Jasmonate and auxin perception: how plants keep F-boxes in check

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Highlight

Increased knowledge about protein-protein interactions, posttranslational modifications and modifying ligands of plant receptor F-box proteins, will enable uncoupling of phytohormone responses and a more precise coordination of vital plant processes.

Abstract

Phytohormones regulate the plasticity of plant growth and development, and responses to biotic and abiotic stresses. Many hormone signal transduction cascades involve ubiquitination and subsequent degradation of proteins by the 26S proteasome. The conjugation of ubiquitin to a substrate is facilitated by the E1 activating, E2 conjugating and the substrate-specifying E3 ligase. The most prevalent type of E3 ligase in plants is the Cullin-RING ligase (CRL)-type with F-box proteins (FBPs) as the substrate recognition component. The activity of these SKP-Cullin-F-box (SCF) complexes needs to be tightly regulated in time and place. Here, we review the regulation of SCF function in plants on multiple levels, with a focus on the auxin and jasmonate SCF-type receptor complexes. We particularly discuss the relevance of protein-protein interactions and posttranslational modifications as mechanisms to keep SCF functioning under control. Additionally, we highlight the unique property of $SCF^{TIR1/AFB}$ and SCF^{COI1} to recognize substrates by forming co-receptor complexes. Finally, we explore how engineered selective agonists can be used to study and uncouple the outcomes of the complex auxin and jasmonate signaling networks that are governed by these FBPs.

Keywords

JA-Ile, coronatine, Arabidopsis, ubiquitin proteasome system, ubiquitination, small molecule binding, polyubiquitination, posttranslational modification, heat shock protein, neddylation

Abbreviations

APC/C, anaphase-promoting complex or cyclosome; Aux/IAA, auxin/indole-3-acetic acid; COI1, coronatine-insensitive protein 1; CRL, Cullin-RING ligase; FBP, F-box protein; GA, gibberellic acid; HECT, homology to E6-AP C-terminus; HSP, HEAT SHOCK PROTEINS;JA, jasmonate; JA-Ile,

(+)-7-iso-jasmonoyl-L-isoleucine; JAZ, jasmonate-ZIM domain; LRR, leucine-rich repeat; MAX2, MORE AXILLARY GROWTH 2; NEDD8, neural precursor cell expressed, developmentally down-regulated 8; PPI, protein-protein interaction; PTM, posttranslational modification; RING, Really Interesting Gene; RBX1, RING-box 1; SCF, SKP-Cullin-F-box; TIR1/AFB, TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX; TPL, TOPLESS; UPS, ubiquitin proteasome system

Introduction

Plants face a continuously changing environment, which poses serious challenges. Therefore they, among others, employ distinct phytohormone cascades to launch the most apt and targeted response. Oxylipins, such as jasmonate (JA), represent a class of phytohormones that is intimately involved in modulating how plants respond to various perturbations. The bioactive form of JA, (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), is recognized by CORONATINE-INSENSITIVE PROTEIN 1 (COI1) and JASMONATE-ZIM DOMAIN (JAZ), forming a co-receptor complex (Fig. 1) (Fonseca *et al.*, 2009; Sheard *et al.*, 2010). COI1 is an F-box protein (FBP), typically forming part of an SKP-Cullin-F-BOX (SCF) E3 ubiquitin ligase complex that recognizes and facilitates the ubiquitination of the JAZ proteins, thereby marking them for degradation by the 26S proteasome (Sheard *et al.*, 2010). The JAZ proteins, by themselves or in association with the co-repressor TOPLESS (TPL) and the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA), act as repressors of transcription factors, like MYC2 (Fig. 1) (Chini *et al.*, 2007; Thines *et al.*, 2007; Pauwels *et al.*, 2010; Fernández-Calvo *et al.*, 2011; Pauwels and Goossens, 2011; Chini *et al.*, 2016). More recently it was discovered that MEDIATOR 25 (MED25) physically interacts with MYC2 and COI1, thereby linking the JA receptor to transcriptionally active chromatin (Fig. 1) (An *et al.*, 2017).

The above-mentioned, commonly designated JA-signaling core proteins involved in JA signaling show conservation among land plants (Howe *et al.*, 2018; Monte *et al.*, 2019). Moreover, the analogy among phytohormone signaling pathways is remarkable. Indeed, not only JA signaling depends on FBPs as receptors, but also strigolactone, auxin and gibberellic acid (GA) signaling (Dill *et al.*, 2004; Zhao *et al.*, 2013). COI1's closest homolog, TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) (Fig. 2A), also integrates into an SCF-complex that mediates the ubiquitination and degradation of another family of repressors, the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins, following auxin perception (Gray *et al.*, 2001).

Much research has been devoted to the substrate and ligand specificity of the FBPs. However, as we gain better knowledge of how FBPs and other components regulating phytohormone signaling cascades have evolved, it is imperative to also understand how plants

keep the FBP receptors "in check". In this review, we will therefore highlight the knowledge about the control of the activity of the FBPs involved in plant hormone signaling, with a focus on COI1 and TIR1. For overviews of the regulation of the interaction between plant FBPs and their hormone ligands and protein substrates, we invite the reader to consult the many excellent reviews that already exist in the field.

The ubiquitin proteasome system

The ubiquitin proteasome system (UPS) is highly conserved in all domains of multicellular life, with plant genomes encoding large numbers of UPS components. For instance, 6% of the *Arabidopsis thaliana* genome is predicted to be involved in ubiquitin-mediated signaling pathways (Vierstra, 2009).

Enzymatic steps of ubiquitination

Ubiquitination is a posttranslational modification (PTM) that involves the covalent attachment of a 76-amino-acid protein called ubiquitin to proteins, either as a single ubiquitin moiety ~~protein~~ or a chain of ubiquitin molecules (polyubiquitination). This PTM event is orchestrated by three enzymes: the E1 activating enzyme, the E2 conjugating enzyme and the E3 ligase that act in concert to promote ubiquitination of the substrate (Fig. 1). The E1 enzyme catalyzes the thioester bond formation between the ubiquitin molecule and a cysteinyl sulfhydryl group on the E1 itself (Burch and Haas, 1994). Next, the E1~ubiquitin transfers the ubiquitin to an E2 protein. Subsequently, through direct interaction with the E2~ubiquitin and the substrate, the E3 ligase facilitates the transfer of the ubiquitin to the substrate. The *Arabidopsis* genome, similar to other plant species, carries two genes coding for E1s, 37 for E2s and a massive amount, over 1500, for E3 ligases (Vierstra, 2012).

Ubiquitin linkages and topology affect substrate function

An ubiquitinated protein's fate is dictated by the topology and linkage of the ubiquitin moieties. Proteins are often ubiquitinated on lysine residues or at the N-terminal amino groups (Walton *et al.*, 2016), or, less commonly, on serine or threonine residues (Gilkerson *et al.*, 2015). In

addition to the different ubiquitin additions on the substrate, all seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) found on the ubiquitin protein show the potential for ubiquitin linkage (Kim *et al.*, 2013). Whereas the common K48 linkage results in polyubiquitination and proteasome degradation, not every ubiquitinated protein undergoes proteolysis. For instance, mono-ubiquitinated proteins have been implicated in DNA repair and intracellular trafficking (Barberon *et al.*, 2011; Kasai *et al.*, 2011; Strzalka *et al.*, 2013). Conversely however, linkage on K29, specifically of the DELLA proteins, which are involved in gibberellic acid (GA) signaling, has also been implicated in proteasomal degradation, (Wang *et al.*, 2009).

The SCF complex coordinates the ubiquitination of target proteins

The E3 ligases are normally classified based on the composition of their subunits, on their catalytic domains and, mechanistically, on how they facilitate the transfer of the ubiquitin from the E2 to the substrate (Hua and Vierstra, 2011). Several types exist, but here we will focus exclusively on the SCF complex E3 ligases, because of their well-studied involvement in the regulation of many diverse aspects of plant development and interaction with the environment, through their role as receptors for several phytohormones such as auxins and JAs (reviewed in Lechner *et al.*, 2006; Li *et al.*, 2018).

The SCF complex is classified as a multimeric E3 ligase. Molecularly**,** SCF complexes are comprised of four subunits in Arabidopsis: Cullin1 (CUL1), Arabidopsis SKP1-like (ASK) proteins, RING-box 1 (RBX1) and the FBP, with each subunit aiding in the E3's ability to recognize and ubiquitinate the substrate, thereby marking it for degradation (Fig. 1). The CUL1 protein acts as a scaffold, interacting with RBX1 at its C-terminus, which coordinates the recruitment of the E2 conjugating enzyme (Zheng *et al.*, 2002). Furthermore, CUL1 interacts with ASK proteins at its N-terminus. Through their interaction with the FBPs, ASK proteins act as adaptors. Experimental evidence demonstrated that the individual subunits in the SCF complex are regulated by redox state, PTMs and protein-protein interactions (PPIs) (Dezfulian *et al.*, 2016; Bagchi *et al.*, 2018; Iglesias *et al.*, 2018). Individually regulating posttranscriptionally the subunits of the SCF complex and consequently the degradation of different substrates, may help ensure the swift initiation of the different hormone signaling cascades. It is possible that upon a specific

environmental cue, the abundance of only the FBP is adjusted, without having to rapidly turn over the other proteins within the complex. Possibly offering support of this, weak *coi1* alleles – which have a lower amount of COI1 protein present – demonstrate only partial loss of COI1 function, implying there may be regulatory COI1 dosage-dependent effects that alter COI1 physiological roles (He *et al.*, 2012; Yan *et al.*, 2013). Albeit, the partial loss of COI1 function seen in weak *coi1* alleles is possibly also partially dependent on the *coi1* mutations residing in the leucine-rich repeat (LRR), C-terminal substrate-interacting domain, effecting COI1's ability to interact with a subset of JAZ proteins. Future investigation into dosage dependency of FBPs, possibly through the creation of weak alleles generated by CRISPR, is capable of addressing this question.

The major role of the FBPs is the targeted recognition of substrates for ubiquitination. FBPs are characterized by an ≈60-amino-acid FBP domain, located at their N-terminus, that interacts with ASK proteins (Xu *et al.*, 2009) (Fig. 2A). The C-terminus of the FBP acts as a substrate recognition domain, subdividing the FBPs into 42 families (Xu *et al.*, 2009). The FBA, Kelch and LRR domain families are among the largest (Xu *et al.*, 2009). The hormone receptors TIR1, COI1, SLEEPY1 (SLY1, for GAs) and MAX2 (for strigolactones) have LRR type F-box domains (Fig. 2A). Yan *et al.* (2018) recently demonstrated that COI1 acts as the primary receptor for JA and can bind JA or COR without JAZ present, whereas JAZ was not shown to bind JA in the absence of COI1. Understanding more about the dynamics of hormone perception is helping to reveal new sights into how CRLs and their specific FBP components play essential roles in the activation of plant hormone responses (Tan *et al.*, 2007; Thines *et al.*, 2007; Fu *et al.*, 2012; Verma *et al.*, 2016).

There are roughly 700 FBPs encoded in the *Arabidopsis* genome, approximately ten times more than in humans and *Drosophila melanogaster* (Jin *et al.*, 2004; Schumann *et al.*, 2011). The high number of FBPs is ubiquitous across higher plants, as *Medicago truncatula* has roughly 1100, *Zea mays* and *Populus trichocarpa* around 400 and *Oryza sativa* (rice) more than 900 FBPs, highlighting the evolutionary importance of the expansion of this family (Stefanowicz *et al.*, 2015). The mechanism behind the high number of FBPs is postulated to be due to tandem duplication events (Gagne *et al.*, 2002). Genome analysis uncovered that many rice *FBP*

genes are found in tandem repeats of two to nine genes, and approximately 35% of Arabidopsis *FBP* genes are in tandem repeats of two to seven genes (Gagne *et al.*, 2002; Jain *et al.*, 2007). Although a large number of FBPs are functionally uncharacterized, it is likely that not every *FBP* gene evolved a unique function, because FBPs have been found to show functional redundancy (Xu *et al.*, 2009; Ikram *et al.*, 2014).

In the following paragraphs, we discuss how the abundance and function of hormone receptor FBPs are regulated through PPIs, protein-ligand interactions and PTMs, with a special focus on COI1 and TIR1. Additionally, we highlight new insights into how engineered agonists can be developed to modulate the conformational changes necessary for FBP substrate interaction, which can be utilized to uncouple specific hormone responses.

Regulation of SCF assembly: interactions between FBPs, CUL1, RBX1 and ASK proteins

Above we have described that FBPs are components of the SCF complex functioning in polyubiquitin-mediated protein degradation. In the following paragraphs, we focus on how the activity of FBPs are kept in check by the availability and activity of the other members of the complex.

Cullin is the scaffold

RBX1 recruits the E2 conjugating enzyme, with CUL1 providing the horseshoe-shaped scaffold that bridges the interaction with ASK and the FBP (Fig. 1). Essential for the formation and activity of the complex is the (de)neddylation of CUL1 (Scott *et al.*, 2014). Analogous to ubiquitination, neddylation entails the covalent addition of the small protein NEDD8 (neural precursor cell expressed, developmentally down-regulated 8, also known as RELATED TO UBIQUITIN (RUB)) to a substrate protein in plants (reviewed in Mergner and Schwechheimer, 2014). Thus, these two very similar PTMs are directly linked to each other, with neddylation controlling ubiquitination and consequently protein degradation. The transfer of NEDD8 accordingly occurs by NEDD8 E1 activating enzymes (NAE) and E2 conjugating enzymes (NCE) (Mergner and Schwechheimer, 2014). DCN1 was found to be a NEDD8 E3 ligase, because it is sufficient for facilitating the transfer of NEDD8 to its substrate, typically the CUL

proteins (Kurz *et al.*, 2008). However, it is likely that there are more NEDD8 E3 ligases. Furthermore, CUL1 also may undergo deneddylation, which can be achieved by CSN5, a subunit of the CSN (COP9 signalosome) complex (Dohmann *et al.*, 2005; Franciosini *et al.*, 2015). In its deneddylated stage, CUL1 physically interacts with Cullin-ASSOCIATED AND NEDDYLATION-DISSASOCIATED (CAND) 1 (Feng *et al.*, 2004). In animals, it has been shown that CAND1 acts as a protein exchange factor promoting the exchange of FBPs within the SCF complex (Pierce *et al.*, 2013). Neddylation of the proteins enables proper SCF function but whether deneddylation of CULLIN inhibits the SCF complex remains to be determined because studies show a necessity for 'dynamic' deneddylation (Fig. 2) (Schwechheimer *et al.*, 2001; Jin *et al.*, 2018).

Interestingly, in addition to the usual view of ASK1 forming a bridging protein between the FBP and CUL1, the human crystal structure of the SCF^{SKP2} complex revealed that the N-terminus of the FBP directly interacts with that of CUL1 (Zheng *et al.*, 2002). Insight into the biological relevance of CUL1 interacting directly with a plant FBP, TIR1, was gained through mutating residues in the conserved first helix (H1) of the F-box domain, responsible for CUL1–FBP interaction. This resulted in enhanced levels of TIR1, but phenotypically resembled auxin-resistant mutants, mainly because the increase in TIR1 protein levels is thought to help stabilize the Aux/IAA repressor proteins by inhibiting their degradation (Fig. 2) (Yu *et al.*, 2015). Similarly, in Arabidopsis the higher abundance of the homologous AFB1 protein compared to that of TIR1 is related to a natural variation in AFB1's H1 domain that affects AFB1's ability to interact with CUL1. This may be a mechanism that extends to regulating protein abundance of other plant FBPs but some questions remain. Notably, TIR1's closest homolog COI1 shows an increased stability through its assembly with the SCF complex (Yan *et al.*, 2013). Also, how assembly into the SCF complex decreases stability of the FBP needs to be further validated, although, it most likely resembles the autocatalytic mechanism in animals and fungi (Yu *et al.*, 2015).

RBX1 forms the catalytic core

RBX1 forms the catalytic core of the SCF complex. A specific interactor of RBX1, Glomulin (GLMN), seems to interfere with SCF activity in mammals (Duda *et al.*, 2012) and, very recently, its Arabidopsis homolog, ABERRANT LATERAL ROOT FORMATION4 (ALF4), was also shown to

(Bagchi *et al.*, 2018). The interaction between ALF4 and RBX1 in Arabidopsis inhibits SCF^{TIR1} activity, resulting in the accumulation of Aux/IAA repressors (Fig. 2) (Bagchi *et al.*, 2018). It remains unknown if ALF4 (or a potential homolog) also interacts with RBX1 of SCF^{COI1} and would thus modulate JA response as well. Additionally, RBX1 regulates FBPs by promoting the neddylation of CUL (Gray *et al.*, 2002).

ASK acts as adaptor

Compared to CUL1 and RBX1, the 21 ASK proteins from a larger family in Arabidopsis (Ogura *et al.*, 2008). This allows many combinatorial ways of SCF assembly, although many FBPs have no known ASK adaptor partner. Multiple groups have sought to uncover the specificity of ASK proteins for FBPs, employing bimolecular fluorescence complementation, yeast two-hybrid and microarray gene expression profiles across various tissues (Dezfulian *et al.*, 2012; Kuroda *et al.*, 2012). The different ASKs display variable affinities and interaction capacities for the multiple FBPs and vice versa. (Wang *et al.*, 2006; Kuroda *et al.*, 2012). It remains to be understood why certain FBPs are unable to interact with ASKs in yeast two-hybrid assays, when this system is able to validate many FBP–ASK interactions. It may be that, because it is a heterologous system, specific PTMs or missing 'unknown' factors are required for interaction between ASKs and FBPs. Another heterologous system used to validate ASK interactors is baculovirus-insect cells. Using this system, it was demonstrated that ASK(1/2/11/13) protein presence was necessary for degradation of JAZ proteins by COI1 (Li *et al.*, 2017a). This revealed the necessity of ASK protein interactions for FBP biological activity.

The best-described ASKs, ASK1 and ASK2, are detectable in all tissues independent of the stage of development, and able to interact with over 40 FBPs, including TIR1, COI1, SLY and MAX2 (Risseeuw *et al.*, 2003; Stirnberg *et al.*, 2007; Kuroda *et al.*, 2012; Marzec, 2016). Yet, other ASK proteins display a more tissue- and developmental stage-specific expression (Dezfulian *et al.*, 2012; Kuroda *et al.*, 2012). While there are not many examples, it is possible that ASK proteins with a more specific expression profile have more explicit functions, as it has been shown in *Lilium longiflorum* (lily) that pollen tube-specific ASK proteins are essential for pollen tube maturation (Chang *et al.*, 2009). Noteworthy, the general abundance of the FBPs

seems to be positively correlated with ASK abundance, e.g. in *ask1* mutants it has been shown that levels of the JA receptor COI1 decreased (Yan *et al.*, 2013). Furthermore, COI1's closest homolog TIR1 was also found to have an increased stability in the presence of ASK1 (Dezfulian *et al.*, 2016). It is postulated that the interaction between COI1 and ASK1 or ASK2 blocks the ubiquitination of K297 on COI1 (Fig. 2A), which otherwise would lead to its degradation (Yan *et al.*, 2013).

Stability of FBPs is also affected by direct interactions with non-SCF components: the SGT1-HSP complex

Two independent forward genetic screens attempting to identify regulators of phytohormone signaling in Arabidopsis uncovered the co-chaperone protein SUPPRESSOR OF G2 ALLELE OF SKP1 b (SGT1b) as a regulator of both the JA and auxin signaling pathways (Fig. 3). On the one hand, Lorenzo *et al.* (2004) revealed that the *jai4* mutation, despite being in a background that shows enhanced JA sensitivity, caused JA insensitivity in root growth inhibition assays and mapped to the *SGT1b* locus. Likewise, Gray *et al.* (2003) found that the *eta3* mutation also mapped to the *SGT1b* locus and caused enhanced auxin insensitivity in the *tir1-1* background (Fig. 3). More recently, the molecular rationale behind this genetic complementation was revealed, when it was shown that both COI1 and TIR FBPs are client proteins of the SGT1b chaperone complex that also comprises the heat shock proteins (HSP)70 and HSP90, and can thus interact with SGT1b (Zhang *et al.*, 2015) (Fig. 3).

The Arabidopsis genome encodes two SGT1 proteins, SGT1b and SGT1a. *SGT1a* loss-of-function lines do not show an altered JA or auxin response, nor other distinguishable phenotypes from wild-type lines, contrary to loss-of-function *SGT1b* lines. Accordingly, COI1 and TIR1 proteins accumulated to wild-type levels in the *sgt1a* mutant lines, whereas these FBPs showed significantly lower accumulation levels in the *sgt1b* mutant lines. Nonetheless, SGT1a could also associate with COI1 and TIR1 FBPs in planta in co-immunoprecipitation assays (Zhang *et al.*, 2015). It was postulated that SGT1a has a minor role in the regulation of FBP stability, for instance by maintaining residual COI1 levels in *sgt1b* mutants, which is supported by the complementation of certain *sgt1b* mutant phenotypes by the overexpression of *SGT1a* in

a dosage-dependent manner (Azevedo *et al.*, 2006). Yet, the *sgt1a-1 sgt1b-1* double mutant is embryo lethal, which complicates unraveling their specific functions within the plant (Azevedo *et al.*, 2006). A broader role was suggested for SGT1a and SGT1b in hormone responses that utilize FBPs as receptors, such as JA (COI1), auxin (TIR1) and GA (SLEEPY1 or SLY1), but not in hormone responses with other receptor modules that do not involve FBPs, such as brassinolides and abscisic acid (Zhang *et al.*, 2015).

So, what could be the molecular function of SGT1b in the context of hormone signaling? SGT1b has been shown to interact with and thought to act as a co-chaperone for HSP70 and HSP90 (Takahashi *et al.*, 2003; Catlett and Kaplan, 2006; Clément *et al.*, 2011) (Fig. 3). The HSPs are highly conserved in eukaryotes, acting as chaperones and assisting in protein stabilization, folding, translocation and complex formation under both biotic and abiotic stress (reviewed in Park and Seo, 2015). The HSP90 and HSP70 families consist of at least seven and eighteen members, respectively, that are differentially expressed, for instance pending developmental and environmental cues, and localize to different subcellular compartments (reviewed in Krishna and Gloor, 2001; Lin *et al.*, 2001).

Interaction between HSP90 and SGT1b has been experimentally validated multiple times, through co-immunoprecipitation experiments as well as genetic interactions (Takahashi *et al.*, 2003; Botër *et al.*, 2007; Hubert *et al.*, 2009). Functionally, SGT1b is thought to act as a scaffold for client transfer from HSP90 or HSP70, whereas HSP90 would work as a chaperone in an ATP-dependent manner to assist in client protein folding during stress (Hubert *et al.*, 2009; Park and Seo, 2015). For instance, it has been shown that increased temperature can rapidly promote TIR1 accumulation in a HSP90-dependent manner, and that within this process both HSP90 and SGT1b interact with TIR1 (Fig. 3). Loss of HSP90 activity results in TIR1 degradation and consequent defects in several auxin-mediated growth processes at distinct temperatures (Wang *et al.*, 2016). Together this demonstrates that TIR1 is an HSP90 client and suggests that the HSP90–SGT1 chaperone complex integrates temperature and auxin signaling, and thereby modulates plant growth in changing environments.

PTM crosstalk adds complexity to SCF complex activity

PTMs of proteins are diverse. Several hundreds of different types of modified amino acids have been registered for eukaryotic proteins in UniProt. These PTMs affect protein activity, stability, localization and interactions, also of proteins that themselves have PTM activity, including the FBPs. Phosphorylation and the less common sumoylation have already been reported for plant FBPs, as well as *S*-nitrosylation. Finally, also the FBPs themselves can get ubiquitinated, often in regulatory feedback loops.

Phosphorylation crosstalk

Crosstalk between phosphorylation and ubiquitination, including by, but not necessarily limited to, SCF complexes and FBPs, is most renowned in humans, where phosphorylation of (the) phosphodegron(s) in a protein modulates its ubiquitination in a cis-regulatory manner, including of SCF targets (Filipčík *et al.*, 2017). In contrast, several studies indicate that in Arabidopsis (de)phosphorylation does not affect SCF^{TIR1} and Aux/IAA protein interaction, and is thus not critical for this element of the auxin response (Dharmasiri *et al.*, 2003; Kepinski and Leyser, 2004). Recently however, an auxin signaling mechanism was discovered, which acts in parallel to the canonical auxin pathway based on TIR1 and the other AFB auxin receptors. This signaling mechanism involves TRANSMEMBRANE KINASE 1 that interacts with and phosphorylates the non-canonical Aux/IAA proteins Aux/IAA32 and 34, which lack the TIR1 interacting domain, thereby increasing their stability and modulating plant growth (Cao *et al.*, 2019). Involvement of (de)phosphorylation events in the establishment of COI1–JAZ interaction has not been established yet. Conversely however, although less well-illustrated by experimental data, the FBPs themselves can be modulated by phosphorylation. In Arabidopsis this has been reported for the FBPs SKIP18 and SKIP31, the phosphorylation of which was critical for interaction with and degradation of 14-3-3 proteins (Hong *et al.*, 2017).

S-*Nitrosylation crosstalk*

PTMs can be modulated via the redox state of the cell, which fluctuates based on various environmental signals. A common redox modification is *S*-nitrosylation, which is the addition of a nitric oxide moiety on a sulfhydryl group, typically of a cysteine. Hormone responses have

demonstrated redox sensitivity (Ndamukong *et al.*, 2007; Bashandy *et al.*, 2010; Marquez-Garcia *et al.*, 2014). Interestingly, several subunits of the SCF complex have been experimentally validated to undergo *S*-nitrosylation, thereby allowing the plant to utilize changes in the cellular redox state to modulate SCF complexes and their activity. First, Arabidopsis ASK1 can be *S*-nitrosylated *in vitro* in C37 and C118, two essential residues for proper activation of the auxin signaling pathway *in planta* (Fig. 2A). Accordingly, ASK1 *S*-nitrosylation was found to enhance ASK1 binding to CUL1 and TIR1/AFB2, which is required for $SCF^{TIR1/AFB2}$ assembly. Mutations of these specific *S*-nitrosylation cysteine residues in ASK1 lead to a reduced interaction between ASK1 and TIR1/AFB2 in a yeast two-hybrid screen (Iglesias *et al.*, 2018). As such, aberration of ASK1 *S*-nitrosylation leads to a reduction in auxin-responsive genes, indicating that *S*-nitrosylation of ASK1 is necessary for proper FBP function (Iglesias *et al.*, 2018).

The FBPs themselves can also be *S*-nitrosylated, which has been shown for instance for TIR1. Similarly to *S*-nitrosylated ASK1, *S*-nitrosylation of TIR1 enhances interaction with its substrate, the Aux/IAA proteins (Terrile *et al.*, 2012). *S*-nitrosylation of TIR1, as well as of ABP2, occurs at the conserved residue C140 and C135, respectively (Fig. 4) (Terrile *et al.*, 2012). Hence, following the more recent finding that this residue enhances the ability of TIR1 to oligomerize, which in turn is necessary for its capacity to interact with and degrade the Aux/IAA proteins, it was postulated that TIR1 *S*-nitrosylation contributes to its oligomerization (Dezfulian *et al.*, 2016). Interestingly, COI1 was also found to oligomerize but how this affects its ability to act as the JA receptor remains unstudied (Dezfulian *et al.*, 2016). Likewise, possible *S*-nitrosylation of the conserved cysteine residue (Fig. 4) remains to be explored.

Sumoylation crosstalk

Conjugation of small ubiquitin-related modifier (SUMO) occurs mostly on nuclear proteins, particularly as a rapid response to biotic and abiotic stress (reviewed in Augustine and Vierstra, 2018; Vu *et al.*, 2018). Again, sumoylation has already been reported for some FBP targets, such as the DELLA protein REPRESSOR OF GA (RGA), in which GA-independent targeting of the K65 residue by sumoylation affects GA-mediated ubiquitination and degradation (Conti *et al.*, 2014).

Interestingly, the E3 SUMO SIZ1 also modulates GA in Arabidopsis by sumoylating SLEEPY1 (SLY1), the FBP element in the SCF^{SLY1} complex that specifically targets DELLAs, such as RGA1 (Kim *et al.*, 2015). Sumoylation of other plant FBPs has not been reported yet, but nonetheless, sumoylation has been found implicated in other FBP-dependent phytohormone signaling cascades, including that of JA, in which SUMO-conjugated JAZ proteins were found to inhibit the JA receptor COI1 from mediating non-sumoylated JAZ degradation (Srivastava *et al.*, 2018).

(Auto-)Ubiquitination of FBPs

Finally, FBP-type ubiquitin ligating enzymes can be conjugated to ubiquitin themselves. In many cases, this may be the mere result of auto-ubiquitination. For instance, once assembled, the CUL/RBX1 core in the CRL may start ubiquitinating their adaptors instead of the substrate, particularly when the substrate is absent. Thereby, the turnover of the adaptor is increased, which provides the plant with a mechanism to dampen the activity of CRLs when they are not needed but without compromising the CUL/RBX1 core (Hotton and Callis, 2008; Hua and Vierstra, 2011). To our knowledge, auto-ubiquitination of FBPs has not been reported in plants yet. Notwithstanding, Arabidopsis TIR1 destabilizes in mutants deficient in the general assemblage of SCF complexes and also increases in abundance when the proteasome inhibitor MG-132 is administered (Fig. 2), suggesting it is subjected to PTM and proteasome-mediated degradation (Stuttmann *et al.*, 2009). Furthermore, in COI1, the K297 residue was identified as an active ubiquitination site (Fig. 4A). Accordingly, COI1 was shown to be degraded through the ubiquitin/26S proteasome pathway, albeit in a SCF^{COI1}-independent manner (Yan *et al.*, 2013). Likewise, circadian phase-specific accumulation of the clock-associated FBP ZEITLUPE (ZTL) is controlled through different circadian phase-specific degradation rates by the ubiquitin/26S proteasome system (Kim *et al.*, 2003).

Transcriptional and translational regulation of *FBP* expression

The underpinning behind the transcriptional regulation of *COI1* and *TIR1/AFBs* expression remains fairly elusive. Nonetheless, there are several scattered and interesting observations that plants also evolved transcriptional and/or translational regulatory systems to keep FBPs,

such as COI1 and TIR1, in check. For instance, using genome-wide transcriptional profiling, many auxin-induced and auxin-signaling genes, including *AFB5*, have been found to be under clock regulation (Covington and Harmer, 2007). Transcriptional regulation through the circadian clock has similarly been seen for *COI1,* whose transcript level oscillations are TIC (TIME FOR COFFEE)-dependent (Shin *et al.*, 2012). *COI1* and *AFB5* are not the only *FBP* genes that are transcriptionally regulated by the circadian clock. Bioinformatics analysis highlighted that transcripts of approximately one third of all Arabidopsis genes are modulated in a circadian manner, with many transcripts corresponding to FBPs that exhibit rhythmic oscillations (Covington *et al.*, 2008). The circadian clock regulating transcript abundance may be reflective of how a number of stresses that elicit responses from FBP hormone receptors are themselves time of day dependent. Similarly, hormone levels themselves display rhythmic oscillations (reviewed in Spoel and van Ooijen, 2014). It will be interesting to see what transcription factors or other regulatory proteins act downstream of the clock to modulate the circadian responses.

Additionally, the transcriptional regulation of *COI1* may be directly linked to the presence of the hormone it perceives, because *COI1* transcript levels show a moderate reduction upon application of JA (Shin *et al.*, 2012). However, typically, hormone application does not involve a change in the expression of its receptor. For instance, there was no alteration in the root transcript levels of *TIR1/AFB*, after addition of IAA (Parry *et al.*, 2009). Nevertheless, *TIR1* transcript levels have been shown to be transcriptionally regulated, because the transcription factor *AGL15* can repress *TIR1* expression (Zheng *et al.*, 2016). It will be interesting to uncover more concise transcriptional regulatory networks for *COI1* and *TIR1.* It cannot be excluded that there may be a common, possibly combinatorial, transcriptional regulatory network to govern expression of multiple *FBP*-encoding genes. This assumption is triggered by consultation of the ATTED-II A Plant Coexpression Database (Obayashi *et al.*, 2018). Indeed, when we use *COI1* as the bait in this database, a marked co-expression is observed with a subset of FBPs, including AFB3, AFB5 and ZTL, among many others. Whether this observation is a mere result of accidental grouping because of a common lack of strong differential expression, or indeed reflects the existence of a coordinated transcriptional regulatory network, remains to be investigated.

Finally, the regulatory discourse of FBPs can be extended beyond the transcriptional level, because posttranscriptional mechanisms seem to be involved as well. The translational regulation of *TIR1* and *AFB2/3* has been shown to be dependent on small RNAs, because the microRNA 393 (miR393) was found to cleave their corresponding mRNAs, thereby reducing their abundance. This cleavage has been found to occur in response to various developmental and environmental cues (Navarro *et al.*, 2006; Parry *et al.*, 2009; Vidal *et al.*, 2010; Si-Ammour *et al.*, 2011). The regulatory action of miR393 on *TIR1/AFB* transcripts was found to be conserved in the plant kingdom, as illustrated by its discovery in rice and cucumber, among others (Bian *et al.*, 2012; Xu *et al.*, 2017). MicroRNA-based cleavage of *COI1* transcripts has not yet been revealed, but for other FBP transcripts, it has already been reported. For instance, the FBPs LEAF CURLING RESPONSIVENESS (LCR) and MAX2 are regulated by miR394 and miR528, respectively (Lima *et al.*, 2011; Song *et al.*, 2012). This suggests that posttranscriptional regulation by small RNAs may be a widespread mechanism to keep *FBP* expression in check.

The interaction between the FBPs and substrates is promoted by small molecules

So far, we discussed how TIR1/AFBs and COI1 are regulated at a transcriptional and posttranscriptional level and by PPIs and PTMs. These FBPs are kept in check by an additional way unique to plants: the recognition of their substrates is dependent on the presence of a small molecule. Only in the presence of an auxin-like IAA, TIR1/AFBs associate with Aux/IAAs and only in the presence of an oxylipin such as JA-Ile, COI1 associates with JAZ proteins (Tan *et al.*, 2007; Sheard *et al.*, 2010). By controlling the presence of these hormones in the nucleus, plants have an elegant way of fine-tuning F-box function. This can be achieved by (i) hormone biosynthesis, (ii) intercellular transport, (iii) intracellular compartmentalization and transport, (iv) modification of the small molecule and finally (v) catabolism (reviewed in Ma and Robert, 2014).

Intercellular polar auxin transport has been studied for decades and is an essential part in the function of auxin to control plant development (reviewed in Zažímalová *et al.*, 2010). Evidence for cell-to-cell transport of endogenously produced oxylipins on the other hand is only recently emerging using micrografting of JA-deficient mutants (Gasperini *et al.*, 2015; Nguyen *et*

al., 2017). The ABC transporter protein JASMONATE TRANSPORTER 1 (JAT1) was only recently identified as a plasma membrane-localized JA exporter, but it is also localized intracellularly at the nuclear envelope and involved in transport of JA-Ile to the nucleus (Li *et al.*, 2017b). Regulation of nuclear hormone concentrations via intracellular compartmentalization and transport has also been found in auxin signaling (Mravec *et al.*, 2009; Ranocha *et al.*, 2013; Simon *et al.*, 2016). Endoplasmic reticulum-localized PIN-LIKES (PILS) proteins are suggested to store auxin in the endoplasmic reticulum and, hence, indirectly control nuclear auxin signaling (Barbez *et al.*, 2012; Feraru *et al.*, 2019).

One of the most elegant ways of controlling the activity of auxins and JA-Ile is by chemical modification in order to make them inactive, more active or more selective. In a more advanced form of selectivity engineering, a chemically modified IAA was synthesized that is only active in combination with a mutant TIR1 protein (Uchida *et al.*, 2018). In addition to IAA, different auxin agonists, such as 2,4-D, picloram and 1-naphthaleneacetic acid, can stimulate interaction between TIR1 and Aux/IAA proteins but with different strengths (Hayashi *et al.*, 2014; Eyer *et al.*, 2016). Moreover, the potency of a small molecule to facilitate interaction varies between the different F-box/substrate co-receptors in case of auxins and JA-Ile (Takaoka *et al.*, 2018; Vain *et al.*, 2019). There are ten JAZ proteins capable of COI1 interaction in Arabidopsis (Takaoka *et al.*, 2018) and a myriad of possible combinations between the six TIR1/AFBs and 29 Aux/IAA family proteins (Calderon-Villalobos *et al.*, 2010). This opens up the possibility of identifying selective agonists that only promote the formation of certain subsets of co-receptors. Using rational design, a selective JA agonist was engineered that stabilizes specifically the interaction with COI1 and JAZ9/JAZ10 (Fig. 5) (Takaoka *et al.*, 2018). In another approach, RubNeddin4 was characterized as a selective agonist of auxin to promote interaction of TIR1 and a subset of Aux/IAA proteins (Vain *et al.*, 2019). In both cases, these selective agonists were used to dissect these very complex pathways and to uncouple different downstream effects. Existence of endogenous selective agonists has not been reported so far, but undoubtedly await discovery. In this regard, it is thought-provoking to look at the variation in the structure of oxylipin signals within and across plant species, as well as in their attackers. For instance, in Arabidopsis catabolism of JA-Ile is modulated by catalytic enzymes like the

CYP94 Cytochrome P450 proteins (Heitz *et al.*, 2012; Koo *et al.*, 2014; Aubert *et al.*, 2015). Depending on the P450, JA-Ile can be oxidized to 12-OH-JA-Ile or 12-COOH-JA-Ile that do not mediate interaction between COI1 and JAZ, leading for instance to an impaired defense response to *Botrytis cinerea* (Koo *et al.*, 2014; Aubert *et al.*, 2015). Conversely, the natural antagonist coronatine (COR), produced by *Pseudomonas syringae,* competitively binds COI1 (Katsir *et al.*, 2008). Building on this natural compound, conjugates of COR and various non-polar amino acids (Met, Leu, Val) showed different abilities to activate late or early transcriptional JA response genes (Yan *et al.*, 2016). The corresponding JA conjugates could also be detected in rice and tomato, demonstrating a possible conserved means to modulate JA response. COI1 in the liverwort *Marchantia polymorpha* is unable to recognize JA-Ile and instead recognizes dn-*iso*-OPDA and dn-*cis*-OPDA (Monte *et al.*, 2018). In higher plants, COI1 evolved specific residues for it to function as a JA-Ile receptor, possibly enabling it to gain novel regulatory roles. Conversely, Arabidopsis COI1 still recognizes dn-*cis*-OPDA, although it remains to be elucidated if specific signaling functions were retained, as multiple OPDA derivatives can be found in Arabidopsis (Chini *et al.*, 2018; Monte *et al.*, 2018).

Perspectives

Angiosperms display an enormous variety of species-specific traits and developmental niches. Given the vast amount of FBPs encoded in their genomes, it is plausible to assume that FBPs, which facilitate a wide range of PPIs that induce proteolytic degradation of numerous, yet specific substrates, have contributed to this versatility. In this regard, it is as relevant to understand how FBPs themselves, and not only their substrates, are regulated. As our knowledge of FBP regulation corresponding to their physiological function increases, it will allow more precise understanding of how to control development, growth and metabolism in plants. A current area of interest lies in determining what external cues prime the FBPs, as not all FBPs serve as receptors. For example, ZTL has a light, oxygen or voltage (LOV) domain and is activated by a chemical bond facilitating a conformational change, triggered by a lack of light (Pudasaini *et al.*, 2017). But for those FBPs that may serve as receptors but with yet unknown ligands, how may we efficiently identify their ligands? Indeed, the design of (high-throughput)

screens allowing to readily detect the interaction between FBPs and small molecules – or other compounds or triggers that can act as a ligand – remains a challenging technical aspect. When overcome, it may greatly help to reveal specific tissue-specific, developmental and external elements that serve to activate FBPs. More detailed knowledge about FBP function and regulation and their ligands may also find applications beyond the plant kingdom. For instance, the auxin-TIR1-Aux/IAA and JA-Ile-COI1-JAZ systems have been adopted by animal, Drosophila, yeast and medical scientists to create conditional synthetic genetic switches (Nishimura *et al.*, 2009; Brosh *et al.*, 2016; Natsume *et al.*, 2016; Trost *et al.*, 2016).

The tremendous technological advances in proteomics are enabling a vastly wider scope and greater resolution of analysis of PTM occurrences, such as of ubiquitination or *S*-nitrosylation on a substrate or their direct/indirect interactors, including the mapping of the PTM site (Walton *et al.*, 2016). In combination with base editing, for instance using CRISPR/Cas9 variants with cytidine or adenine deaminase activity facilitating transitions of C-G to T-A or A-T to G-C, respectively (Zong *et al.*, 2017; Kang *et al.*, 2018), precise targeted alteration of nucleotides corresponding to the PTMs can be achieved, which will allow revealing unprecedented insights into the functional role and relevance of the PTM. A more cohesive view on the role of PTMs and PPIs will enable the rational design of more or superior agonists or antagonists. As discussed above, the uncoupling of regulatory processes by rational-designed agonists has already been demonstrated in hormone receptor FBPs involved in growth and defense. The future will tell us how desired outputs can be enhanced even more while diminishing unwanted effects of FBP signaling. It may also help resolving the relevance of the apparent functional redundancy found in the JA and auxin regulatory networks, specifically with regard to the respective substrates of COI1 and TIR1/AFBs, that form families of 13 JAZ and 29 AUX/IAA proteins, respectively. Research thus far is supporting the idea of specificity for the different JAZ and AUX/IAA proteins, which leads to activation of a defined subset of JA or auxin signaling responses (Chini *et al.*, 2018).

Further, it remains to be determined to what extent the PPI and PTMs identified in model plant species such as Arabidopsis are ubiquitously found in other plant species, particularly crops and medicinal plants. Tandem duplication events are postulated to be

responsible for the large number of plant FBPs (Gagne *et al.*, 2002), and multiple FBP homologs may exist in species-specific manners, like three COI1s in rice (Lee *et al.*, 2013) and one in Arabidopsis or the liverwort *M. polymorpha* (Monte *et al.*, 2018). In parallel to working with these more complicated models, working with basal land plants, like the liverwort *M. polymorpha* that exhibits low genetic redundancy in most regulatory pathways (Bowman *et al.*, 2017), including the JA signaling pathway with just one COI1 and one JAZ protein (Monte *et al.*, 2018; Monte *et al.*, 2019), will enable to reveal fundamental regulatory roles of plant FBPs as well as identifying their ligands.

As mentioned already above, revolutionary genome editing tools, such as CRISPR/Cas9, can be utilized to alter structural motifs that effect PTMs of the FBP and/or to engineer orthogonal receptor/ligand strategies (reviewed in Helander *et al.*, 2016). Much of the current knowledge of FBP regulation involves regulation of specific SCF complex subunits, and targeting the subunits leads to many additional undesired effects. Hopefully, in the future a combination of engineered agonists and targeted gene editing will enable maximum efficiency in controlling FBP-dependent signaling cascades that govern developmental, growth and metabolic outputs in plants.

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Figure legends

Fig. 1. SCF-mediated protein degradation. The E1 activating enzyme forms a thioester bond, in an ATP-dependent reaction with the ubiquitin (Ub) protein. The E1 enzyme, either UBA1 or UBA2 in Arabidopsis, transfers the ubiquitin to the E2 conjugating enzyme. The CUL1 protein of the SCF^{COI1} E3 ligase complex acts to bridge the RBX1 protein, which coordinates the recruitment of the E2 conjugating enzyme. The N-terminus of the FBP, here exemplified by the JA receptor COI1, contains a F-Box domain (brown rectangle) that interacts with ASK1/2, whereas the C-terminus of COI1 acts as the recognition domain for the substrate, here exemplified by JAZ1. The E3 ligase facilitates the proximal interaction between the E2 conjugating enzyme. Although it has not been experimentally validated yet for COI1, UBC8 was experimentally shown to have high activity with a large number of FBPs, including TIR1 (Kraft *et al.*, 2005; Winkler *et al.*, 2017), necessary for ubiquitination of the substrate. COI1 also directly interacts with MED25 and thereby brings COI1 to MYC2 target promoters (An *et al.*, 2017). The activity of the SCF-type E3 ligase complex is controlled by the neddylation of CUL1 with the small protein RUB. Finally, the (poly)ubiquitinated substrate can be recognized by the 26S proteasome and undergo proteolysis. The ubiquitin proteins can be reused for the ubiquitination of additional substrates. Once MYC2 is released from the JAZ-NINJA-TPL repressor complex, MED25 transcriptional machinery recruitment is facilitated and the JA response activated.

Fig. 2. Interaction of COI1 and TIR1 with SCF subunits. Thick black lines represent direct interaction of the respective FBP with the corresponding protein or pertaining to having the PTM. Dotted gray lines refer to an indirect interaction between the PTM or the protein. Dashed gray lines refer to the potential for the protein to have the PTM or interaction. The corresponding effect of the JA or auxin response as well as the effect the PTM or PPI has on the stability of the FBP is shown with green and dark blue respectively indicating a positive and negative effect on the FBP stability or hormone response. White indicates that there is no experimental data to support a response or effect whereas yellow refers to the fact that some

interactions or modifications are dynamic and can have both positive and negative effects. Supplementary table 1 depicts all of this information with references in a table format.

Fig. 3. Interaction validation of the SCF subunits with SGT1 and HSPs. The chart depicts experimentally validated interactions between the four subunits of the SCF and SGT1 and HSPs. The different validation strategies are indicated at the right: genetic interaction, in vitro or in planta PPIs, and interaction in yeast (typically Y2H), as these are highly conserved proteins. *The interaction between SGT1 and CUL1 was found in *Hordeum vulgare* (barley). Because there is only one *SGT1* gene in barley, a MUSCLE alignment was performed showing it shares a 62.3% amino-acid sequence conservation with SGT1b and 64.6% with SGT1a from Arabidopsis. **There is one *SGT1* gene in *Saccharomyces cerevisiae*, with a 26.1% amino-acid sequence conservation with SGT1b and 28.1% with SGT1a from Arabidopsis. ***The close homologs to Arabidopsis CUL1 in *S. cerevisiae* are CDC53 and CUL3, which both interact with SGT1.

Fig. 4. Posttranslational modifications of F-box proteins. (A) The phylogenetic tree of FBPs was constructed by the PhyML program (one click mode, Dereeper *et al.*, 2008) using the MUSCLE alignment (Chojnacki *et al.*, 2017), which was manually edited using Jalview (Waterhouse *et al.*, 2009). The sequence of ZTL was used as an outgroup. Simplified domain architecture of the FBP/ASK and PTMs are depicted. (B) Selected part of the Jalview alignment of amino-acid sequences depicting the conserved cysteine residue which is *S*-nitrosylated in both TIR1 and AFB2. (C) The protein structure of COI1 (Sheard *et al.*, 2010) (3ogk/pdb) with the analysis of the conserved amino-acid residues mapped on the protein surface using the Consurf program (Ashkenazy *et al.*, 2016) based on the MUSCLE alignment and the Phyml-calculated phylogenetic tree. In the van der Waals representation, the conserved cysteine (C148) depicting the location of a possible S-nitrosylation is shown. The ligand, JA-Ile, is shown in the balls-and-sticks representation colored in green. (D) The protein structure of TIR1 (Hayashi *et al.*, 2008) (3c6o/pdb) with the analysis of the conserved amino-acid residues mapped on the protein surface using the Consurf program (Ashkenazy *et al.*, 2016) based on the MUSCLE alignment and the Phyml-calculated phylogenetic tree. In the van der Waals representation, the

conserved cysteine (C140) is shown depicting the location of the S-nitrosylation. The ligand, (2S)-2-(1H-indol-3-yl)hexanoic acid, is shown in the balls-and-sticks representation colored in green.

Figure 5. Selective agonists of FBP/substrate interactions. (A) Phytohormones such as JA-Ile (light green circle) mediate binding of an array of substrate JAZ repressor proteins with the cognate FBP COI1. This leads to their polyubiquitination, degradation by the proteasome and subsequent activation of downstream responses. Here, distinct JAZ repressor isoforms are visualized by the different color. (B) Selective agonists (red circle) only promote interaction of a subset of repressor proteins, here exemplified by the dark green isoform JAZ10, with COI1 leading to activation of only a subset of downstream responses.