F-box proteins in flowering plants

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Abstract **In eukaryotes, the ubiquitin-mediated protein degradation pathway has been shown to control several key biological processes such as cell division, development, metabolism and immune response. F-box proteins, as a part of SCF (Skp1-Cullin (or Cdc53)-F-box) complex, functioned by interacting with substrate proteins, leading to their subsequent degradation by the 26S proteasome. To date, several F-box proteins identified in *Arabidopsis* and *Antirrhinum* have been shown to play important roles in auxin signal transduction, floral organ formation, flowering and leaf senescence. Arabidopsis genome sequence analysis revealed that it encodes over 1000 predicted F-box proteins accounting for about 5% of total predicted proteins. These results indicate that the ubiquitin-mediated protein degradation involving the F-box proteins is an important mechanism controlling plant gene expression. Here, we review the known F-box proteins and their functions in flowering plants.**

Keywords: SCF complex, F-box protein, proteolysis, auxin signal transduction.

Selective protein degradation by the ubiquitin-proteosome pathway is a key regulatory mechanism in a variety of cellular processes. In general, the formation of ubiquitin-protein conjugates involves three classes of enzymes. Ubiquitin is first activated by the formation of a thiolester bond between a glycine within its C terminus and a cysteine residue within the ubiquitin-activating enzyme (Uba1; E1), and this activated ubiquitin is then transferred to a serine residue of an ubiquitin-conjugating enzyme (Ubc; E2). Ultimately, with the aid of ubiquitin ligase (E3), ubiquitin is covalently attached to an ε-NH2 group of a lysine residue within a substrate protein. A polyubiquitin chain is subsequently formed by the addition of ubiquitin monomers via an internal lysine residue within the ubiquitin. Polyubiquitination typically leads to proteolysis[1].

In general, enzyme diversity increases through the ubiquitin transfer cascade. In yeast, Uba1 is the major form of E1, which can activate thirteen E2. E2 enzymes then couple to a large, but unknown number of E3[2]. E1 and E2 are easily recognized because of their high degree of sequence conservation, whereas the few characterized E3 enzymes are structurally divergent, a characteristic that has hindered their identification in database[3]. In many cases, the E3 participates directly in the transfer reaction, forming an intermediate thioester with ubiquitin. Occasionally, the E3 juxtaposes the substrate and the E2 enzymes allow direct transfer of ubiquitin from the E2 to the substrate[4]. At present, four unrelated classes of E3 enzymes have been identified: HECT, Ubr1p, Apc/c and SCF complex[5—7]. In this review, we focus on some recent progress in the SCF complex in flowering plants.

1 F-box domain, F-box protein and SCF complex

F-box was initially identified as a region of homology among Cdc4, β-TrCP, Met30, Scon2 and MD6, all of which contain WD (Trp-Asp) repeats[8]. Implication of the homology was not appreciated until Bai et al. recognized that the F-box was a widespread motif that was required for protein-protein interaction[9]. The name F-box was given by Bai et al. on the basis of the presence of the motif in cyclin F.

Members of the F-box protein family contain a conserved 40—50 amino acid F-box motif. As can be seen from the consensus sequence, they have very few invariant positions; the least variables are positions 8 (leucine or methionine), 9 (proline), 16 (isoleucine or valine), 20 (leucine or methionine) and 32 (serine or cysteine) (fig. 1)[10]. This lack of a strict consensus makes identification by eye difficult, it is therefore necessary to use search algorithms to detect F-boxes. Currently, the two best search algorithms are found in the Prosite and Pfam databases (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html).

The SCF complex was identified first in yeast and is composed of four subunits: Skp1, Cullin (Cdc53 in yeast), Roc1/Rbx1/Hrt1 and an F-box protein (the underlined capital letters indicate SCF)[11]. The first three proteins form a common scaffold onto which different F-box proteins can be assembled, conferring specificity to the complex. SCF complex are designated by their associated F-box protein, for example, SCF^{cdc4} and SCF^{Grr1} (Cdc4 and Grr1 are F-box proteins).

The F-box motif itself is generally found in the amino-terminal half of proteins and participates in the interaction with Skp1. There are many F-box proteins in eukaryotes. A yeast two-hybrid screen using Skp1 as a bait identified a family of 26 human F-box proteins[12]. In *Arabidopsis*, a two-hybrid screen with the Skp1-related protein Ask1 also resulted in the recovery of 20 different F-box proteins[13]. Some F-box proteins can interact with different substrates, such as Grr1. Grr1 captures phosphorylated G1 Cyclins for ubiquitination by the core SCF machinery. Grr1 has other targets, including activators of polarized cell growth called Gic1 and Gic2[14].

The F-box domain is perhaps a modular one as different F-box motifs can be functionally interchanged between different proteins across species. The *Neurospora crassa* sulfur circuit features a set of regulatory genes acting to modulate gene expression due to environmental

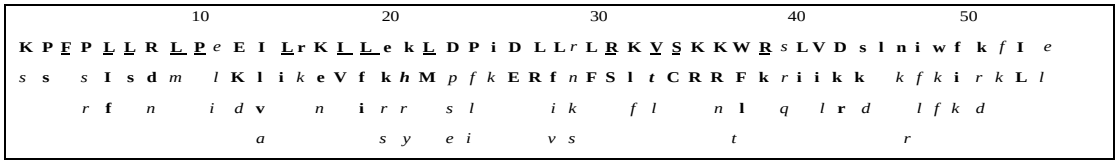

Fig. 1. F-box consensus sequence. The consensus was derived from alignment of a total of 234 sequences. Single-letter amino-acid code is used. Underlined capital letters signify residues found in over 40% of the F-box sequences; non-underlined capital letters signify residues found in 20%—40% of the F-boxes; lower case letters indicate residues found in 15%—19% of the F-boxes; italic lower case letters indicate residues found in 10%—14% of the F-boxes[10].

sulfur conditions. These sulfur regulatory genes include *cys-3*$^{+}$, which encodes a basic leucine zipper transcriptional activator, as well as the negative regulatory gene *Scon-2*$^{+}$. Through site-directed mutagenesis of the Scon-2 F-box, Kumar et al. generated a sulfur auxotrophic phenotype[8]. They found that a series of chimeric Scon-2 proteins containing swapped F-box domains from the yeast transcriptional inhibitor Met30p and the *Candida albicans* cell cycle regulator Cdc4p can restore partial wild-type sulfur regulation *in vivo*, indicating that a universal nature of this motif[8].

Apart from the conserved motif in the amino-terminus, F-box proteins often include additional carboxyl-terminal motifs capable of protein-protein interaction, which recruit specific substrates to the SCF complex. Three F-box classes have been classified based on the three motifs: FBXL denotes a protein containing an F-box and LRRs, FBXW a protein with an F-box and WD repeats, and FBXO a protein with an F-box and either additional motif, e.g, leucine zippers, ring fingers, proline-rich motifs etc., or unknown motif .

F-box proteins are widely distributed in eukaryotes, such as yeast, nematodes, flies, humans and flowering plants. About 326 predicted F-box proteins have been identified in *C. elegans*, representing a largest collection in eukaryotes. However, in the recently completed *Arabidopsis* genome, over 1000 F-box motif-containing proteins have been identified[15].

Evolutionary constraints are higher for certain classes of F-box proteins: all of the human FBXW or FBXL proteins have counterparts in *C.elegans* with the most conserved in yeast, but only about half of the human FBXO class of proteins is conserved in *nematodes* or yeast[10]. The F-box proteins found to function in SCF complexes have so far been those that have WD repeats or LRRs in their carboxyl termini, through which phosphorylated substrates bind to the complex. Whether other SCF complexes also bind specifically to phosphorylated substrates remains unknown.

Cullin family includes at least cullins (cullin1-cullin5) and APC2 in humans. In addition to SCF complex, cullins participate in other E3s, such as APC[16]. Interestingly, Cul1 in mammalian cells assembles into SCF complexes, and is able to complement a Cdc53-2 temperature sensitive *S. cerevisiae* strain, indicating that cullins are conserved among eukaryotes[17]. The best-characterized cullin is yeast Cdc53. Its sequences in the carboxyl-terminal region are required for interaction with Cdc34 (an E2). A second domain, located close to the carboxyl terminus, is implicated in the posttranslational attachment of the ubiquitin-like protein Rub1 to the cullin. Finally, a conserved region in the N-terminal part of cdc53 participates in the interaction with the SCF component Skp1[11]. Skp1 constitutes a bridge between the cullin and the F-box proteins[11]. In fact, Skp1 was originally identified in a complex with cyclin/Cdk2 and the F-box Skp2 in human cells. There are at least 17 putative Skp1 homologs in the *C. elegans* genome，at least 10 Skp1-like proteins in the *Arabidopsis* genome[18]. Rbx1 was the last component of the SCF complex to be identified which contains a RING-H2 finger domain. In spite of its small size, Rbx1 is at the hub of SCF complex. The proposed function of Rbx1 is to recruit the E2 to the complex and to promote transfer of ubiquitin from the E2 to the targets[19]. The F-box proteins serve as critical substrate recognition subunits of the SCF complex and generally recognize substrates after they have been phosphorylated[7]. Phosphorylation is one of the major mechanisms used by cells to rapidly transduce signals. The F-box protein thus links protein phosphorylation networks to the proteolytic degradation and controls the abundance of many crucial cellular proteins[20]. Nonetheless, other proteins can associated with the complex, including the recently described Sgt1 protein, which required for the G1/S and G2/M transitions, suggesting that the SCF plays a role in kinetochore function[21].

2 Function of SCF complex

The first SCF pathway was identified through the analysis of the G_1-S phase transition in the yeast *S. cerevisiae*. In yeast, the initiation of DNA replication requires the mitotic cyclin(Clb)-cyclin-dependent kinase (cdk) activity, which can not function until a cdk inhibitor called Sic1 is destroyed. Sic1 degradation in the late G_1 phase is triggered upon phosphorylation by G_1 cyclin (Cln)-CDK kinases and requires an E3 ubiquitin ligase complex composed of the subunits Skp1，Cdc53，Rbx1 and Cdc4. This class of E3 is generally referred to as an SCF complex[22].

plex[22]. One of the main functions of SCF complex is the regulation of cell-cycle phase transitions through the phosphorylation-dependent elimination of inhibitors and activators of various processes in the cell cycle[7].

In addition to regulating the cell cycle, recent findings suggest that SCF complex have many non-cell-cycle functions. At least three major metabolic pathways in yeast are regulated by different SCF complexes: glucose induction is mediated by SCF^{Grr1}, methionine repression by SCF^{Met30}, and, at least in part, repression of amino acid biosynthesis genes by SCF^{Cdc4}[14,23,24].

SCF complexes have many different functions in animals. Such as in *C. elegans* and *Drosophila*, SCF^{SEL-10} may negatively regulate Notch/LIN-12 signaling, which mediates cell-to-cell communication that induces equivalent cells to adopt different fates; in *Drosophila*, SCF^{Slimb} may regulate the hedgehog (Hh) pathway which regulates limb development in *Drosophila* by controlling the expression of wingless and decapentaplegic[25,26]; and in mammals E2F-1 transciption factor may be targeted by SCF^{Skp2} complex which is important in mammalian cell cycle[23].

As in other eukaryotic organisms, plants possess the E1/E2/E3 activities responsible for activation and conjugation of ubiquitin. Evidence has shown that many crucial biological processes in plants, such as hormone response, stress response, circadian rhythm, photomorphogenesis and flower development are governed by proteolysis (table 1)[27]. Among plant F-box proteins, the role in the SCF complex in auxin response has been the most explicit. Here, we focus on recent progress in this field. In addition, there are many putative F-box motifs in the recently completely *Arabidopsis* and rice genomes[15, 28, 29], which may have important unknown functions[27].

The plant hormone indole-3-acetic acid (IAA or auxin) controls many aspects of the plant growth and development. Some of the best-characterized effects include stem elongation, lateral root branching, apical dominance, phototropism and gravitropism[30]. It is assumed that the machinery of auxin signal transduction and auxin responses is expected to be complicated. Recent studies with *Arabidopsis thaliana* have revealed that the SCF complex plays a central role in the auxin-response pathway (fig. 2)[31]. The strategy that has been employed to identify genes involved in auxin-mediated growth and development is the recovery of mutants that exhibit resistance or reduced response to applied auxin. In this way, several mutants have been isolated and phenotypic analyses indicate that one of them, TIR1, is important for auxin signaling. TIR1 encodes an F-box protein containing leucine-rich repeats; the phenotype caused by mutation in TIR1 includes defects in hypocotyl elongation and lateral root formation[32]. TIR1 together with Skp1-like protein ASK1 and Atcul1 (*Arabidopsis cullin-like*) form a ubiquitin ligase complex called SCF^{TIR1}, which has been shown to function in auxin[33].

What are the substrates recognized by TIR1 in auxin response? One group of potential targets is Aux/IAA proteins. The Aux/IAA genes form a gene family that encodes short-lived transcriptional regulatory proteins and can be rapidly and specially induced by auxin. The *Arabidopsis thaliana* genome contains at least 24 Aux/IAA genes, most of them have a relative molecular mass of 20—35 kD[34]. Molecular and genetic evidence suggests that Aux/IAA proteins are SCF^{TIR1} substrates[33]. The rapid turnover of Aux/IAA proteins by SCF^{TIR1} appears to be an integral feature of auxin response, and their increased half-life in the SCF^{TIR1} loss-of-function mutants is the basis of its auxin phenotype[33].

From the mutants that display reduced response to auxin, a gene called *AXR1* was obtained[35]. Mutations in *AXR1* confer an auxin-resistance phenotype that is similar to that caused by mutations in TIR1, suggesting that TIR1 and AXR1 function in a common pathway connected with a ubiquitin-like protein (Rub1)[35]. The AXR1 protein is

Table1 F-box proteins in plants

Abbreviation	F-box proteins	C-terminal motifs*	Function	Organism	References
COI1	Coronatine insensitive 1	LRR	Injury response	*Arabidopsis*	[36]
FIM	Fimbriata	Unknown	Floral differentiation	*Antirrhinum*	[37]
FKF1	Flavin-binding, Kelch Repeat, F-box I	Kelch	Control flowering time	*Arabidopsis*	[38]
TIR1	Transport inhibitor response 1	LRR	Auxin response	*Arabidopsis*	[32, 33]
UFO	Unusual floral organs	Unknown	Floral differentiation	*Arabidopsis*	[39]
ZTL	Zeitlupe	Kelch	Control of flowering time	*Arabidopsis*	[40]
ORE9	Oresara 9	LRR	Regulation of leaf senescence	*Arabidopsis*	[41]
EID1	Increased sensitivity to light responses	LZ	Function of phytochrome A	*Arabidopsis*	[42]
SLF	S locus F-box	Unknown	Self-incompatibility	*Antirrhinum*	[43]

* LRR, Leucine-rich repeats; Kelch, Kelch Repeats; LZ, Leucine zipper.

related to the N-terminal half of ubiquitin-activating enzyme (E1) and interacts with ECR1 (E1 C-terminus-related 1) to form a bipartite enzyme that activates the ubiquitin-like proteins Rub1[35]. Once activated, Rub1 is transesterified to the Rub1-conjugating enzyme (E2). *In vitro* experiment indicated that in absence of E3, the activated Rub1 can be transferred to substrates[33].

Recent results indicate that Rub1 is then conjugated to AtCul1[44]. Atcul1 is a component of SCF^{TIR1}. A recent observation suggests that COP9 signalsome may promote Rub1 deconjugation[44]. The COP9 signalsome is an evolutionary conserved multiprotein complex of an unknown function that acts as a negative regulator of photomorphogenic seedling development in *Arabidopsis*. Plants with reduced COP9 signalsome levels had decreased auxin response similar to loss-of-function mutants of the E_3 ubiquitin ligase SCF^{TIR1} and COP9 signalsome can interact with SCF^{TIR1} *in vivo*, suggesting that the COP9 signalsome is required for protein degradation mediated by SCF^{TIR1}. Further investigation indicates that the mutants of COP9 signalsome accumulate preferentially Rub1-modified AtCul1 and the COP9 signalsome promotes Rub1 deconjugation. Thus, the essentially antagonistic steps of AXR1-mediated Rub1 conjugation and its subsequent COP9 signalsome-promoted deconjugation are both required for proper auxin response and act together toward the degradation of SCF^{TIR1} substrate[45].

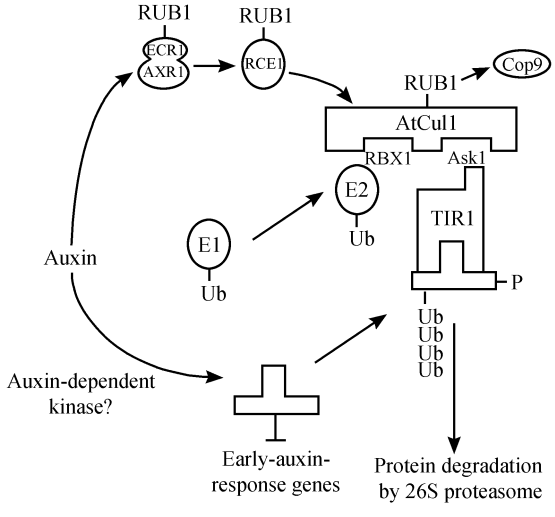

Fig. 2. A model for auxin response[31].

In recent years, a number of ubiquitin-like proteins (Ubls) have been identified. They are conjugated to a lysine residue of target proteins by a mechanism very similar to ubiqutin conjugation. However, in marked contrast to ubiquitin, Ubl conjugation does not generate a polyubl chain and does not appear to affect target protein stability. The SUMO-1 protein (for small ubiquitin-related modifier, also known as PIC1/Ubl1/Sentrin) is approximately 20% identical to ubiquitin and is conjugated to several proteins, including RanGAP1, PML and I_kB in mammals. In several cases, modification by SUMO-1 appears to regulate subcellular localization of the target protein. *Arabidosis* contains at least three members of the Rub family (NEDD8 in mammals) which is 50%—60% identical to ubiquitin[46].

Recently, in order to identify possible candidates for pollen self-incompatibility gene, we investigated the genomic structure of the *S* (self-incompatibility) locus in *Antirrhinum* and identified a novel gene family with an F-box structure (SLF, *S* locus F-box) (table 1). AhSLF-S_2 is one member of this family and is specifically expressed in the microspores and pollen grains, which indicates its possible function in self-incompatibility[43]. Through the analysis of the genomes of other organism, we found no homologs of AhSLF-S_2 in yeast, nematodes, flies and humans, but we identified about 100 homologs in *Arabidopsis* and rice (unpublished) which have unknown function. This indicates that AhSLF-S_2 belongs to a plant specific gene family. Currently, we are dissecting roles of members of SLF family in plant growth and development by a combinatorial approach including genetics, molecular biology and biochemistry.

3 Perspectives

Ubiquitin-mediated protein degradation is a key step in many important cellular processes. In recent years, through the identification of plant F-box proteins and the study of SCF^{TIR1} in auxin response, much more are known about this process in flowering plants. Although we have known the function of F-box protein through the work on SCF^{TIR1}, it is imperative to identify the function of other SCF complexes. Further, a systematic functional study on over 1000 predicted F-box motif-containing proteins in the completed *Arabidopsis* genome represents an important challenge to explore their unknown function. Recently, US scientists have initiated a genome-wide study on *Arabidopsis* F-box proteins including the production of loss-of-function or gain-of-function mutants and protein-protein interaction. This will give us a deeper understanding how plant development, signal transduction pathways and other cellular processes are regulated by ubiquitin-mediated proteolysis.

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