are N-terminally fused with D3-CTH. The D3-CTH region in chain A is omitted. The GR24 D ring (sticks) is shown together with the surround 2Fo − Fc electron-density map calculated before the compound modelled in and contoured at 0.8σ. b, A close-up view of the GR24 D ring (sticks, green) and its electron density, calculated as in a. c, Overall structure of D14 (magenta) bound to D3-CTH (orange), with a GR24 D ring (green sticks). The GR24 hydrolysis product D-OH (cyan sticks)—revealed in the D14-D-OH structure (PDB 3WIO)—is shown on the basis of superposition analysis. d, Kinetics of YLG hydrolysis by free D14 and D14 fused to D3-CTH. Experiment repeated three times. e, Comparison of the interface that D14 (magenta and brown) makes upon binding to D3-CTH (orange) versus upon binding to ASK1–D3 (blue). The lid domain (brown) of D14 adopts open and closed conformation upon binding to D3-CTH and ASK1–D3, respectively. f, Electrostatic-potential surface map of D14 bound to D3-CTH (orange). The dashed line indicates the C-terminal region of D3 that would otherwise be free, if D3-CTH were not fused to another copy of D14 in the crystal. g, Conformational changes in the lid domain of D14, induced by D3-CTH binding, as revealed by superposition analysis between D3-CTH-bound (magenta) and apo D14 (grey, PDB 4IH9). Arrows indicate the rotation of the lid domain of D14, induced by D3-CTH (orange), relative to the catalytic triad shown in sticks. h, Superposition analysis of apo D14 (PDB 3W04) and D14 bound to D3-CTH, which highlights a possible allosteric pathway that connects Leu707 of D3-CTH to the catalytic triad of D14. Arrows indicate conformational changes within D14 that are induced by binding to D3-CTH.

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Extended Data Fig. 6 | The formation of the D3–D14–D53 complex is mediated by the D2 domain of D53. a, Pull-down assay using recombinant ASK1–D3, His–D14, and GST-tagged N domain (D53-N), D1 domain (D53-D1) or D2 domain of D53. b–d, Size-exclusion chromatography analyses of the interaction between: full-length GST–D53, D14–GR24 and ASK1–D3 (b), D14–GR24 and either ASK1–D3 or ASK1–D3(ΔCTH) (c), and D14–GR24 and D53-D2 with ASK1–D3(ΔCTH) (d). All gels were resolved by SDS–PAGE and analysed by western blot using anti-GST and anti-His antibodies (as indicated under a) or Coomassie blue staining (b–d). All experiments shown in a–d were repeated independently at least three times.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | D3-CTH facilitates the binding of the D2 domain of D53 to D14–D3.  

**a**, GST pull-down assay using GST–D53-D2 or the GST-tagged D2 domain of the d53 (GST–D53-D2) mutant with non-tagged D14, in the presence or absence of D3-CTH as indicated.  

**b**, AlphaScreen data showing the ability of the D3-CTH peptide (28 amino acids, D3(693–720)) to promote the interaction between D53-D2 and D14 in a dose-dependent manner; D3(693–707) (15 amino acids) and D3(708–720) (13 amino acids) peptides did not stimulate binding. DMSO (indicated as ‘no peptide’) served as control (data are mean ± s.d. of biological triplicates).  

**c**, GST pull down using recombinant GST–D53-D2 and His–D3-ASK1 in the presence of recombinant D14 wild type (WT), D14(A223E), D14(S224E) and GR24 as indicated.  

**d**, GST pull down in the presence of the D3-CTH peptide with or without GR24, and in the presence of GST–D14 wild type or GST–D14(S224E). BSA was used in the assay to prevent non-specific interactions. MG132 was added as indicated. Proteins were resolved using SDS–PAGE, and were visualized by Coomassie blue staining or western-blot using anti-GST antibodies. The D3-CTH peptide contains four amino acid mutations that were designed to disrupt the D14–D3-CTH interface: E700R, L707R, D719R and D720R.  

**e, f**, Degradation of GST–D53-D2 in the Col-0 (e) or max2-1 (f) *A. thaliana* cell-free extract system. GST–D53-D2 was resolved at the indicated time in the presence or absence of the wild-type D3-CTH peptide (e, top) or a mutant (MT) (e, bottom), and in the presence of D3 and either D14 wild type or the D14(S224E) mutant (f).  

**g**, Time-dependent degradation of GST–D53-D2 and GST–d53-D2 in *Arabidopsis* seedlings of Col-0 extracts. Proteins were resolved by SDS–PAGE, and analysed by western blot using anti-GST antibody. MG132 indicates the addition of proteasome inhibitor.  

**h**, Size-exclusion chromatography analysis of complex formation among D53-D2, ASK1–D3 and D14 in the presence of YLG.  

**i**, Kinetics of YLG hydrolysis by D14 in the presence of ASK1–D3 and D53-D2 at two concentrations. Gels were resolved by SDS–PAGE and analysed by western blot using anti-GST and anti-His antibodies as indicated under c, e–g. All experiments were repeated independently at least three times.
Extended Data Fig. 8 | A model for strigolactone perception and signalling. A model of the activity cycle that underlies strigolactone-induced and SCF<sup>D3-D14</sup>-mediated D53 polyubiquitination. D3 adopts two conformational states with a structurally variable CTH (left). With a dislodged CTH, D3 binds and inhibits D14 in its open conformation, until D53 is loaded (top). D53 binding re-activates D14, which can hydrolyse strigolactones after or while D53 is polyubiquitinated. The strigolactone hydrolysis intermediate then stabilizes the closed conformation of D14, which converts D3-CTH into its engaged form. The resulting complex can ubiquitinate D14 and feed D3 back to the activity cycle (right). CLIM-bound D14 might participate in D53 polyubiquitination or in an alternative path (bottom). It remains unknown how many strigolactone molecules are hydrolysed during the polyubiquitination of each D53 molecule.
Extended Data Table 1 | Data collection and refinement statistics

| Data collection                                | ASK1-D3 (form 1) | ASK1-D3 (form 2) | ASK1-D3 (form 3) | D3-CTH-D14-GR24 |
|------------------------------------------------|------------------|------------------|------------------|-----------------|
| Space group                                    | C2               | C2               | P2₁              | P6₃             |
| a, b, c (Å)                                    | 233.7 79.7 153.4 | 237.4 79.8 151.7 | 79.4 130.4 94.3  | 77.9 113.3 92.8  | 183.8 183.8 153.6|
| α, β, γ (°)                                    | 90 128.6 90      | 90 129.7 90      | 90 99.4 90       | 90 99 90        | 90 90 120          |
| Resolution (Å)                                 | 50.00 - 2.50     | 50.00 - 2.50     | 50.00 - 2.40     | 50.00 - 2.40    | 50.00 - 2.40     |
| Rmerge (°)                                     | 0.126 (0.403)    | 0.128 (0.539)    | 0.179 (0.939)    | 0.129 (0.658)   | 0.172 (0.699)     |
| Completeness (%)                               | 98.8 (88.4)      | 95.9 (71.6)      | 98.9 (97.8)      | 96.6 (97.8)     | 100 (99.8)        |
| Redundance                                      | 4.7 (3.6)        | 8.1 (6.3)        | 7.2 (5.6)        | 3.7 (3.5)       | 11.7 (8.9)        |
| Refinement                                     |                  |                  |                  |                 |                 |
| Resolution (Å)                                 | 2.50             | 2.40             | 3.00             | 2.40            |
| No. reflections                                | 75577            | 73464            | 31050            | 115753          |
| Rmerge / Ramal (%)                             | 20.0/22.5        | 19.2/21.8        | 22.0/25.6        | 24.8/30.0       |
| No. atoms                                      | 11832            | 12231            | 10771            | 17166           |
| Protein                                        | 11366            | 11737            | 10771            | 16607           |
| Ligand/ion                                     | 0                | 0                | 0                | 8               |
| Water                                          | 466              | 494              | 0                | 551             |
| B-factors                                      |                  |                  |                  |                 |                 |
| Protein                                        | 39.3             | 35.5             | 65.6             | 32.4            |
| Ligand/ion                                     | N/A              | N/A              | N/A              | 38.6            |
| Water                                          | 34.9             | 31.9             | N/A              | 24.1            |
| R.m.s. deviations                              |                  |                  |                  |                 |                 |
| Bond lengths (Å)                               | 0.010            | 0.010            | 0.008            | 0.011           |
| Bond angles (°)                                | 1.48             | 1.40             | 1.31             | 1.27            |
| Ramachandran favored (%)                       | 97.9             | 98.5             | 96.5             | 96.0            |
| Ramachandran allowed (%)                       | 2.1              | 1.5              | 3.5              | 4.0             |
| Ramachandran outliers (%)                      | 0                | 0                | 0                | 0               |
| PDB ID                                         | 6BRO             | 6BRP             | 6BRQ             | 6BRT            |

This table describes the data collection, phasing and refinement statistics of ASK1-D3 crystals in three forms, as well as crystals of D3-CTH-D14-GR24. Values in parentheses are for the highest-resolution shell.
Reporting Summary

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### Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
|     | ✓         |
| The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement | ✓ |
| An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly | ✓ |
| The statistical test(s) used AND whether they are one- or two-sided | ✓ |
| Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| A description of all covariates tested | ✓ |
| A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons | ✓ |
| A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) | ✓ |
| For null hypothesis testing, the test statistic (e.g. \(F, t, r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted | ✓ |
| Give \(P\) values as exact values whenever suitable. |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | ✓ |
| For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes | ✓ |
| Estimates of effect sizes (e.g. Cohen's \(d\), Pearson's \(r\)), indicating how they were calculated | ✓ |
| Clearly defined error bars | ✓ |
| State explicitly what error bars represent (e.g. SD, SE, CI) | ✓ |

Our web collection on [statistics for biologists] may be useful.

### Software and code

| Policy information about: availability of computer code |
|--------------------------------------------------------|
| Data collection                                       |
| Crystallography: HKL2000 for X-ray data collection. Leica SP8 laser scanning confocal: Leica Application Suite X and ImageJ version 2.0.0 |
| Data analysis                                         |
| Prism 7.00 was used to analyze and produce graphs.     |
| Protein structure statistics were produced, processed and analyzed by HKL2000, CCP4, Phenix 1.13, COOT 0.8, 8 and PyMOL 1.8.6.0 |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

### Data

| Policy information about: availability of data |
|-----------------------------------------------|
| All manuscripts must include a data availability statement. This statement should provide the following information, where applicable: |
| - Accession codes, unique identifiers, or web links for publicly available datasets |
| - A list of figures that have associated raw data |
| - A description of any restrictions on data availability |

The proteins coordinate and atomic structure factors have been deposited in the Protein Data Bank (PDB) under accession number 6BRP, 6BRT, 6BRO, 6BRQ. All other data are available from the corresponding author upon reasonable request.
Field-specific reporting

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☑ Life sciences   ☐ Behavioural & social sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were determined based on prior literature and best practices in the field; no statistical methods were used to pre determine sample size |
|-------------|----------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded |
| Replication | Each experiment was reproduced at least three times on separate occasions. Experimental findings were reliably reproduced. |
| Randomization | Animal experiments were not performed in this study, so no randomization was needed. |
| Blinding | Animal experiments were not performed in this study, so Investigators were not blinded to the experiment |

Materials & experimental systems

Policy information about availability of materials

n/a

Involved in the study

☒ ☐ Unique materials
☒ ☒ Antibodies
☐ ☒ Eukaryotic cell lines
☐ ☒ Research animals
☒ ☒ Human research participants

Antibodies

Antibodies used

Antibodies were used for Western-Blotting:
- Anti-Glutathione-S-Transferase (GST) antibody- produced in rabbit (Sigma, G7781)
- Monoclonal Anti-polyHistidine, antibody produced in mouse (Sigma, M1029)
- Amersham ECL Mouse IgG, HRP-linked whole Ab (from sheep) Lot 9793520
- Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) Lot 12219044

Validation

All Antibodies used in this study were certified and validated by manufacturers and vendors

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

SF9 and High Five insect cells were used for recombinant protein expressions only

Authentication

Cells have been authenticated by the vendors. No further authentication was performed for commercially available cell lines.

Mycoplasma contamination

Cells were not tested for mycoplasma contamination.

Commonly misidentified lines

(See ICLAC register)

no commonly misidentified cell lines were used
| Method-specific reporting |
|---------------------------|
| n/a | Involved in the study |
|    | □ ChIP-seq |
|    | □ Flow cytometry |
|    | □ Magnetic resonance imaging |