COMMENTARY

Plant ubiquitin ligases as signaling hubs

Nitzan Shabek & Ning Zheng

The past decade has witnessed an explosion in the identification of ubiquitin-ligase complexes as the missing receptors for important small-molecule hormones regulating plant growth and development. These breakthroughs were initiated by genetic approaches, with structural analysis providing mechanistic insights into how hormone perception and signaling are coupled to protein ubiquitination. Although there are still many unknowns, plants have imparted valuable lessons about the pharmacology of ubiquitin modification.

Plants are amazing organisms: they shape the biosphere on earth and provide oxygen, food, shelter and medicine to humans. Moreover, since the dawn of modern biology, they have helped scientists understand the foundations of life. After all, it was plants that gave away the secret of the cell to Robert Hooke and offered Gregor Mendel a phenotype-rich storytelling system as he pioneered modern genetics. At the turn of the twenty-first century, the field of ubiquitin research has had its own opportunity to learn from plants, which are adept at leveraging the full power of the ubiquitin-proteasome system (UPS) to regulate their physiology.

Plant UPS

When the ubiquitin polypeptide was first isolated in 1975 from calf thymus as the 'ubiquitous immunopoietic polypeptide', it was also detected in tissues of higher plants, including celery stalk, eggplant fruit and carrot root[1]. In fact, as it does in animals, the UPS has a fundamental role in virtually every aspect of growth and development in plants. In the UPS, ubiquitin is covalently attached to target proteins through the action of three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase)[2]. Depending on the type and extent of this modification, ubiquitination either targets the substrate proteins for destruction by the 26S proteasome or alters their biochemical properties and subcellular localization. In the plant model organism *Arabidopsis thaliana*, more than 6% of the proteome is composed of UPS components, with potentially thousands of additional UPS-associated proteins and target substrates[3,4]. Similarly to animals, *A. thaliana* has two E1 isoforms and at least 40 E2s[5], but its genome is predicted to encode more than a thousand potential E3s, far exceeding those in other eukaryotes[4]. This large number of ubiquitin E3 ligases hints at an expanded and unique usage of the UPS in the plant kingdom. Notably, one of the largest gene families in *A. thaliana* encodes nearly 900 F-box proteins, which function as the substrate-receptor module of an evolutionarily conserved multisubunit E3 family, the Skp–cullin–F-box complex, or SCF (**Fig. 1**). A recent phylogenetic comparison of the F-box genes within the plant kingdom has revealed substantial variations in the size and composition of the superfamily even among closely related species[6]. Therefore, the F-box E3 genes have been, and probably still are, rapidly evolving in land plants.

Nitzan Shabek and Ning Zheng are in the Department of Pharmacology, Howard Hughes Medical Institute, University of Washington, Seattle, Washington, USA.
e-mail: nzheng@uw.edu

Plant UPS and photomorphogenesis

As the energy source for photosynthesis, light is one of the most important environmental factors that control plant developmental processes. Under light, plant seedlings adopt a developmental program known as photomorphogenesis. In the 1990s, extensive genetic screens for photomorphogenesis repressors identified a group of genes whose defects led to constitutive light-grown phenotypes in darkness[7]. Remarkably, almost all of those genes have an immediate connection to the UPS: *COP1* encodes a RING-type E3; *COP10* (*FUS9*) encodes a classic E2 variant lacking the active site cysteine; and the other COP genes encode subunits of the COP9 signalosome (CSN), which is homologous to the lid subcomplex of the 19S proteasome. Thanks to the synergy between plant and animal studies, it is now understood that both the COP1 and COP10 proteins, as well as the protein product of another photomorphogenesis repressor gene, *DET1*, are part of the SCF-related Cul4A–DDB1 E3 complex[8], which regulates transcription. Furthermore, CSN modulates the ligase activities of CUL4A–DDB1, SCF and

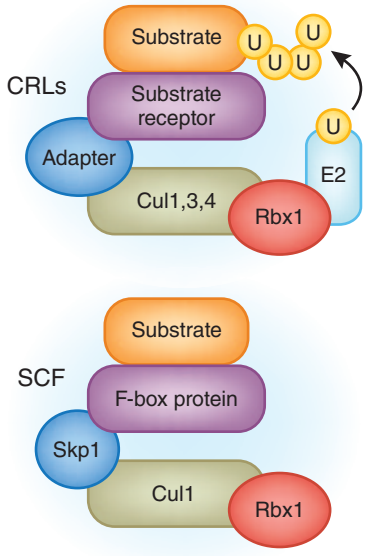

Figure 1 The general modular architecture of CRLs and SCF E3 ligase complexes. The multisubunit CRL E3s are organized by a catalytic core complex formed between a cullin protein (Cul1, Cul3 and Cul4 (Cul1,3,4) in plants) and the RING-domain protein Rbx1. Specific substrates are recruited to each family of CRLs via a unique group of interchangeable substrate receptors. In all CRLs except CRL3, an adaptor protein connects the catalytic core complex to the substrate-receptor subunits. In the CUL1-based SCF (bottom), the F-box proteins are the substrate receptors, and Skp1 serves as the adaptor.

other related E3 complexes, collectively known as the cullin–RING ligases (CRLs).

In all eukaryotes, CRLs constitute the largest superfamily of E3 complexes and share a similar modular architecture[4]. The cullin proteins (CUL1, CUL3 and CUL4 in plants) serve as an elongated scaffold, holding the E2-docking catalytic subunit RBX1 at one end and interchangeable substrate-receptor subunits at the other, often via a bridging adaptor (**Fig. 1**). Conserved from yeast to humans, CSN is believed to regulate the assembly of the CRL E3s by cleaving off a ubiquitin-like protein, Nedd8, which modifies all cullins[9].

The TIR1 F-box protein as auxin receptor

As sessile organisms, plants have to constantly adapt their growth and architecture to survive and thrive in the fluctuating environment. To meet this challenge, plants have evolved dynamic signaling networks enabling rapid communication and coordination among different body parts. These networks are controlled by a chemically diverse set of endogenous small molecules, known as plant hormones or phytohormones. Of these, auxin was the first to be discovered, and this was followed by discovery of gibberellins, cytokinins, abscissic acid, ethylene, jasmonates (JAs), brassinosteroids and, recently, strigolactones (SLs)[10].

Auxin has long been recognized as a pivotal plant hormone. It regulates many aspects of plant growth and development, such as embryo patterning, root and shoot branching, tropic growth responses and apical dominance. At the cellular level, auxin modulates cell division, differentiation and elongation through transcription reprogramming. Despite the long history of auxin research, it was not until the 1990s that several core components of the auxin signaling pathway were finally identified. Out of a collection of auxin-resistant mutants, the first cloned gene, *AXR1*, immediately pointed to the UPS: the AXR1 protein shares high sequence homology with the ubiquitin-activating E1 enzyme[11]. In fact, AXR1 was later identified as a subunit of the E1 enzyme complex responsible for activating the cullin modifier Nedd8. Rapid progress ensued, with the mapping of several auxin-related mutants to CUL1 and the F-box protein TIR1 delineating the critical role of the entire SCF complex containing TIR1 (SCF^{TIR1}, with superscript denoting the substrate-receptor subunit of the SCF complex) and its regulators in auxin signaling[12]. In the presence of auxin, the SCF^{TIR1} E3 promotes the ubiquitination and degradation of a family of transcription repressors, termed AUX/IAAs, thereby activating the expression of auxin-responsive genes. The most unexpected and most striking discovery made at the peak of this line of research was the final identification of the auxin receptor as the TIR1 F-box protein itself[13,14]. Whereas most yeast and animal F-box proteins recognize only post-translationally modified substrates[15], the plant F-box protein TIR1 binds auxin and interacts with its substrate proteins in a hormone-dependent manner (**Fig. 2a**). This groundbreaking finding established a new paradigm of plant hormone signaling in which the receptor function is fulfilled by ubiquitin-ligase machinery.

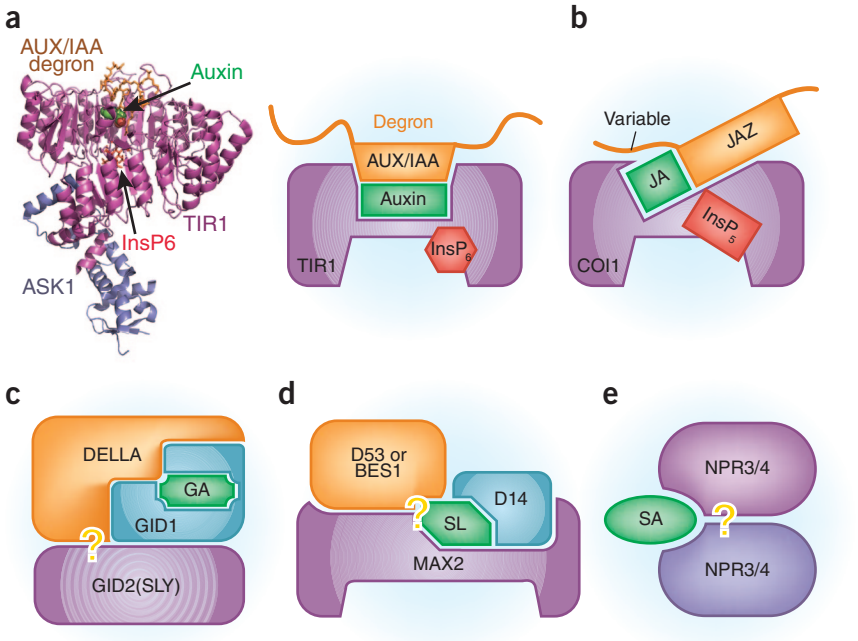

Figure 2 Plant cullin–RING ligases and mechanisms for hormone-dependent substrate recognition. (**a**) Auxin binds directly to the F-box protein TIR1 and promotes its interaction with the substrate proteins, AUX/IAAs. Left, structural model of the complex (ref. 27, PDB 2P1Q); ASK1 is the SCF^{TIR1} adaptor. A conserved degron sequence within AUX/IAAs makes direct contacts with the auxin–TIR1 complex, which also contains an inositol hexakisphosphate ($InsP_6$) cofactor. (**b**) Jasmonic acid (JA) regulates COI1-JAZ interaction via a similar molecular-glue mechanism, but the hormone contacts a variable region of the JAZ degron. Inositol pentakisphosphate ($InsP_5$) acts as a cofactor potentiating the JA co-receptor. (**c**) Binding of gibberellin (GA) to GID1 is necessary for GID1 association with the DELLA proteins. By a yet-unclear mechanism, the F-box protein GID2 (SLY1) recruits the GID1–GA–DELLA complex to the SCF E3 to promote DELLA ubiquitination. (**d**) Recognition of strigolactones (SLs) by D14 enables their interaction with the F-box protein MAX2. How the D14–SL–MAX2 complex is formed and recruits potential substrates, such as D53 and BES1, remains elusive. (**e**) Salicylic acid (SA) is recognized by the CRL3 substrate–receptor proteins, NPR3 and NPR4 (NPR3/4), via an unknown mechanism.

CRLs as signaling hubs

The identification of the TIR1 F-box protein as the auxin receptor greatly accelerated the study of several other key hormones. JAs mediate pathogen defense and stress responses in plants and form amino acid conjugates. The receptor for JA–isoleucine (JA–Ile) and other JA conjugates was mapped to COI1, an F-box protein with high sequence homology to TIR1 (**Fig. 2b**)[16]. Analogously to auxin, JA–Ile enables SCF^{COI1} to catalyze the ubiquitination and degradation of the JAZ family of transcriptional repressors to trigger the expression of JA-responsive genes[17]. Signal transduction of gibberellins (GAs), a hormone family important for plant growth responses, is also mediated by an SCF complex, albeit via a variant scheme[18]. GAs are sensed by a soluble protein receptor called GID1 that subsequently binds to the DELLA family of transcriptional repressors (**Fig. 2c**). Once engaged, this tripartite GID1–GA–DELLA complex is targeted to the $SCF^{GID2\ (SLY1)}$ E3 ligase, and this results in the ubiquitination and degradation of the DELLA proteins. In this system, the hormone is perceived by a binding partner of the F-box protein, which acts as an extended arm of the E3.

Given the large number of uncharacterized F-box proteins in plants, the prevalence of SCF in hormone perception and signaling is probably still underappreciated. Indeed, the receptor function of the most recently identified class of phytohormones, SLs, has now been formally linked to yet another F-box protein, MAX2 (D3). SLs are synthesized in the plant root and have a crucial role in regulating shoot branching and broad developmental processes.

Genetic analyses of SL signaling have identified MAX2 (D3) and a GID1-like protein, D14, as the most-downstream components of the pathway[19]. Recent studies suggest that these two proteins might function as the co-receptors of SLs and facilitate the degradation of at least two distinct target proteins: D53 (SMAX2), a Clp ATPase family member, and BES1, a transcriptional effector (**Fig. 2d**)[20–23]. Interestingly, SCF^{MAX2}, together with the D14-like protein KAI2, has also been implicated in the signaling of karrikins, a group of smoke-generated compounds that share structural similarity to SLs and are able to stimulate seed germination[24].

Besides SCF, the CRL3 E3 complexes take part in a variety of plant signaling functions. Among several different CRL3s involved in phototropism, abscisic acid and ethylene signaling[4], $CRL3^{NPR3}$ and $CRL3^{NPR4}$ has recently been identified as the receptor complex for salicylic acid (SA)[25], a plant immune signal generated in response to local pathogen infection. From a genetic screen for SA-insensitive mutants, NPR1 was first identified as a central regulator of SA signaling[26]. Recent studies have revealed that SA is directly sensed by two NPR1-homologous proteins, NPR3 and NPR4 (**Fig. 2e**), which target NPR1 and themselves for ubiquitin-dependent proteasomal degradation[25]. This finding expands the examples of CRL-based plant hormone receptors beyond the SCF E3s and raises the possibility that other CRL subfamilies might be able to function as hormone receptors or signal sensors.

Mechanistic insights from structures

The discovery of the CRL-associated plant hormone receptors has triggered a cascade of structural studies in the past few years. The crystal structure of the TIR1–auxin–AUX/IAA complex revealed new and surprising mechanistic insights[27]. First, the structure shows that auxin does not induce conformational changes in its receptor upon binding. Instead, auxin nestles at the interface of TIR1 and AUX/IAA, filling a gap and helping to nucleate a hydrophobic core between the two proteins (**Fig. 2a**). This indicates that auxin activates the SCF^{TIR1} E3 by acting as a 'molecular glue' to mediate productive interactions between the F-box protein and the substrate proteins. Auxin, chemically known as indole-3-acetic acid, is a tryptophan derivative. Structural analysis suggests that auxin enhances protein interactions by functionally mimicking tryptophan, a residue frequently found at protein-protein interfaces.

Because auxin is almost completely buried at the E3-substrate interface, high-affinity hormone binding is likely to be achieved by a co-receptor system, which consists of both the ubiquitin ligase and the substrate polypeptide. This concept was also suggested by the subsequently reported structure of a complex of COI1, JA–Ile and JAZ and validated by radiolabeled ligand-binding assays for both systems[28,29].

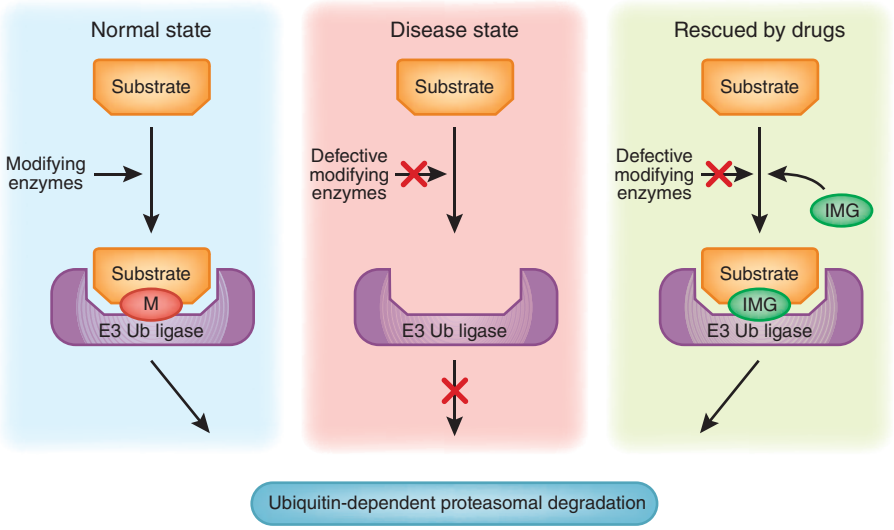

Figure 3 A strategy for plant hormone–inspired drug discovery. Left, under normal conditions, a human substrate protein undergoes post-translational modification (M) by upstream functional modifying enzymes before it is recognized and ubiquitinated by an E3 ubiquitin (Ub) ligase. Middle, under pathophysiological conditions, failure in substrate modification, either due to defects of the modifying enzymes or to mutations in the substrate, can lead to abnormal substrate accumulation, which is involved in disease pathogenesis. Right, a strategy to rescue the impaired substrate-E3 interaction in the absence of substrate post-translational modification could be the development of compounds that function as interfacial molecular glues (IMG). These small molecules have the potential to exert therapeutic effects by restoring the normal levels of the protein targets.

The *A. thaliana* genome encodes nearly 30 AUX/IAAs, which are ubiquitinated by TIR1, and five additional TIR1-like F-box proteins (AFB1–AFB5). Interestingly, the different auxin co-receptor pairs formed with AUX/IAAs and TIR1 (AFB) proteins displayed differential sensitivities to hormone concentration, thus suggesting that auxin might be able to control protein ubiquitination over a wide dynamic range of concentrations[29]. Such a unique property might underlie the multitude of physiological effects elicited by this single hormone with spatiotemporal specificity. All AUX/IAAs share a short consensus recognition motif, or 'degron', which directly engages with auxin-loaded TIR1[27]. However, AUX/IAA regions outside the degron appear to contribute to the differential hormone binding affinity among different combinations of AUX/IAAs and F-box proteins. Strikingly, the COI1-substrate proteins, JAZs, seem to have this property confined to a condensed degron sequence, which features a variable region in direct contact with the COI1-anchored JA–Ile molecule[28].

Unexpectedly, the crystal structure of the auxin–receptor complex revealed the presence of a second small molecule, inositol hexakisphosphate ($InsP_6$), which was copurified with the TIR1 F-box protein[27]. Also known as phytic acid, $InsP_6$ has long been thought to be a storage form of phosphorus in plants. In complex with TIR1, $InsP_6$ is embedded right underneath the hormone-binding pocket, thus indicating that it might be a functionally important cofactor that supports the activity of the hormone receptor (**Fig. 2a**). Remarkably, a slightly different cofactor molecule, inositol pentakisphosphate ($InsP_5$), was also copurified with the JA receptor COI1 (ref. 28). In a reconstitution assay, it became clear that $InsP_5$ indeed has a critical role in potentiating the F-box protein to recognize JA–Ile and its substrate (**Fig. 2b**). In those studies, the two recombinant F-box proteins were purified from insect cells via an identical procedure, and yet each displayed high specificity toward its inositol polyphosphate molecules, which are interconvertible forms. Thus, these small-molecule cofactors might represent a second signal integrated at the ubiquitin-ligase machinery, mediating cross-talk between the two antagonizing hormones.

The mechanism by which GA is recognized by GID1 has been revealed by the crystal structures of GA-bound GID1 in both free and DELLA-bound forms[30,31]. Distinct from auxin and JA–Ile, GAs are exclusively recognized

by GID1, which features an N-terminal lid domain that sequesters the hormone in a pocket presented by the core domain (**Fig. 2c**). Binding of GAs to GID1 allows the DELLA proteins to engage with the N-terminal lid domain of the hormone receptor. Although the structural state of the apo form of GID1 remains unknown, it has been proposed that its N-terminal lid domain is largely disordered in the absence of GAs, and it might act as a conformational switch that senses the hormone[30]. Despite the distinct sensing mechanism for GAs, their action at the structural level is very much reminiscent of auxin. Both hormones can be considered to be interfacial molecules mediating either protein-protein interaction or domain-domain engagement. However, the question as to how the F-box protein GID2 (SLY1) recognizes the ternary complex of GID1–GA–DELLA to ubiquitinate the DELLA proteins remains unanswered.

Lessons from plants and outlook

Despite the progress described here, the complete mechanism of action remains elusive for several key phytohormones, including GAs, SA and SLs (**Fig. 2c–e**). Importantly, the majority of the plant F-box proteins, as well as the substrate receptors of other CRLs, have not been characterized. New mechanisms used by CRLs to regulate plant signaling will certainly be revealed in future studies. In this regard, several light-sensitive F-box proteins have been identified that control the circadian clock and photoperiodic flowering in plants[4]. Mechanistic studies of their functions will shed light on how a photoreceptor module is integrated into these fascinating E3 ligase complexes. Finally, although the functions of E3 ligases in plant signaling are well appreciated, the roles of other proteins of the UPS, including deubiquitinating enzymes, ubiquitin-associated and ubiquitin-like proteins, remains to be explored.

Dysregulation of the human UPS is associated with numerous pathophysiological conditions, such as cancer, neurological and immune disorders. Proteasome inhibitors have had clinical success, but there has been little progress in developing small molecules targeting the upstream E3s, to potentially achieve higher specificity. In that regard, it is stunning that nature has long evolved effective strategies to directly manipulate the plant UPS with diverse hormonal compounds, including one as simple as auxin. As discussed above, these naturally occurring small molecules almost always act as interfacial molecules, promoting and stabilizing protein-protein interactions. For small molecules with desirable pharmacokinetic properties, such a task could be more achievable than disrupting protein complexes, the direction taken by most drug-discovery efforts to date. Because defective E3-substrate interactions are associated with several human disease–related signaling pathways, it is conceivable that an auxin-inspired strategy could be used to develop therapeutic agents capable of resurrecting impaired binding of a substrate to its E3 (**Fig. 3**). This concept could be further extended to active compounds that confer a gain of function for E3s to ubiquitinate new substrates. In fact, the antitumor activity of lenalidomide, a member of the thalidomide class of drugs, has been recently attributed to such a mechanism on the $CRL4^{CRBN}$ E3 (refs. 32,33).

Plants never cease to surprise us. Deciphering how they take advantage of the entire UPS to thrive and to dominate earth holds promise for both deepening understanding of biology and improving human health.

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1. Goldstein, G. *et al. Proc. Natl. Acad. Sci. USA* **72**, 11–15 (1975).
2. Hershko, A. & Ciechanover, A. *Annu. Rev. Biochem.* **67**, 425–479 (1998).
3. Kim, D.Y., Scalf, M., Smith, L.M. & Vierstra, R.D. *Plant Cell* **25**, 1523–1540 (2013).
4. Hua, Z. & Vierstra, R.D. *Annu. Rev. Plant Biol.* **62**, 299–334 (2011).
5. Kraft, E. *et al. Plant Physiol.* **139**, 1597–1611 (2005).
6. Hua, Z., Zou, C., Shiu, S.H. & Vierstra, R.D. *PLoS ONE* **6**, e16219 (2011).
7. Serino, G. & Deng, X.W. *Annu. Rev. Plant Biol.* **54**, 165–182 (2003).
8. Lau, O.S. & Deng, X.W. *Trends Plant Sci.* **17**, 584–593 (2012).
9. Lyapina, S. *et al. Science* **292**, 1382–1385 (2001).
10. Santner, A., Calderon-Villalobos, L.I. & Estelle, M. *Nat. Chem. Biol.* **5**, 301–307 (2009).
11. Leyser, H.M. *et al. Nature* **364**, 161–164 (1993).
12. Dharmasiri, N. & Estelle, M. *Trends Plant Sci.* **9**, 302–308 (2004).
13. Dharmasiri, N., Dharmasiri, S. & Estelle, M. *Nature* **435**, 441–445 (2005).
14. Kepinski, S. & Leyser, O. *Nature* **435**, 446–451 (2005).
15. Skaar, J.R., Pagan, J.K. & Pagano, M. *Nat. Rev. Mol. Cell Biol.* **14**, 369–381 (2013).
16. Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. & Turner, J.G. *Science* **280**, 1091–1094 (1998).
17. Browse, J. *Annu. Rev. Plant Biol.* **60**, 183–205 (2009).
18. Sun, T.P. *Curr. Biol.* **21**, R338–R345 (2011).
19. Domagalska, M.A. & Leyser, O. *Nat. Rev. Mol. Cell Biol.* **12**, 211–221 (2011).
20. Jiang, L. *et al. Nature* **504**, 401–405 (2013).
21. Wang, Y. *et al. Dev. Cell* **27**, 681–688 (2013).
22. Zhou, F. *et al. Nature* **504**, 406–410 (2013).
23. Stanga, J.P., Smith, S.M., Briggs, W.R. & Nelson, D.C. *Plant Physiol.* **163**, 318–330 (2013).
24. Nelson, D.C. *et al. Proc. Natl. Acad. Sci. USA* **108**, 8897–8902 (2011).
25. Fu, Z.Q. *et al. Nature* **486**, 228–232 (2012).
26. Dong, X. *Curr. Opin. Plant Biol.* **7**, 547–552 (2004).
27. Tan, X. *et al. Nature* **446**, 640–645 (2007).
28. Sheard, L.B. *et al. Nature* **468**, 400–405 (2010).
29. Calderón Villalobos, L.I. *et al. Nat. Chem. Biol.* **8**, 477–485 (2012).
30. Murase, K., Hirano, Y., Sun, T.P. & Hakoshima, T. *Nature* **456**, 459–463 (2008).
31. Shimada, A. *et al. Nature* **456**, 520–523 (2008).
32. Krönke, J. *et al. Science* **343**, 301–305 (2014).
33. Lu, G. *et al. Science* **343**, 305–309 (2014).