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Ubiquitin-mediated control of protein stability is central to most aspects of plant hormone signaling. Attachment of ubiquitin to target proteins occurs via an enzymatic cascade with the final step being catalyzed by a family of enzymes known as E3 ubiquitin ligases, which have been classified based on their protein domains and structures. While E3 ubiquitin ligases are conserved among eukaryotes, in plants they are well-known to fulfill unique roles as central regulators of phytohormone signaling, including hormone perception and regulation of hormone biosynthesis. This review will highlight up-to-date findings that have refined well-known E3 ligase-substrate interactions and defined novel E3 ligase substrates that mediate numerous hormone signaling pathways. Additionally, examples of how particular E3 ligases may mediate hormone crosstalk will be discussed as an emerging theme. Looking forward, promising experimental approaches and methods that will provide deeper mechanistic insight into the roles of E3 ubiquitin ligases in plants will be considered.

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E3 ubiquitin ligases: key regulators of hormone signaling in plants

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Abstract

Ubiquitin-mediated control of protein stability is central to most aspects of plant hormone signaling. Attachment of ubiquitin to target proteins occurs via an enzymatic cascade with the final step being catalyzed by a family of enzymes known as E3 ubiquitin ligases, which have been classified based on their protein domains and structures. While E3 ubiquitin ligases are conserved among eukaryotes, in plants they are well-known to fulfill unique roles as central regulators of phytohormone signaling, including hormone perception and regulation of hormone biosynthesis. This review will highlight up-to-date findings that have refined well-known E3 ligase-substrate interactions and defined novel E3 ligase substrates that mediate numerous hormone signaling pathways. Additionally, examples of how particular E3 ligases may mediate hormone crosstalk will be discussed as an emerging theme. Looking forward, promising experimental approaches and methods that will provide deeper mechanistic insight into the roles of E3 ubiquitin ligases in plants will be considered.

Roles of E3 ligases in phytohormone signaling

Ubiquitin E3 ligases are conserved among eukaryotes and fulfill a myriad of regulatory functions by facilitating the covalent attachment of ubiquitin to target proteins. Attachment typically occurs on lysine residues and can occur singly (monoubiquitination) or in chains (polyubiquitination). The types of attachments and degree of ubiquitination varies considerably among substrates and may be context dependent. This enzymatic reaction is carried out by a family of proteins called E3 ubiquitin ligases, which act at the end of a three-enzyme cascade to transfer ubiquitin from an E2 ubiquitin conjugating enzyme to a specific substrate(s). Ubiquitination of substrates is a tightly regulated process and can result in several functional outcomes, including protein degradation, changes in subcellular localization, and protein activation. In plants, numerous ubiquitin E3 ligases act as central regulators in phytohormone signaling pathways including auxin, brassinosteroid (BR), cytokinin (CK), ethylene, gibberellic acid (GA), jasmonate (JA), salicylic acid (SA), and strigolactone (SL) (for recent detailed reviews see (1–5)). This review will highlight up-to-date findings that have defined new E3 ligase-substrate interactions that mediate phytohormone signaling pathways, discuss examples of how some E3 ligases mediate hormone crosstalk, and touch on emerging approaches that will help us gain deeper mechanistic insight into these proteins.

There are over 1,500 E3 ubiquitin ligase proteins encoded by the Arabidopsis genome which can be subdivided into different families (6). This includes the HECT (homologous to the E6AP carboxyl terminus) type, RING (really interesting new gene) family, Kelch-type and U-box containing ubiquitin protein ligases. The Cullin-RING ligase (CRL) family can be further subdivided into five subfamilies based on subunit

organization and conserved domains: 1) SKP1-Cullin-F-box (SCF) type, 2) broad complex/tramtrack/bric-a-brac (BTB) type, 3) DDB1-binding/WD-40 domain containing proteins (DWD) type, 4) VON-HIPPEL LINDAU (VHL) type, and 5) SUPRESSOR OF CYTOKINE SIGNALING (SOCS) type (6). While CRLs have been called "molecular hubs" in plant hormone signaling pathways because of their central roles in hormone perception mechanisms and far-reaching cellular signaling effects, there are several examples of other types of E3 ubiquitin ligases playing roles in phytohormone signaling. Regardless of E3 ligase type, one common theme among these plant E3 proteins is that they interact with their substrates in a hormone-dependent manner (3). This is especially interesting given that both E3 ubiquitin ligases and plant hormones are diverse chemical structures with complex evolutionary histories.

The exact roles of E3 ligases in hormone perception and/or signaling have been well established for most known cases (Figure 1). The first hormone discovered to utilize ubiquitin E3 ligases as receptor molecules was auxin, which is perceived by SCF^{TIR1} and related Auxin F-box (AFB) family members (7, 8). Perception of auxin by $SCF^{TIR1/AFB}$ triggers rapid degradation of the Aux/IAA family of transcriptional repressors (9–13). Among all the various types of E3 ligases, the SCF type play prominent roles in perception of most phytohormones including jasmonic acid (14), gibberellin (15–17), ethylene (18), salicylic acid (SA) (19, 20), and strigolactones (21–25); also reviewed in (1). Other types of E3 ligases have also been linked to hormone signaling, including BTB type regulating ethylene biosynthesis (26–28) and CRL3-based E3 ligases modulating ABA signaling (Figure 1B). RING type E3 ligases play key roles in ethylene biosynthesis (29) and several aspects of ABA pathways including biosynthesis, transcriptional regulation, and signaling (30–34) (Figure 1C). U-box type E3s have been linked to ABA biosynthesis and downstream responses such as stomatal closure (35–37) (Figure 1D). Finally, an E3 ligase and autophagy receptor protein have recently described to regulate BR signaling during stress (38, 39) (Figure 1E). Overall, the number of E3 ligases linked to ABA signaling thus far is greater than for any other phytohormone, demonstrating the diversity and extent to which substrate ubiquitination can regulate ABA biosynthesis, signaling and downstream responses (1).

Detailed biochemical studies have provided new insights into E3-substrate complex assembly and composition. For instance, protein structure studies on TIR1-auxin-AUX/IAA and JA-COI1-JAZ (JASMONATE ZIM-DOMAIN) complexes have revealed that small molecule co-factors are directly involved in SCF^{TIR1} and SCF^{COI1} complexes (inositol hexakisphosphate ($InsP_6$) and inositol pentakisphosphate ($InsP_5$) respectively) (14, 40). Additionally, several of the key E3 ligases involved in auxin, JA and SL perception have been proposed to function as "co-receptor" complexes, whereby high-affinity hormone binding is facilitated by both the E3 ubiquitin ligase and substrate. This has been observed for the TIR1-auxin-AUX/IAA complex, the COI1-JA-Ile-JAZ complex, the ABI1 PUB12/13/U-box E3 ligase complex and the MAX2/D3-SL-D14 complex (11, 14, 41–45). These molecular interactions along with distinct naturally occurring forms of these hormones and co-receptor pairs could provide for increased complexity and specificity via varied combinatorial configurations, which may underlie particular downstream responses mediated by auxin, JA, ABI and SL.

Substrate recognition has been mapped to minimal amino acid sequence motifs for several E3 targets related to auxin and JA signaling through careful biochemical

studies; such sequences are not identifiable based solely on primary amino acid sequence and thus are not amenable to bioinformatics based approaches. For instance, all Aux/IAAs contain a short consensus recognition motif, or 'degron', which directly engages with auxin-loaded TIR1 (13, 40, 46). However, regions outside the degron appear to contribute to differential hormone binding affinity among Aux/IAAs and F-box proteins (11, 46). JAZ proteins also contain a condensed degron sequence, which is a variable region in direct contact with the COI1-anchored JA-Ile molecule (14). Notably, this mechanism of interaction has been co-opted into a yeast assay for monitoring ubiquitin-mediated protein degradation in yeast called the auxin-inducible degradation (AID) system (46–49). This assay enables versatile conditional protein depletion and can be applied to various eukaryotic proteins that function as part of SCF complexes and/or substrates.

Novel E3 ligase-substrate interactions involved in hormone signaling

A number of new interactions have recently been described for several E3 ubiquitin ligases, which play various roles in hormone signaling. In particular, several novel E3-substrate interactions have been described in BR pathways. One of the most surprising recent findings describes how BES1 (BRI1 EMS SUPPRESSOR 1) can be ubiquitinated by SINAT (SINA of Arabidopsis thaliana) E3 ligases, which promotes BES1-DSK2 (DOMINANT SUPPRESSOR OF KAR 2) interactions and subsequent degradation via selective autophagy (38, 39). Thus, both the proteasome and selective autophagy are involved in degrading BES1 while SINAT E3s ubiquitinate BES1 during starvation and light response. While it is not clear why a single transcription factor would need to be degraded through several independent pathways, although it has been proposed that such multiple regulatory checkpoints could regulate BES1 levels to allow for integration of morphogenesis with distinct environmental cues such as light or stress. This work also points to an interesting possibility that other E3 ubiquitin ligase substrates could be degraded via selective autophagy mechanisms, but the signals that direct ubiquitinated proteins to autophagy versus proteasome mediated degradation need to be studied further. A novel F-box protein, KINK SUPPRESSED IN BZR1-1D (KIB1) was recently shown to mediate BR-induced ubiquitination and degradation of the glycogen synthase kinase-3 (GSK3)-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) (50). Also, plasma membrane localization of the BRI1 receptor is regulated by ubiquitination but the E3 ligase(s) responsible are still unknown (51). Additionally, two different U-box type E3 ubiquitin ligases have been implicated as positive regulators of BR signaling in rice, ERECT LEAF 1 (ELF1) (52) and Taihu Dwarf (TUD1) (53) but further studies are required to identify the substrates of these ligases and their role(s) in BR-mediated plant growth.

Recent studies have continued to build on our understanding of how ABA pathways are regulated post-translationally. For example, the RING E3 SDIR targets SDIR1-INTERACTING PROTEIN1 for degradation to modulate ABA signaling (54). Additionally, degradation of the ABA receptor ABI1 occurs by the PUB12/13 U-box E3 ligases (41). Perception of ABA by the pyrabactin resistance (PYR)/PYR1-like (PYL)/regulatory components of ABA receptor (RCAR) proteins with the co-receptor protein phosphatase type 2Cs facilitates activation of Snf1-related protein kinase 2 (SnRK2) kinases, which are in turn ubiquitinated and degraded by $^{SNFAtPP2\text{-}B11}$ (55). AtPP2-

B11 is an newly described F-box protein that functions as part of a canonical SCF E3 ligase complex to negatively regulate plant responses to ABA (55). Adding to the complexity of this pathway is ECERIFERUM9 (CER9), which encodes a putative RING domain-containing E3 ubiquitin ligase that is a novel negative regulator of ABA biosynthesis and ABA signaling during seed germination. CER9 is similar to Doa10 in *S. cerevisiae*, which targets substrates for degradation via the UPS. Further work on CER9 will be required to identify target protein(s) and their role in ABA signaling (56).

Other examples of recent ligase-substrate interactions include description of two novel E3 ligases involved in JA signaling, RING DOMAIN LIGASE 3 (RGLG3) and RGLG4 (57), and DEFECTIVE IN ANTHER DEHISCENCE1-Activating Factor (DAF) (58); the direct substrates of these ligases are not currently known and await further studies. Additionally, the plant U-box protein (PUB10) was recently found to regulate MYC2 degradation while de-ubiquitination enzymes UBP12 and UBP13 positively influence MYC2 stability (59, 60). Identification of UPB12 and UBP13 highlights our limited understanding of the roles of de-ubiquitination enzymes in plant hormone pathways and suggests that this aspect of ubiquitination could be better explored in plants. Finally, SA was one of the last major phytohormones without a known receptor. A recent study provides further evidence that the E3 ligase Nonexpressor of Pathogenesis-Related genes 1 (NPR1) gets degraded via interaction with the closely related NPR3/NPR4 substrate adaptor proteins (19, 61), thus NPR1 or NPR1-related proteins (NPR3 and NPR4) are the long-sought-after SA receptors (62)

New insights into ubiquitin attachment to substrates

Typically, ubiquitin is covalently attached to substrate proteins on lysine residues but there are several examples of non-canonical attachment mechanisms in other eukaryotes (63). With respect to E3 substrate proteins involved in phytohormone signaling the AuxIAA family is the best studied to date (13, 46, 47, 64, 65). A major outstanding question in the field was which residue(s) served as ubiquitin attachment sites and thus participate in auxin perception. Recent biochemical studies have provided surprising findings and underscore the need for further work related to ubiquitin attachment sites in plants. Specifically, biochemical and peptide mass spectrometry studies have recently demonstrated that conserved and variable lysine residues on AuxIAA proteins are ubiquitinated (65) but in the absence of lysine residues attachment can occur on serine and/or threonine positions (64). Taken together these studies demonstrate that ubiquitination of AuxIAA proteins can occur in several exposed flexible regions (i.e. “ubiquitination zones”) in a dose dependent manner. Additionally, several alternative linkage topologies may occur on AuxIAA proteins, including poly-mono-ubiquitination and/or multi-, poly-ubiquitination (65). By applying similar approaches to other well characterized substrates (Figure 1) it will be possible to determine if such complex and/or flexible attachment properties are common or restricted to Aux/IAAs.

In other eukaryotes, post-translational modification (for e.g. phosphorylation) of substrate proteins is a common mechanism that regulates E3-substrate interactions, but the extent to which this occurs in plants is not clear. One unique example to date is the demonstration that s-nitrosylation of ABA INSENSITIVE 5 (ABI5) facilitates the subsequent degradation by the E3 ligase KEEP ON GOING (KEG) (66). Further studies

focused on how E3 substrate proteins are modified (or not) will help address this discrepancy.

Making connections: E3 ligases and targets coordinate phytohormone crosstalk

Recently, a number of studies on E3 ligases in plants have demonstrated potential crosstalk mechanisms between various phytohormones. This finally moves hormone crosstalk out of the realm of transcriptional regulation and into the proteome, which potentially can impact hormone signaling in a more rapid and integrated fashion. For example, Dwarf and short grain 1 (DSG1) encodes U-box E3 that is collectively regulated by BR, ET, auxin and SA and functions to positively regulate cell division and elongation (67).

Another putative E3 ligase that integrates dueling hormone pathways to control photomorphogenesis is HIGH EXPRESSION OF OSMOSTICALLY RESPONSIVE GENES 1 (HOS1) (68, 69). HOS1 regulates hypocotyl expansion via auxin signaling and leaf expansion via ethylene, suggesting that this RING E3 ligase can regulate multiple hormone pathways in a tissue specific manner. Studies from the rice ortholog show that OsHOS1 directly regulates the stability of two ETHYLENE RESPONSE FACTOR transcription factors, rice ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN 1 (OsEREPB1), and OsEREBP2, which thereby regulates the JA mediated root curling response (70).

Hormone antagonism between ABA and SA also appears to involve E3 ligase activity, specifically via the regulation of NONEXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1). ABA and SA act antagonistically to influence NPR1 levels: while ABA promotes NPR1 degradation via the CUL3(NPR3/NPR4) complex, SA stabilizes NPR1 from ABA-promoted degradation through phosphorylation (71). The ABA pathway also integrates with JA signaling via the RING E3 ligase KEG modulating JASMONATE ZIM-DOMAIN (JAZ) protein stability (72).

A final example of potential hormone crosstalk involves targets of the SCF^{MAX2} E3 ubiquitin ligase, which is central to strigolactone (SL) signaling. Multiple proteins have been suggested as targets for SCF^{MAX2} including SUPPRRESSOR OF MORE AXILLARY GROWTH-LIKE (SMXL) family members, BRI1-EMS-SUPPRESSOR (BES1) and DELLAs (43, 73, 74). Thus, perception of SL could lead to alterations in BR and GA signaling that converge to regulate shoot development. Additionally, MAX2 can oppositely regulate GA and ABA biosynthesis to promote photomorphogenesis (75). Further work is required to determine the extent to which SL signaling directly regulates protein degradation in each of these individual hormone pathways, but SCF^{MAX2} holds a unique regulatory position among hormone crosstalk mechanisms.

Regulation of hormone related E3 stability

Substrate stability has been an intense area of study, but E3 ligases have also been shown to have variable stability. A wealth of evidence demonstrates that various factors can contribute to E3 ligase stability, such as self-ubiquitination and protein-protein interactions. Considering the central roles F-box proteins play in phytohormone perception, it is of interest to know how these proteins themselves are regulated such that it impacts hormone perception and/or other downstream signaling events. Biochemical and genetic studies have recently explored stability of different types of E3 ligases

involved in auxin, ABA and ethylene signaling. For example, the auxin receptor TIR1 has been recently found to undergo autocatalytic degradation based on key residues that affect binding to CUL1 that were identified from yeast two-hybrid assays (76). Furthermore, direct associations between SCF^{TIR1} and HEAT SHOCK PROTEIN 90 (HSP90) have been recently shown to regulate the stability of TIR1 in response to temperature, which contributes to auxin responsiveness (77). Additionally, oligomerization of TIR1 appears to contribute to SCF^{TIR1} function and auxin signaling (78), suggesting that the relative levels of TIR1 may influence auxin perception mechanisms as well. Another example comes from studies on ABA-regulated degradation of KEG; biochemical experiments have recently identified phosphorylation of KEG driven by Calcineurin B-like Interacting Protein Kinase 26 (CIPK26) as being important for KEG activity (79). Finally, the Ethylene Overproducer1 (ETO1)/ETO1-like family of E3 ligase proteins are negatively regulated by light such that ethylene biosynthesis is promoted during photomorphogenesis (80). Continued efforts in these areas to define protein-protein interactions, post-translational modifications and/or other contributing factors regulating stability of particular E3 ligases involved in phytohormone signaling should provide fresh insight into how particular complexes may be modified.

Looking forward: methods & approaches to gain deeper mechanistic insights

To date, ~100 proteins have been shown to be involved in hormone-mediated E3 ligase activity in model plant systems through detailed genetic and biochemical studies. There are still many outstanding questions in the field that could be addressed using peptide mass spectrometry-based approaches, including improved identification of plant ubiquitinated proteins. Various affinity purification techniques together with mass spectrometry have been fruitful in identifying ubiquitinated proteins in response to light and during seedling development (81, 82) but similar studies have yet to be performed following hormone treatments. Thus, we have only captured a portion of the plant ubiquitinome to date and new approaches and methods will need to be applied to go deeper. In other eukaryotic systems, the use of antibodies that recognize the Lys-E-Gly-Gly (diGLY) remnant that is generated following trypsin digestion of ubiquitinated proteins have been successful at quantitatively describing ubiquitination sites under various cellular conditions (83–85). This approach has been used in rice and wheat to describe ~400 ubiquitinated in rice and ~300 ubiquitinated proteins in wheat (86, 87), demonstrating that it could be effectively applied to other model plant species to perform comprehensive profiling of hormone-dependent ubiquitinated proteins.

Another successful approach to identify ubiquitinated proteins is the so-called StUbEx method which relies on replacing endogenous ubiquitin in human U2OS cell lines with a modified version that amenable to purification and identification via peptide mass spectrometry (88). Arabidopsis and other key plant species (*Physcomitrella patens*, *Zea mays*, *Marchantia polymorpha*) have >10 copies of ubiquitin genes which is a hurdle to reduction and replacement of this regulatory protein. However, *Chlamydomonas reinhardtii* is an algal model species that only contains four copies of ubiquitin and is amenable to targeted DNA replacement (89). Thus, application of the StUbEx approach to *Chlamydomonas reinhardtii* would be possible and perhaps allow deeper identification of ubiquitinated proteins related to plant hormone signaling. Altogether such high-throughput proteomics approaches could greatly expand our depth of understanding with

respect to phytohormone signaling and aid in identification in exact sites of modification on substrates.

The recent identification of ubiquitination sites on Aux/IAA proteins (65) is a reminder that we do not know the exact sites of ubiquitination for most of the other known substrates in phytohormone signaling pathways. A sensor-based proteomic approach modeled after the Vx3K0 K63 polyubiquitin-specific sensor identified 107 proteins in juvenile Arabidopsis plants with K63 polyubiquitination events (90). This approach is based on three repetitions of ubiquitin interaction motifs from *Saccharomyces cerevisiae* VPS27 subunit of the Endosomal Sorting Complex Required for Transport (termed Vx3K0) combined with a helical linker that spaces the UIMs for selective binding to K63-linked polyubiquitin chains (90). Similar polyubiquitin-sensor methods have revealed localization a linkage type dependence of ubiquitin signaling events in mammalian cell lines (91, 92). With this method (90) now developed for Arabidopsis, further studies could be performed on various mutants and/or following hormone treatments in order to provide better resolution of known hormone regulated E3 substrates. Other types of linkages may require different approaches for identification in plants, but such data will greatly increase our understanding of ubiquitinated proteins.

An additional challenge in the field has been defining E3-target interactions. There are still hundreds of E3 ligases in *Arabidopsis* without known substrates and several known substrates without known ligases. Given the large number of E3 ligases with known roles in hormone pathways it would be reasonable to assume there are more UPL proteins involved in hormone signaling that have not been captured via traditional genetic or biochemical approaches. Because these enzymes are part of large gene families in plants with high degrees of functional overlap it can be challenging to perform functional studies; for instance, there are >700 F-box proteins encoded by the *Arabidopsis* genome. Additionally, interactions between E3 ligases and their substrates are often transient and rapid, which can make detection difficult. One approach to mitigate these issues involves expression of affinity-tagged dominant negative F-box proteins which allow *in vivo* substrate identification (93). A recent application of this method to generate a F-box "decoy" to trap and identify *in planta* substrates has been applied to circadian regulated F-box targets (94). In this method the authors removed the F-box domain from a small family of partially redundant paralogous F-box proteins in order to allow these proteins to interact with their cognate substrates without triggering ubiquitination. These mutant proteins were fused with a dual affinity tag (3xFLAG-6xHis) to allow for affinity-purification followed by mass spectrometry (AP-MS) to detect ligase-substrate interactions. This approach yielded a number of novel interacting proteins that were validated using heterologous systems including yeast two-hybrid and expression and co-immunoprecipitation in mammalian cell cultures. If this approach were applied to additional F-box proteins involved in phytohormones signaling further progress could be made towards substrate identification and mapping of ubiquitination sites. Additionally, the application of other successful AP-MS approaches (95, 96) to these studies may help to uncover substrate proteins for E3 ligases that have remained elusive.

Another strategy for mapping ligase-substrate interactions could involve high-throughput protein-protein interaction assays such as yeast two-hybrid screens. The excellent genome annotation in *Arabidopsis* and available rapid cloning techniques make

such screens readily feasible. Given the large number of transcriptional regulators known to be E3 targets (1) it would be logical to screen existing transcription factor libraries (97) against a custom library of E3 ligases to identify potential ligase-substrate interactions; while some interactions may require additional molecules such as hormones, other protein-protein interactions will be detectable by such high-throughput screens. Additionally, researches can validates these omic results and overcome genetic redundancy amount E3 ligases by using multiplexed CRISPR/Cas9 techniques developed for Arabidopsis (98). Altogether the application of high-throughput omics approaches will deepen our understanding of E3 ligases involved in hormone signaling and provide novel insights to answer several outstanding questions.

Figure 1. Several different types of E3 ubiquitin ligases have known roles in various aspects of phytohormone pathways. Illustrated here are representative E3-subtrate interactions from each class of E3 ligase with corresponding hormone(s) shown below. (A) SCF type E3 ligases have been linked to several hormone pathways, including auxin, JA, GA, ethylene, strigolactones, cytokinins and ABA. (B) BTB-type E3 ligases play roles in ethylene, SA and ABA pathways. (C) RING-type E3 ligases are important regulators of ABA and ethylene signaling. (D) One U-box type E3 ligase contributes to ABA biosynthesis. (E) A novel mechanism for selective autophagy via the SINAT E3 ligases and ubiquitin receptor DSK2 has recently been linked to BR signaling.

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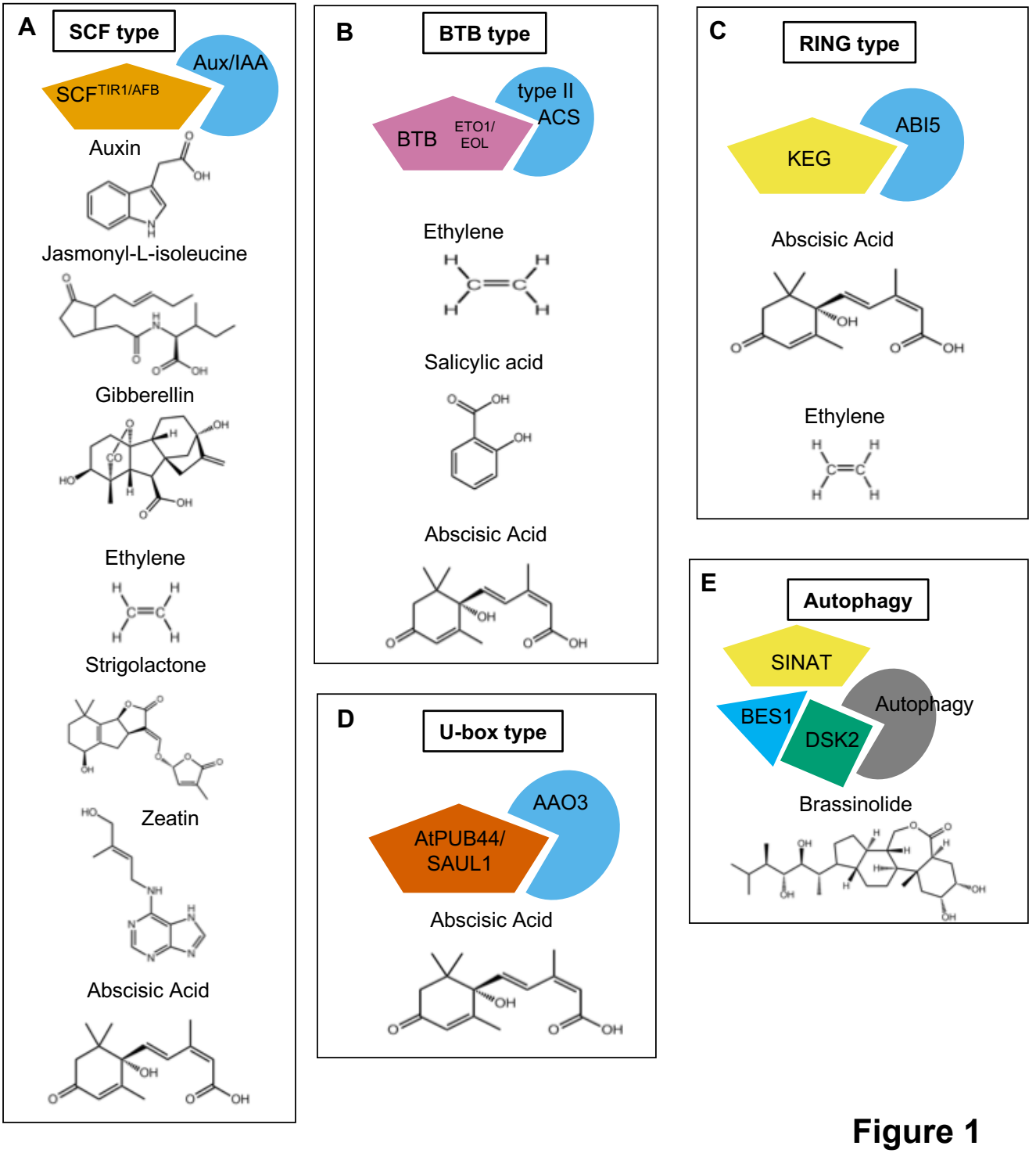
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type II ACS
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ETO1/ EOL
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Abscisic Acid
C
RING type
ABI5
KEG
Abscisic Acid
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BES1
DSK2
Autophagy
Brassinolide

Figure 1