Jan Hejátko · Toshio Hakoshima *Editors*

Plant Structural Biology: Hormonal Regulations

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Foreword

In terms of the intensity with which information can be interpreted, there is nothing better than a picture, and this helps structural biology stand at the forefront of interpretation for biochemistry. Plant structural biology is strong and is growing in importance even though there is a distinct lack of plant protein structures in the databases. With the number of structures arising from human proteins currently close to 1500, the highest ranked plant (*Spinacea oleracea*) has 50, but the long list of other species separating these two reduces the total contribution of plant protein structures to just a few percent of structures solved (based on RCSB PDB entries 2018). At a time when plant genomes are being sequenced at a fantastic rate, our understanding of what gene products do and the annotation necessary to help make sense of the genomic data are lagging behind. However, here lies opportunity, and the history of plant protein structural biology can be proud of its successes. This volume captures some of these successes in the field of hormone signalling.

Some readers may be entering the world of protein structures for the first time and others may be seeking to immerse themselves in the details of molecular signalling mechanisms, and both will find this volume rewarding. There are chapters introducing the various methodologies for solving protein structures, from X-ray crystallography as probably the most familiar to the increasingly popular cryo-electron microscopy. However, the bulk of the contributions are overviews of the structures of hormone receptors. In this, we are fortunate. Structures are introduced and explained for all the most important receptors and some of their interactors and, importantly, these structures are interpreted in terms of, e.g. the residues, loops and features which contribute mechanistically to function. As noted above, structural biology is visually strong and each article displays beautiful images to help explain how hormone perception works.

Most of the proteins described have been solved using crystallography. Fortunately, many plant hormone receptors are soluble proteins which have made their structural biology more amenable, but a subset are membrane-bound such as the receptors for ethylene and cytokinins. No structural biology project should be undertaken lightly given the immense efforts required to purify sufficient protein,

but difficulties for those interested in membrane proteins is amplified by the need to maintain protein integrity during extraction. It is with intrinsic membrane proteins that cryo-electron microscopy techniques are starting to play a vital role as its resolution gets better and better.

Collectively, the articles in this volume provide a welcome and exciting prelude to what I hope will be a wave of biochemical illuminations.

School of Life Sciences, University of Warwick, Coventry, UK

Richard Napier

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Part I
Introduction

Chapter 1
Overview of Proteins in Plant Hormone Signaling

Toshio Hakoshima

1.1 Plant Hormones

Plant hormones (or phytohormones) play central roles in the integration of diverse environmental cues with signaling networks and the genetic programs of plants. In animals, hormones comprise any member of classes of signaling molecules produced by glands in multicellular organisms that are transported by the circulatory system to distant target organs so as to regulate physiology and behavior. Although plants do not possess glands that secrete hormones, they do possess several tissues that produce and also respond to hormones. Plant hormones comprise a set of structurally unrelated small organic compounds (Fig. 1.1), including auxin, jasmonic acid (JA), gibberellin (or gibberellic acid, GA), abscisic acid (ABA), brassinosteroid (BR), cytokinin, ethylene, strigolactone (SL), and salicylic acid (SA). Although karrikin (KAR) is not produced by plants, it is included as a plant hormone given its function as an "endocrine disruptor" or "environmental hormone".

In terms of chemical structure, some of the plant hormones could find animal counterparts produced from common precursors (Table 1.1) (Chow and McCourt 2006). Animals produce several steroid hormones that display similarity to BR. ABA and SL are produced from carotenoids, which are precursors of retinoic acid in animals. Plant JA and animal prostaglandins are both produced from fatty acids. Auxin is an indole acetic acid derived from indole, and its animal counterpart could be melatonin, which is produced in the pineal gland and regulates sleep and wakefulness. All of these animal counterparts seem not to possess similar functions as with the plant hormones. Generally, plant hormones possess a much broader range of functions compared to animal hormones, and a single plant cell can respond to more than one hormone, and a single hormone can affect different tissues in different

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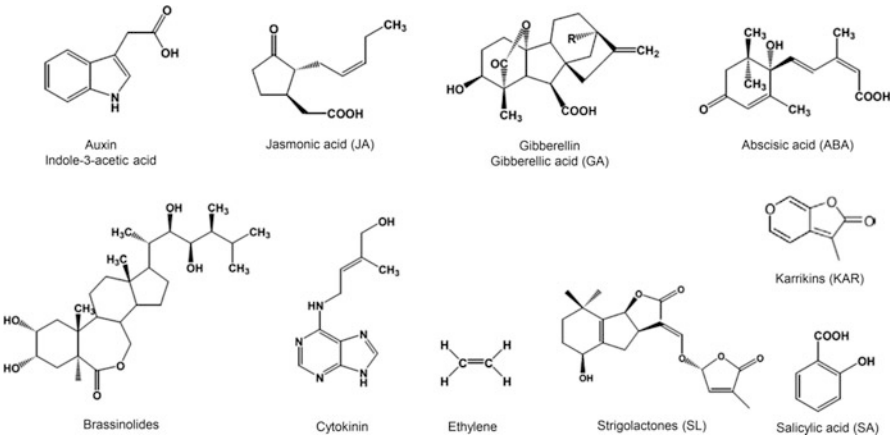

Fig. 1.1 Chemical structures of plant hormones

Table 1.1 Biosynthetic precursors of plant and animal hormones

Precursor	Plant	Animal
Sterol	Brassinosteroid (BR)	Progesterone
Fatty acid	Jasmonic acid (JA)	Prostaglandin
Indole	Auxin	Melatonin
Carotenoid	Abscisic acid (ABA)	Retinoic acid
	Strigolactone (SL)	
Geranylgeranyl-PP	Gibberellin (GA)	–
Purine	Cytokinin (CK)	–
Methionine	Ethylene	–
Phenylalanine	Salicylic acid (SA)	–

ways. GA is produced from geranylgeranyl-phosphates, cytokinin from purines, and ethylene from methionine, and these have no counterparts in animals. Animals do not produce SA, although SA exhibits remarkable pharmacological activity.

The number of recognized plant hormones may increase in the future, particularly if we are reminded that only 9 years have passed since SL was accepted as a plant hormone (Umehara et al. 2008; Gomez-Roldan et al. 2008). In addition to plant hormones comprised of organic compounds, we now know that signaling peptides also play prominent roles in regulating plant physiology by short-range intercellular communication mechanisms, and have established the concept of peptide hormones just as in the case of animals. The Arabidopsis genome encodes more than 1000 putative small signaling molecules, yet only a few polypeptides have been functionally characterized (Grienenberger and Fletcher 2015). Elucidation of the molecular functions and structures of these small peptides and their receptors is a frontier of current plant and structural biology.

1.2 Plant Hormone Nuclear Receptors

Animals possess a conserved nuclear receptor superfamily of transcription factors that perceive a variety of hormones. Humans have 48 nuclear receptors that contain orphan receptors that perceive unknown compounds and receptors that perceive compounds other than hormones, such as certain lipids (Evans and Mangelsdorf 2014). The genomic data, however, suggest that plants have no such nuclear receptor homologs. Instead, plants co-opt proteins from different protein families to perceive plant hormones (Table 1.2). One prominent characteristic found in plant hormone signaling is the central role played by the ubiquitin-proteasome system (UPS). In particular, cullin-RING ubiquitin ligase (CRL) complexes play a key role in the

Table 1.2 Plant hormone receptors

Hormone	Receptor	Receptor type
Nuclear receptors		
Auxin	TIR1 (TRANSPORT INHIBITOR RESPONSE 1)	LRR-type F-box protein: Ub-substrate[a] receptor
Jasmonic acid (JA)	COI1 (CORONATINE INSENSITIVE 1)	LRR-type F-box protein: Ub-substrate receptor
Gibberellin (GA)	GID1 (GIBERELLIN INSENSITIVE DWARF 1)	F-box protein adaptor (α/β hydrolase-superfamily)
		Ub-substrate receptor
Strigolactone (SL)	D14 (DWARF 14)	Hormone hydrolase (α/β hydrolase-superfamily)
		F-box protein adaptor: Ub-substrate receptor
Karrikin (KAR)[a]	KAI2 (KARRIKIN INSENSITIVE 2)	Hormone hydrolase (α/β hydrolase-superfamily)
	/D14L (D14-like)	F-box protein adaptor: Ub-substrate receptor
Salicylic acid (SA)	NPR3,4 (NONEXPRESSOR OF PR GENES 3, 4)	CUL3 adaptor (BTB domain protein)
		Ub-substrate receptor
Abscisic acid (ABA)	PYR/PYLs (PYRABACTIN RESISTANCE 1)	Phosphatase inhibitor (START-superfamily)
		Ub-substrate receptor
Receptor kinases		
Brassinosteroid (BR)	BRI1 (BRASSINOSTEROID INSENSITIVE 1)	LRR-type receptor kinase (Ser/Thr protein kinase domain)
Cytokinin (CK)	AHK2,3,4 (ARABIDOPSIS HISTIDINE KINASE 2,3,4)	Histidine kinase (single TM helix[b])
Ethylene	ETR1 (ETHYLENE RESPONSE1), ERS1 (ETHYLENE RESPONSE SENSOR 1),	Histidine kinase (3–4 TM helices)
		ETR2, EIN4, ERS2

[a]Ubiquitylation substrate (Ub-substrate)
[b]TM helix, transmembrane helix

signaling pathways of major plant hormones including auxin, JA, GA, SL, KAR, SA, ABA, and ethylene (details are reviewed in Chap. 2). A significant proportion of plant genomes is devoted to the encoding of UPS components. For example, inspection of the Arabidopsis genome suggests the presence of 500–600 F-box proteins, which is a large number when compared to the 78 F-box proteins in humans (Cardozo and Pagano 2004). The auxin and JA receptors, in fact, are the F-box proteins TIR1 and COI1, respectively. These F-box proteins possess leucine-rich repeat (LRR) domains, which recognize substrate proteins for ubiquitylation in a hormone-dependent manner (Chaps. 4, and 5). The GA, SL, and KAR receptors are members of the α/β hydrolase superfamily and act as F-box protein-bound adaptor proteins that recognize substrate proteins for ubiquitylation in a hormone-dependent manner. The GA receptor GID1 has no catalytic activity due to replacement of the catalytic His residue of the Ser-His-Asp catalytic triad with a Val/Ile residue (Chap. 6). In sharp contrast to GID1, the SL and KAR receptors possess a conserved catalytic triad system. These catalytic residues are essential for the action of SL and KAR receptors, and the SL receptor obviously exhibits catalytic activity with respect to SL hydrolysis, which is essential for SL function (Chap. 7).

Identification of the SA receptor remained elusive for quite some time. Recently, NPR (nonexpressor of PR genes) proteins have been reported to function as SA receptors in developing pathogen-induced systemic acquired resistance (SAR). One of these proteins, NPR1, was reported to function as a transcriptional co-activator (Wu et al. 2012), whereas two other proteins, NPR3 and NPR4, function as ankyrin-repeat-containing CRL3 E3 substrate receptors for ubiquitylation of NPR1 followed by degradation (Fu et al. 2012). NPR1, NPR3, and NPR4 contain a conserved BTB domain, share sequence homology, and were reported to bind SA. The precise manner by which SA binding differentiates the molecular functions of these three NPRs and regulates the interplay with each other and other transcription factors responsible for SAR-induced gene expression remains unclear, and will require structural studies of these proteins in an effort to delineate the mechanisms involved.

Unlike other plant hormone receptors that contain only a single or few members, the ABA receptor family contains multiple members (14 in Arabidopsis), and implies functional differentiation of receptor members. The ABA receptors PYR/PYLs belong to a protein family containing a steroidogenic acute regulatory protein-related lipid transfer (START) domain, and also belong to the Bet v 1 superfamily containing the birch pollen allergen Bet v 1a. The ABA receptors act as protein phosphatase inhibitors against type 2 Ser/Thr phosphatases (PP2Cs), ABI1, 2, HAB1, 2, HAI1, 2 and 3, and AHG1 (Chap. 8). This inhibition elevates the protein kinase activity of Subfamily III of Snf1-related kinases (SnRKs), which belong to the AMP-dependent protein kinase (AMPK) superfamily. The ABA signaling mediated by some ABA receptors is down-regulated by CRL4-mediated ubiquitylation and degradation (Chap. 2).

1.3 Plant Hormone Receptor Kinases

The other three plant hormones, BR, cytokinin, and ethylene, are perceived by receptor kinases located at plasma membranes. Among these, the BR receptor BRI1 is a receptor kinase possessing an LRR ectodomain, a single transmembrane (TM) helix, and a cytoplasmic domain containing an Ser/Thr protein kinase domain. The auxin and JA receptors also utilize LRR domains incorporated into the F-box proteins for hormone perception as mentioned above. LRR domains appear frequently in receptors such as peptide hormone receptors or Toll-like receptors (TLRs) in innate immune systems for the recognition of a variety of small compounds. In plants, receptor kinases form the largest family of membrane receptors and ca. 600 putative receptor kinases possessing Ser/Thr protein kinase domains have been identified in the Arabidopsis genome, although most of these receptors remain uncharacterized to date. Of these, the number of receptors that act as peptide hormone receptors is unknown at present. Considering that ca. 1000 putative small signaling molecules are encoded in the Arabidopsis genome, we expect a plethora of peptide hormone receptors to be identified (details are reviewed in Chap. 3).

The cytokinin and ethylene receptors are histidine kinases, which comprise the upstream module in the His-Asp phosphorelay (or two-component) signaling system. The downstream module of the signaling system comprises response regulators, which possess a conserved receiver domain containing an Asp residue for accepting a phosphate group. Thus, phosphoryl transfer occurs from ATP to the His residue of the histidine kinase and from the His residue to the Asp residue of the response regulator. Cytokinin and ethylene receptors are hybrid kinases that possess both histidine kinase and receiver domains. The cytokinin receptors AHK2, 3, and 4 comprise a CHASE ectodomain for cytokinin binding, a single TM helix, and a cytoplasmic hybrid kinase domain. The downstream elements of the receptor are AHP proteins with a conserved HPt domain for phosphoryl transfer (Kato et al. 1997). The ethylene receptors are divided into subfamily 1 (ETR1, ESR1) acting as His kinases with all hallmark residues conserved, and 2 (ETR2, ESR2 EI N4), which lack one or more hallmark residues and adopted Ser/Thr kinase activity. Ethylene perception is accomplished by the N-terminal copper ion-containing transmembrane domain comprised of three (subfamily 1) or four (subfamily 2) TM helices. It remains unknown how the gaseous nonpolar molecule is perceived by the protein. The copper ion likely participates in ethylene binding by mediating characteristic intermolecular interactions such as charge transfer between ion charges and π electrons of ethylene. However, no structural or biophysical information is currently available. Structural studies of the ethylene receptor remain to be the most challenging subject in structural plant biology.

1.4 Plant Hormone Perception

Hormone receptors possess two major molecular functions. One is hormone perception and the other is transfer of the hormone signal to downstream molecules. Plant hormones are bipartite molecules possessing both nonpolar hydrocarbon/aromatic

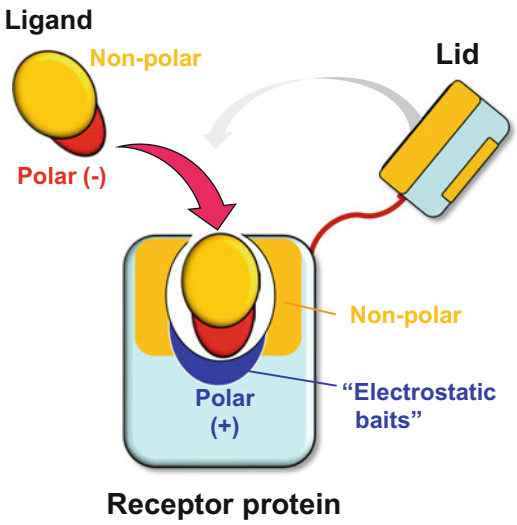

Fig. 1.2 Electrostatic guidance found in GA and other acid hormone binding

moieties and polar groups, but are mostly hydrophobic compounds exhibiting low solubility. Therefore, the hormone-binding sites of the receptors comprise primarily hydrophobic pockets with polar patches to facilitate specific interactions with the polar groups of hormones. Therefore, hormone perception inside the pockets is mediated by both nonpolar and polar intermolecular interactions, which contain hydrophobic contacts, salt bridges, and direct and water-mediated hydrogen bonds. A prominent and common polar interaction is found in the hormone-binding pockets for auxin, JA, GA, and ABA. These hormones are acids that possess one negatively charged carboxyl group. In the receptor-bound state, the carboxylic acid group is anchored to the positively charged Arg/Lys residues located at the bottom of the deep binding pockets of the receptors, and the negative charge of the hormone molecule is neutralized by the formation of salt bridges (Fig. 1.2). The anchoring of the carboxylic acid groups of plant hormone molecules by electrostatic interaction is reminiscent of the electrostatic guidance mechanism by which animal hormones enter the binding pocket of their nuclear receptors (Renaud et al. 1995). It is most likely that the carboxyl group enters the pocket first and is drawn down through the hydrophobic pocket to the anchoring site displaying an electrostatic bait. For the polar interaction, the GA receptor utilizes the region corresponding to the active site of the α/β hydrolase fold containing the oxyanion hole. SL/KAR receptors also comprise one α/β hydrolase domain with all active residues of the Ser-His-Asp triad, and the hormone molecules are hydrolyzed (Chap. 7). Compared to the deep hormone-binding pockets found in the auxin, JA, GA, ABA, and SL/KAR receptors that occlude the hormone molecules, the BR and cytokinin receptors provide surface pockets for hormone binding and receptor-bound BR and cytokinin molecules are still accessible from the solvent region.

1.5 Signal Transfer by Plant Hormone Receptors

When a hormone molecule binds the pocket of the receptor, perception of the hormone induces local and/or global structural changes in the receptor molecule. These changes switch on the interactions with the downstream effector molecule. The GA receptor GID1 displays a drastic conformational change on GA binding, referred to as the "close-the-lid" mechanism (Murase et al. 2008). In the first step of binding, the GA molecule still has an exposed hydrocarbon moiety to the solvent region since the carboxyl group of the GA molecule is placed first inside the deep binding pocket by the "electrostatic guidance" mechanism. This binding state induces a conformational change in the N-terminal switch region, which is conformationally flexible and forms no stable structure in the GA-free state. On GA binding, the N-terminal switch region is folded into three α-helices to form the lid of the binding pocket so as to contact the hydrocarbon moiety of GA and completely occlude the GA molecule (Fig. 1.3). The GA-induced lid also provides an active molecular surface containing nonpolar residues to recognize the effector proteins, DELLA proteins, which are the ubiquitylation substrates. ABA receptors possess a mechanism referred to as the "gate-latch-lock" mechanism with ABA as an allosteric inducer mechanism (Melcher et al. 2009). In this case, two loops, a gate and a latch, located at the entrance of the binding pocket, undergo marked conformational changes on ABA binding to form an interaction platform to trap the effector proteins, PP2C phosphatases. The auxin receptor TIR1 shows another mechanism by which the receptor, an F-box protein, recognizes its effector proteins, substrate proteins for ubiquitylation by the "molecular glue" mechanism in a hormone-

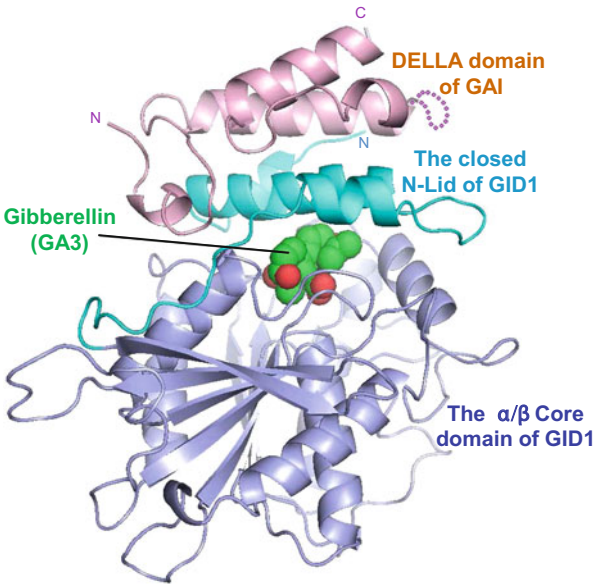

Fig. 1.3 The "closed-the-lid" mechanism found in the GA3-GID1-DELLA complex. The lid also provides binding site for DELLA protein

dependent manner (Chap. 4) (Tan et al. 2007). In this mechanism, the receptor-bound auxin molecule interacts directly with the ubiquitylation substrate protein bound to the auxin receptor. This binding mode shows that the bound substrate protein plays a role in the lid of the auxin-binding pocket. A similar mechanism is also seen in the JA receptors. Intriguingly, the BR receptor kinase and certain peptide receptor kinases adopt the "molecular glue" mechanism for co-receptor binding. BR possibly also cytokinin binding to receptor kinases defines the configuration of the receptor kinase dimer so as to activate the kinase domain located at the cytoplasm via transmembrane helices. As with animal receptor kinases, the nature of the conformational changes that take place and are essential for kinase activation remains obscure, and structural studies of receptor kinases as membrane proteins represent challenging projects in the future.

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Chapter 2
Overview of Protein Degradation in Plant Hormone Signaling

Domnita V. Rusnac and Ning Zheng

2.1 Introduction to the Ubiquitin-Proteasome System

Protein degradation is a proteolytic process, which counteracts protein synthesis and determines the half-lives of all proteins in the cell. Although some proteins can be extremely long lived, the majority of cellular proteins has a measurable half-life, ranging from minutes to days (Toyama and Hetzer 2013; Hershko and Ciechanover 1998). Early studies of protein breakdown in animals and plants emphasized on its roles in protein quality control and amino acid reutilization, which help eukaryotic cells to cope with cellular and environmental stress as well as nutrient starvation. Recent advances, however, have unraveled an unexpected regulatory function of protein degradation in actively controlling the abundance of a variety of intracellular proteins, thereby, modulating their activities (Hershko and Ciechanover 1998).

The ubiquitin-proteasome system (UPS) is the central pathway for intracellular protein degradation and is evolutionarily conserved in all eukaryotes, including plants (Vierstra 2009; Callis 2014). In an ATP-dependent manner, the UPS is programmed to respond to diverse cellular cues and selectively label target proteins for rapid breakdown. Thanks to the groundbreaking work by Avram Hershko, Aaron Ciechanover, Irwin Rose, Alfred Goldberg, Alexander Varshavsky, and many other pioneers in the field, most of the key components of the UPS have now been identified and biochemically characterized in great details (Wilkinson 2005). Our mechanistic understanding of the UPS function has also benefited tremendously from the extensive structural studies in the past two decades. This chapter offers a brief overview of the UPS and its major constituents in eukaryotes and highlights its unique involvements in various hormonal signaling pathways in plants.

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2.1.1 Ubiquitin and Ubiquitin-Like Proteins

Ubiquitin is a 76-amino acid protein universally found in all eukaryotic species and broadly expressed in different tissues of animals and plants. It has a highly conserved polypeptide sequence, which differs by three amino acids between the yeast and human orthologues. Ubiquitin is characterized by a compact β-grasp fold and a flexible C-terminal tail terminated by a di-Gly motif after maturation (Fig. 2.1a). In the UPS, ubiquitin serves as a protein posttranslational modifier, whose C-terminal carboxyl group is covalently conjugated to the ε-amino group of a substrate lysine residue via an isopeptide bond. As ubiquitin itself also has seven lysine residues, polyubiquitin chains can be formed when the carboxyl terminus of one ubiquitin molecule is linked to a lysine residue of another copy (Fig. 2.1a, b). Depending on which ubiquitin lysine residue is involved in chain elongation, polyubiquitin chains can be built with different linkages either in a homogeneous or branched fashion (Komander and Rape 2012; Meyer and Rape 2014). Among different types of ubiquitin chains, the Lys-48-linked tetraubiquitin chain has long been established as the minimal signal for proteasome targeting (Fig. 2.1b) (Thrower et al. 2000).

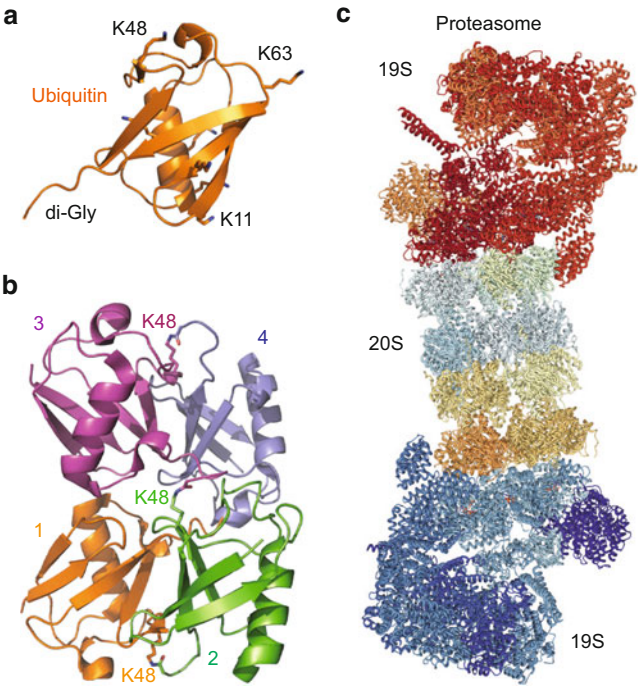

Fig. 2.1 (**a**) Ubiquitin with seven lysine residues (sticks) and a C-terminal di-Gly motif. Three commonly modified ubiquitin lysine residues are labeled. (**b**) Lys-48 linked tetraubiquitin chain (PDB:2O6V). (**c**) 26S proteasome with the 20S core particle and the 19S regulatory particle (PDB:5GJR)

In most, if not all, eukaryotic organisms, several proteins have been found to share sequence homology with ubiquitin and adopt the same ubiquitin fold, but do not serve as proteasome-targeting signal. These ubiquitin-like proteins (UBLs), such as NEDD8 and SUMO, also feature a C-terminal di-Gly motif after precursor processing and function as protein modifiers in regulating diverse cellular processes, including the UPS (van der Veen and Ploegh 2012). In most cases, these UBLs modify the substrate proteins in a monomeric form and elicit their effects by altering the structural topology, protein network, or cellular localization of the targets.

2.1.2 Proteasome as a Protein Degradation Machinery

The 26S proteasome is an intracellular multi-subunit proteolytic machinery, which is localized in both cytosolic and nuclear compartments and acts as the most downstream component of the UPS (Coux et al. 1996). Due to its protein destruction nature, the 26S proteasome has been evolved to safeguard its proteolytic activity at both architectural and functional levels (Tomko and Hochstrasser 2013). To achieve tight regulation of its protease function, the 26S proteasome is composed of two parts, the 20S core particle, which carries the catalytic activities, and the 19S regulatory particle, which controls the access of the active sites hidden inside the enzymatic core (Fig. 2.1c). Crystal structures of the 20S core particle revealed a cylindrical architecture, which consists of four stacked rings sequestering a central pore (Kish-Trier and Hill 2013). The inner two rings are each constructed by seven β-subunits, harboring three peptidase activities with the catalytic sites buried in the interior cavity, whereas the outer two rings are each formed by seven α-subunits, whose N-terminal regions converge at the center and together close up the proteolytic chamber of the core particle.

By docking to the outer rings of the 20S particle, the 19S regulatory particle of the proteasome is engaged with the proteasome core on its two ends and feed the degradation machinery with only polyubiquitinated protein substrates. Distinct from the 20S particle, the 19S particle has a highly asymmetric structure, which has historically been divided into two sub-complexes, the lid and the base (Lander et al. 2012). The base of the 19S particle contains six different ATPase subunits, which are assembled into a trimer-of-dimers ring-shaped structure. In addition, it also features three non-ATPase subunits, which have ubiquitin receptor functions. Together, these 19S base subunits are responsible for recognizing polyubiquitinated substrate, opening the gate of the 20S core, unfolding the folded substrate protein, and translocating the linearized polypeptide into the proteolytic chamber. The 19S lid complex, which consists of ten subunits, partially covers the base ATPases and makes direct contacts with the 20S core. Besides contributing to ubiquitin recognition, one important function of the 19S lid is to catalyze the removal of ubiquitin from the substrate before it is fed to the protease core. Recent advances in cryoelectron microscopy (cryo-EM) have not only allowed near atomic resolution structural determination of the entire proteasome, including the 19S regulatory

particle, but also helped reveal the protein degradation machinery in different functional states with substrate and/or nucleotides bound (Bhattacharyya et al. 2014).

2.1.3 The E1-E2-E3 Enzyme Cascade

Ubiquitin conjugation to a protein substrate, a process referred to as ubiquitination (or ubiquitylation), is the hallmark of ubiquitin-dependent protein degradation. Protein ubiquitination is catalyzed by the sequential actions of three enzymes, the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligases (Pickart 2001) (Fig. 2.2a). Free ubiquitin is first activated by the E1 enzyme, which uses ATP-Mg^{2+} to catalyze the acyl adenylation of ubiquitin's C-terminal carboxyl group and then captures the activated ubiquitin tail with its catalytic cysteine via a thiolester bond. Upon binding to a ubiquitin-conjugating enzyme, the ubiquitin-activating E1 enzyme subsequently transfers ubiquitin to the active site cysteine residue on E2 through a trans-thiolesterification reaction. As a highly active enzyme, E1 is responsible for constitutively charging E2 enzymes with ubiquitin in the cell. Vertebrates have two E1 genes, whose protein products, known as UBE1 and UBA6, have been found to preferentially charge different E2s (Jin et al. 2007). In *Arabidopsis thaliana*, two ubiquitin E1 enzymes, UBA1 and UBA2, have also been identified with nonredundant functions (Goritschnig et al. 2007).

In contrast to the small number of E1 enzymes, the ubiquitin-conjugating E2 enzymes are numbered in 30–40 in higher eukaryotes and often act in different cellular pathways (Wenzel et al. 2011b). All E2 enzymes share a conserved ~150-amino acid catalytic core domain, which adopts a classic UBC fold with the active site cysteine tucked in a cleft between two loops. Certain E2s feature additional N-terminal or C-terminal extension sequences, whereas a specific subgroup of E2s contains an internal acid loop close to the active site cysteine. Although E2s were once thought to be simple ubiquitin "carriers," recent studies have shown that they display distinct intrinsic reactivity and often play a critical role in dictating the linkage specificity of a polyubiquitin chain (Stewart et al. 2016). Because many ubiquitin-charged E2s (Ub~E2s) selectively interact and function with specific types of ubiquitin E3 ligases (see below), their active sites can have characteristic reactivity toward different attacking groups, such as the ε-amino group of the lysine side chain and the thiol group of a cysteine residue. Furthermore, with the help of extra sequence elements or binding partners, some E2s can recognize a specific lysine residue on the receiver (proximal) ubiquitin, which accepts the C-terminus of the incoming donor (distal) ubiquitin during chain extension. Interestingly, some E2 variants, which lack the active site cysteine, have been shown to interact with a canonical E2 to confer linkage-specific polyubiquitin chain activities.

Although the thiolester bond in the Ub~E2 conjugate is less stable than the isopeptide bond linking ubiquitin and substrate, transfer of ubiquitin from an E2 to

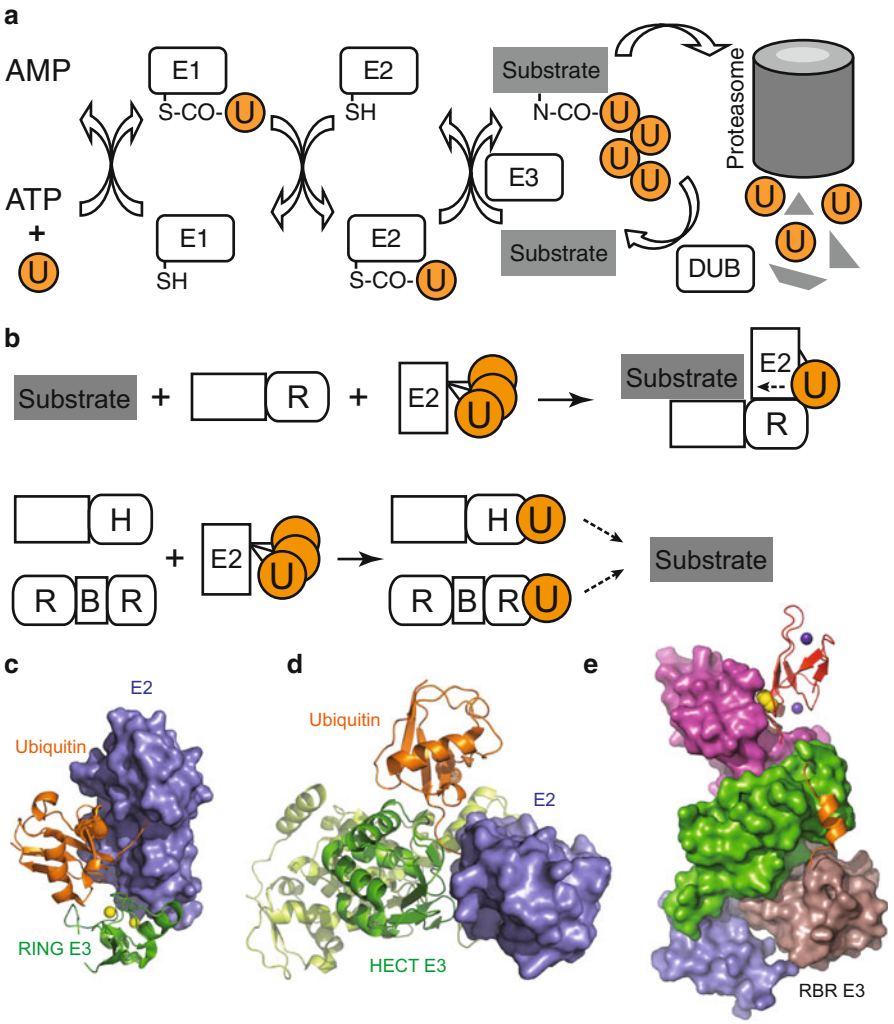

Fig. 2.2 (**a**) Ubiquitin-proteasome system with the E1-E2-E3 enzyme cascade acting upstream of the proteasome and the counteracting deubiquitinases (DUBs). (**b**) The three types of ubiquitin E3 ligases (R, RING; H, HECT; and RBR) and their different ubiquitin transfer mechanisms. (**c**) A RING E3-Ub~E2 complex (PDB:4AP4). (**d**) A HECT E3-Ub~E2 complex (PDB:3JW0). (**e**) An RBR E3 (PDB:4 K95)

a substrate does not occur efficiently until an E3 ubiquitin ligase is present (Pickart 2001). In the three-enzyme cascade, the E3 enzyme performs two critical functions to facilitate substrate ubiquitination. First, E3s stimulate the reactivity of a ubiquitin-charged E2 to accelerate ubiquitin discharge. Second, E3s provide a platform, onto which a specific protein substrate and the ubiquitin-charged E2 are recruited and brought together in close proximity. Ubiquitin E3 ligases, therefore, represent an

ideal class of enzymes favored by evolution for adopting novel functions that can couple protein ubiquitination and degradation with various upstream signals in diverse cellular pathways.

2.1.4 Three Types of Ubiquitin E3 Ligases

The functional importance and versatility of ubiquitin ligases in the UPS are best manifested by the different types of E3s and their sheer number in the eukaryotic genomes in comparison to other UPS enzymes. In *Arabidopsis thaliana*, more than one thousand genes have been identified to encode putative ubiquitin ligases (Vierstra 2009). Although this number varies among other plant species, the prevalence of E3s and their roles in regulating plant physiology are obvious. Intriguingly, plant pathogens are known to produce effector proteins that either mimic or hijack E3 ligases to take advantage of the host UPS and benefit their infection and life cycle (Banfield 2015). Such cross-kingdom functions further highlight the central roles played by ubiquitin ligases in the cell.

In all eukaryotes, three types of E3s have been identified, which are grouped based on their different signature sequence motifs and distinct catalytic mechanisms (Fig. 2.2b). The Really Interesting New Gene (RING) domain defines the largest family of ubiquitin ligases, known as RING-type E3s, which share a common protein fold consisting of two zinc-binding fingers with eight zinc-coordinating cysteine and histidine residues (Deshaies and Joazeiro 2009) (Fig. 2.2c). Besides the RING domain, these E3 ligases either contain a substrate-binding domain in the same polypeptide or belong to a multi-subunit ubiquitin ligase complex, which uses another subunit for recruiting substrate. The RING-type E3s are distinguished from other E3s by catalyzing the direct transfer of ubiquitin from an E2 to the subunit. Recent structural studies have shown that, upon binding to a ubiquitin-charged E2 enzyme, the RING domain makes contacts with both the E2 and the donor ubiquitin and stabilizes the Ub~E2 conjugate in a "closed" conformation (Fig. 2.2c) (Plechanovová et al. 2011; Dou et al. 2012; Pruneda et al. 2012). In doing so, a RING E3 activates the ubiquitin-charged E2 for ubiquitin transfer by presumably optimizing the geometry of the E2 active site for the nucleophilic attack by the side chain of a lysine residue in either a substrate or a receiver ubiquitin molecule.

The homology to E6AP carboxyl terminus (HECT) type of E3s represents a second family of ubiquitin ligases, which are characterized by their common C-terminal catalytic domain, known as HECT domain (Rotin and Kumar 2009) (Fig. 2.2d). With a bilobal structure, the HECT domain harbors an active site cysteine, which forms an obligate thiolester intermediate with ubiquitin to promote substrate ubiquitination (Fig. 2.2b) (Huang et al. 1999; Metzger et al. 2012). The first step of ubiquitin transfer mediated by the HECT E3s involves a trans-thiolesterification reaction, in which ubiquitin is passed from the active site cysteine of the E2 to that of the E3. Due to the nature of this specific reaction, HECT E3s only function with a small subset of E2 enzymes. Akin to single polypeptide RING E3s,

most known HECT E3s recognize their specific substrate through regions outside their catalytic domain. Although the human genome encodes nearly 30 HECT E3s, this family of ubiquitin ligases remains relatively small in plants (Marín 2013).

Remarkably, recent studies have unveiled a third family of E3s, which is named RING-in-Between-rings-RING (RBR) E3s (Spratt et al. 2014; Wenzel et al. 2011a). Despite the presence of several zinc finger-containing RING-like domains, RBR E3s are mechanistically closer to the HECT E3s than the RING E3s. While the RING1 domain of RBR E3s is responsible for recruiting a ubiquitin-charged E2 enzyme, ligation of ubiquitin to the substrate involves the formation of a ubiquitin~E3 intermediate, which is anchored at a strictly conserved catalytic cysteine found in the RING2 domain of the E3s (Fig. 2.2b, e). Similar to the HECT E3s, the RBR E3s relay ubiquitin to the substrate and display strong E2 preferences. Recent structural analyses of several RBR E3s have revealed that these multi-domain ubiquitin ligases almost exclusively adopt an auto-inhibited conformation in isolation (Trempe et al. 2013; Wauer and Komander 2013; Stieglitz et al. 2013; Lechtenberg et al. 2016) (Fig. 2.2e). Activation of these enzymes might be achieved by posttranslational modifications of the E3s or upon interactions with their binding partners, which presumably recruits specific substrates. So far, RBR E3s have been poorly studied in plants (Marín 2010). However, the potential functional connections of a RBR subfamily, Ariadne/HHARI, with the superfamily of cullin-RING E3s, as suggested by recent studies, might implicate a prominent role of the RBR E3 in plant hormone signaling (see below) (Scott et al. 2016).

2.1.5 Deubiquitinases

In the same way as most protein posttranslational modifications, protein ubiquitination is reversible, and the activities of ubiquitin ligases can be counterbalanced by enzymes capable of cleaving ubiquitin-linked isopeptide bonds (Fig. 2.2a). These isopeptidases, also known as deubiquitinases (DUBs), can either trim various ubiquitin chains with specific linkages or catalyze the removal of ubiquitin from substrate (Komander et al. 2009). Their activities not only enable ubiquitin recycling prior to substrate degradation by the proteasome but also provide a mechanism for regulating protein ubiquitination in a dynamic manner. In animals, DUBs are classified into six different subfamilies (USPs, UCHs, OTUs, MJDs, JAMM, and MINDYs) based on their sequence homology. The same six DUB families are also found in plants with a total of ~60 different family members in the *Arabidopsis* genome. Although little is known about their functions, it is expected that their deubiquitinase activities might be involved in fine-tuning the ubiquitination and degradation of many substrate polypeptides, including those implicated in hormone signaling.

2.2 Cullin-RING Ubiquitin Ligases

The cullin-RING ubiquitin ligase (CRL) complexes represent the largest family of multi-subunit E3s in all eukaryotic species (Deshaies 1999; Zhao et al. 2003; Zimmerman et al. 2010). In animals, these E3 machineries regulate diverse cellular functions, such as signal transduction, cell cycle progression, metabolic processes, DNA repair and replication, circadian clock, and stress responses. In plants, they not only participate in many of these functions that are conserved in all eukaryotic cells but also perform numerous plant-specific tasks, particularly, in perceiving and transducing phytohormone signals (Hua and Vierstra 2011). A rapid growing number of studies have now revealed the central roles CRLs in the signaling pathways of the vast majority of known plant hormones, including auxin, jasmonate (JA), gibberellin (GA), strigolactone, salicylic acid (SA), abscisic acid (ABA), and ethylene.

The CRL E3 machineries are built in a modular fashion, in which a common catalytic platform is used by numerous interchangeable substrate receptor subunits for ubiquitinating specific substrates (Li et al. 2003) (Fig. 2.3a). The CRL catalytic platform is formed between a ~80 kDa cullin scaffold protein and a ~20 kDa RING domain protein, RBX1. The cullin scaffold adopts an elongated overall structure with a more globular C-terminal domain (CTD) interacting with Rbx1 via an intermolecular β-sheet (Fig. 2.3b). The CRL substrate receptor subunits assemble with the catalytic platform by either directly interacting with the N-terminal

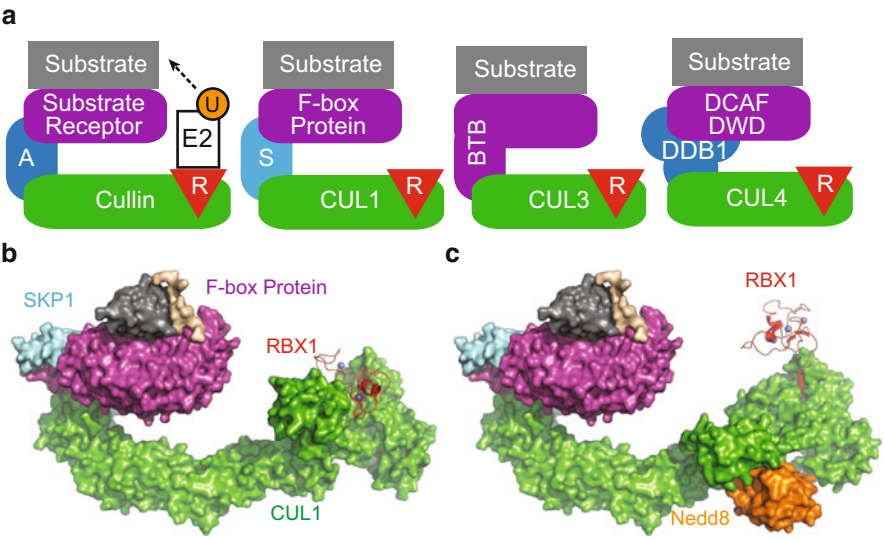

Fig. 2.3 (**a**) The assembly logic of cullin-RING E3 complexes and the three types of CRLs in plants, SCF/CRL1 (S, SKP1; R, RBX1), CRL3, and CRL4. (**b**) Structural model of a substrate-loaded SCF/CRL1 complex. (**c**) Structural model of a substrate-loaded NEDD8-modified SCF/CRL1 complex

domain (NTD) of the cullin scaffold or indirectly through an adaptor protein. When a substrate is presented by the substrate receptor subunit, CRLs promote sequential addition of ubiquitin to the substrate by successively engaging and activating a ubiquitin-charged E2 enzyme. Humans have six closely related cullin proteins (CUL1, 2, 3, 4A, 4B, and 5), which organize five different types of CRLs. By contrast, plants have only three cullin proteins (CUL1, CUL3, and CUL4), which give rise to three major CRL E3s, CRL1, CRL3, and CRL4 (Fig. 2.3a).

2.2.1 An Introduction to SCF/CRL1 E3s

The SCF (SKP1-CUL1-F-box proteins)/CRL1 E3 is the prototype of all CRLs and is composed of the CUL1-RBX1 catalytic core, the cullin adaptor SKP1, and members of the F-box protein family, which serve as the interchangeable substrate receptors (Deshaies 1999). Through a conserved ~40-amino acid F-box motif, F-box proteins interact with the relatively abundant SKP1 adaptor to form a battery of stable SCF substrate-binding modules. In addition to the F-box motif, the F-box proteins contain various protein-protein interaction domains, such as leucine-rich repeats (LRRs) domain or WD40-repeat domain, for binding specific substrates. Early studies of the SCF/CRL1 E3s in fungi and animals showed that F-box proteins often recognize their cognate substrates through a short linear sequence motif, known as degron (Zhao et al. 2003; Skaar et al. 2013). Their high affinity interaction frequently requires phosphorylation of one or two amino acids in the substrate degron by an upstream kinase, a prerequisite effectively linking protein ubiquitination to kinase signaling. Recent studies have shown that certain SCF substrates can not only bypass this requirement but also employ its entire protein fold to interface with the F-box protein (Xing et al. 2013).

In the past decade, research in plant hormone signaling has unraveled a stunning new paradigm of SCF/CRL1 functions, which places the E3 complexes as the central components in the signaling pathways of several key phytohormones (Fig. 2.4). These studies, together with high-resolution structural analyses, help establish novel mechanisms by which the SCF/CRL1 E3s bridge hormone sensing and transcriptional reprogramming via protein ubiquitination and degradation. In the auxin and JA signaling pathways, the F-box proteins, TIR1 and COI1, have been identified as the long-sought hormone receptors, which directly perceive the two phytohormones with a ligand-binding pocket constructed by their LRR domains (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Xie et al. 1998; Tan et al. 2007; Sheard et al. 2010). In a hormone-dependent manner, the two F-box proteins interact with the degron sequences of AUX/IAAs and JAZs, respectively, and promote their polyubiquitination and proteasome-mediated degradation (Fig. 2.4a, b). AUX/IAA and JAZ proteins are transcription repressors in the auxin and JA signaling pathways. Their rapid hormone-triggered degradation alleviates their inhibitory effects and activates the gene expression governed by IAA and MYC2 transcription factors. Interestingly, crystallographic studies of the two systems have revealed a “molecular

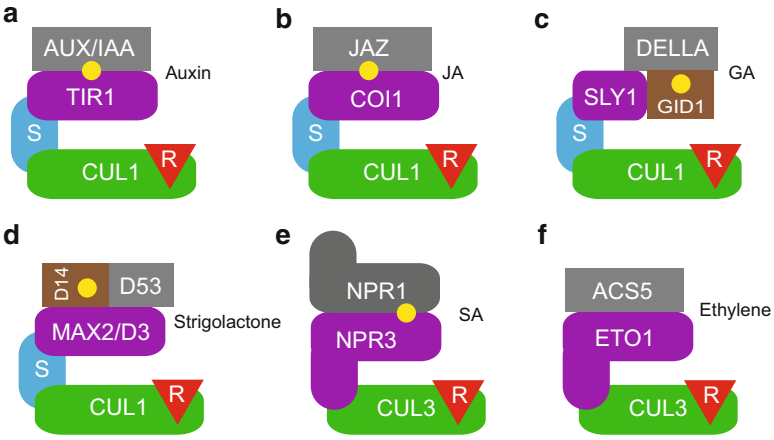

Fig. 2.4 (**a**) SCF^{TIR1} in complex with auxin and AUX/IAA. (**b**) SCF^{COI1} in complex with JA and JAZ. (**c**) $SCF^{SLY1\text{-}GID2}$ in complex with GA and DELLA. (**d**) $SCF^{MAX2\text{-}D14}$ in complex with strigolactone. (**e**) $CRL3^{NPH4}$ in complex with SA and NPR1. (**f**) $CRL3^{ETO1}$ in complex with ACS5. Yellow dots indicate hormone molecules

glue" mechanism by which each of the two hormones enhances the interactions between the F-box protein and its substrates by filling up a gap at their protein-protein interaction interface. The structural studies have also revealed the binding of specific inositol polyphosphate molecules to the two F-box proteins underneath their hormone-binding pockets. These soluble inositol polyphosphates could serve as a second signal for phosphate availability that is recognized by the F-box proteins. The details of the two systems are elaborated in the following chapters of this book.

Remarkably, the plant SCF/CRL1 E3s also play a major role in GA and strigolactone signaling (Shabek and Zheng 2014). These two phytohormones regulate a myriad of plant developmental processes, such as stem elongation (GA), flowering (GA), shoot branching (strigolactone), and symbiotic interactions with arbuscular mycorrhizal fungi (strigolactone). Similar to auxin and JA, GA and strigolactone are perceived by SCF complexes, which feature SLY1/GID2 and MAX2/D3 as the F-box proteins (Fig. 2.4c, d). Distinct from TIR1 and COI1, SLY1/GID2 and MAX2/D3 do not physically recognize the hormone molecules. Instead, these two F-box proteins interact with a cognate α/β hydrolase, GID1 and D14, respectively, which harbors a deep pocket for sensing the hormonal signal. With a nonproductive catalytic triad, GID1 loses its catalytic activity as a hydrolase. GA binding induces major conformational remodeling of the enzyme and enables it to interact with the DELLA transcription regulators. By promoting the ubiquitination and degradation of DELLA proteins, the GA-bound $SCF^{SLY1/GID2\text{-}GID1}$ E3 complex relays the hormonal signal to alter downstream gene expression (Murase et al. 2008; Shimada et al. 2008). Intriguingly, the strigolactone sensor protein D14 retains its hydrolase activity and is capable of slowly hydrolyzing the hormone as a substrate (Nakamura et al. 2013; Hamiaux et al. 2012). Upon binding to the F-box protein

MAX2/D3, D14 undergoes profound conformational changes and blocks the release of the hydrolysis product (Yao et al. 2016). Through its interaction with MAX2/D3, D14 is thought to recruit a class I Clp ATPase protein, D53, and promotes its degradation to inhibit axillary bud outgrowth and branching (Jiang et al. 2013; Zhou et al. 2013).

Besides acting as the sensors of plant hormones, SCF E3s also participate in hormonal signaling by regulating the abundance of key components of the pathways. In ethylene signaling, which dictates fruit ripening and several plant developmental and stress response processes, the F-box proteins EBF1/2 have been shown to destabilize EIN3, which is the transcription factor controlling ethylene responses (Guo and Ecker 2003; Potuschak et al. 2003). Interestingly, EIN3-EBF1/2 interactions can be negatively regulated by ethylene and positively promoted by red light through the action of photoreceptor phyB (Shi et al. 2016; Qiao et al. 2009). Furthermore, the F-box proteins ETP1/2 have been recently identified to promote the proteasomal degradation of EIN2, which is a transmembrane protein acting downstream of the ethylene receptors and upstream of EIN3. Additional examples of plant F-box proteins involved in hormonal signaling include the KISS ME DEADLY (KMD) proteins, which ubiquitinate type-B *Arabidopsis* response regulator (ARR) 2 in the cytokinin signaling pathway, and RCAR3 INTERACTING PROTEIN 1 (RIFP1), which negatively regulates the abscisic acid (ABA) receptor RCAR3 (Kim et al. 2013; Li et al. 2016).

2.2.2 An Introduction to CRL3-BTB E3s

The CRL3 E3s constitute a second large family of plant CRLs (Hua and Vierstra 2011). They differ from SCF/CRL1 complexes by having their cullin adaptor and substrate receptor functions combined into a single polypeptide, named Bric-a-brac, Tramtrack, Broad-complex (BTB)-domain protein. Members of this protein family share a conserved BTB domain that adopts a SKP1-like fold and directly binds CUL3. BTB-domain proteins have also evolved several other substrate-binding domains with characteristic protein folds and sequences such as ankyrin repeats, KELCH, MATH, and ZnF domain. The NPR (nonexpressor of PR genes) proteins constitute one small cohort of ankyrin-repeat-containing CRL3 E3 substrate receptors, which are particularly relevant to hormonal signaling. These proteins have been reported to function as the missing receptors for salicylic acid (SA) in developing pathogen-induced systemic acquired resistance (SAR) in plants (Fu et al. 2012). When a host is under the attack of a microbial pathogen, the cells around the local infection site produce SA, which will dissipate throughout the organism inducing the expression of a wide range of pathogenesis-related proteins (Fu and Dong 2013). NPR3 and NPR4 have recently been shown to directly bind SA and function as CUL3 adaptors to mediate NPR1 degradation (Fig. 2.4e). With different binding affinities toward SA, NPR3 and NPR4 are thought to enable differential responses to the variable cellular SA concentrations in the infected and neighboring cells (Fu et al.

2012). As a master regulator of SAR, NPR1 itself is a BTB-domain protein, which shares sequence homology with NPR3 and NPR4. It interacts with several transcription regulators. Although NPR1 has also been documented to bind SA, how it interplays with NPR3/4 and other transcription factors responsible for SAR-induced gene expression remains unclear (Wu et al. 2012).

Besides NPRs, several other BTB-domain proteins have been implicated in regulating plant hormone functions. Through its interaction with ACS5, a 1-aminocyclopropane-1-carboxylic acid synthase responsible for synthesizing ethylene, the BTB-domain protein ETO1 has been reported to block ethylene biosynthesis by both inhibiting the enzymatic activity of ACS5 and promoting its proteasome-dependent degradation (Fig. 2.4f) (Wang et al. 2004). NAKED PINS IN YUC MUTANTS (NPY) is another BTB-domain protein, which forms a complex with the PINOID kinase and regulates auxin-mediated organogenesis downstream of the auxin efflux carriers, PINs (Strader and Zhao 2016). Last but not the least, a BTB-domain protein, BT2, has been suggested to antagonize ABA signals and enhance certain auxin responses (Mandadi et al. 2009). The BTB protein family has at least 80 members in the *Arabidopsis* genome, and the number is nearly doubled in rice due to a major subfamily expansion (Gingerich et al. 2007). Given the importance of phytohormone in regulating plant physiology, it is highly likely that more BTB-domain proteins are involved in mediating hormonal signaling.

2.2.3 An Introduction to CRL4-DDB1-DCAF/DWD E3s

The CLR4 E3 complexes represent a unique family of CRLs specialized for ubiquitinating substrates mostly in the nucleus (Li et al. 2003). Distinct from other cullins, CUL4 interacts with a large adaptor protein, DDB1, which consists of three β-propeller domains and assembles with a family of WD40-repeat-containing proteins, DCAF/DWDs, as the substrate receptors (Angers et al. 2006; Li et al. 2006; Jin et al. 2006; He et al. 2006). To date, four DCAF/DWD proteins, DWA1, DWA2, DWA3, and ABD1, have been functionally characterized in hormone signaling (Lee et al. 2010; Lee et al. 2011; Seo et al. 2014). All of them negatively regulate ABA signal transduction by recruiting the ABA-responsive transcription factor, ABI5, to the CRL4 E3 for ubiquitination and proteasomal degradation. Interestingly, DDA1, a small noncanonical DCAF/DWD protein lacking a WD40-repeat domain, has also been documented to downregulate ABA signaling. DDA1 interacts with the ABA receptors, PYL8, PYL4, and PYL9, and induces their CRL4-mediated ubiquitination (Irigoyen et al. 2014). With ~80 family members in *Arabidopsis thaliana* and rice, the CRL4 E3s most likely play additional roles in regulating hormone responses beyond the ABA pathway (Hua and Vierstra 2011).

2.2.4 Regulation of CRL E3s by Nedd8 and COP9

AXR1 is one of the first few genes identified in the genetic screens of auxin-resistant mutants (Leyser et al. 1993). With high sequence similarity to the E1 ubiquitin-activating enzyme, AXR1 was later characterized as the E1 enzyme for the UBL protein, NEDD8. Analogous to the activation of kinases by phosphorylation, all CRL E3s are activated by the covalent modification of cullin scaffolds by NEDD8 (also known as RUB1) at a specific lysine residue next to the RBX1-binding site (Deshaies and Joazeiro 2009). Crystal structures of neddylated cullins have revealed an open topology of the catalytic platform, in which the E2-binding RING domain of RBX1 is released from a cullin CTD cleft, potentially allowing the ubiquitin-charged E2 to approach the substrate recruited by the substrate receptor subunit anchored at the cullin NTD (Duda et al. 2008) (Fig. 2.3c).

In addition to enhancing the ligase activities, NEDD8 modification has been suggested to play a role in facilitating the exchange of substrate-binding modules on the shared catalytic platform of CRL E3s (Lydeard et al. 2013). In eukaryotic cells, a 120 kDa HEAT-repeat protein, CAND1, has been identified to bind the unmodified cullin-RBX1 complexes and block the assembly of substrate-binding modules, such as the SKP1-F-box protein complexes and BTB-domain proteins (Goldenberg et al. 2004). Because NEDD8 modification of cullins inhibits CAND1 association, dynamic neddylation and deneddylation are thought to mediate the redistribution and recycling of the CRL catalytic platform among different substrate-recruiting modules (Pierce et al. 2013).

To cleave NEDD8 from cullins, eukaryotic cells have evolved a conserved eight-subunit protein complex, the COP9 signalosome or CSN, which can interact with all cullin-RBX1 complexes (Wei and Deng 2003; Lyapina et al. 2001). Among the eight CSN subunits, CSN5 is the JAMM-type isopeptidase responsible for catalyzing the cullin deneddylation reaction (Cope et al. 2002). Interestingly, each of the CSN subunits shares sequence homology to one of the subunits of the 19S proteasome lid, suggesting that the two complexes have evolutionary, and possibly functional, connections (Wei et al. 1998). Just like the NEDD8 E1 enzyme, CSN was first identified in plants (Chamovitz et al. 1996). Mutations of each CSN subunit led to the same constitutive photomorphogenesis phenotype. Interestingly, the same phenotype is shared by mutants of three other genes—DET1, a DCAF/DWD protein; COP10, a DET1-DDB1-interacting E2 variant; and COP1, which is a RING E3 that also interacts with DET1 (Lau and Deng 2012; Chen et al. 2006; Wertz et al. 2004). Together, these proteins and CSN might act in the same pathway to promote the ubiquitination and degradation of HY5, a key transcription factor regulating photomorphogenic development. Besides mediating light signaling, CSN also participates in hormonal signal transduction by controlling the assembly of several aforementioned CRL complexes.