The E3 Ubiquitin Ligase Gene Family in Plants: Regulation by Degradation

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Abstract: The regulation of protein expression and activity has been for long time considered only in terms of transcription/translation efficiency. In the last years, the discovery of post-transcriptional and post-translational regulation mechanisms pointed out that the key factor in determining transcript/protein amount is the synthesis/degradation ratio, together with post-translational modifications of proteins. Polyubiquitinaytion marks target proteins directed to degradation mediated by 26S-proteasome. Recent functional genomics studies pointed out that about 5% of *Arabidopsis* genome codes for proteins of ubiquitination pathway. The most of them (more than one thousand genes) correspond to E3 ubiquitin ligases that specifically recognise target proteins. The huge size of this gene family, whose members are involved in regulation of a number of biological processes including hormonal control of vegetative growth, plant reproduction, light response, biotic and abiotic stress tolerance and DNA repair, indicates a major role for protein degradation in control of plant life.

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INTRODUCTION

The ubiquitination cascade is one of the main pathways of post-translational regulation of gene expression in eucariotic cells, in which ubiquitin is bond to a lysine residue of a target protein. Ubiquitin itself has different lysine residues: one of them, tipically Lys-48, can be further conjugated by another ubiquitin moiety in a processive manner to form a polyubiquitin chain. Being a reversible form of covalent modification, ubiquitination acts very rapidly on target protein in different ways. The best characterised function of ubiquitination is the degradation of target proteins through the 26S proteasome. Besides the elimination of aberrant or truncated proteins for cellular housekeeping, this pathway regulates the amount of active proteins, which depends on the synthesis/degradation ratio. An example is the human PARKIN protein, involved in autosomal recessive familial Parkinson's disease, which functions as E3 ligase promoting the degradation of a synaptic vesicle-associated septin [1, 2]. Furthermore, other regulatory functions (i.e. protein activation) of ubiquitin have been described over the past few years [3]. The consequences of protein ubiquitination might depend on the number of ubiquitin units in the chain linked to the target protein and on the lysine residue of ubiquitin utilised for the formation of polyubiquitin chain. For example, Lys-63 (K63)-linked polyubiquitin chains have been shown to mediate human protein kinase activation, DNA repair and vesicle trafficking. Monoubiquitination has also shown to be involved in nuclear targeting of human proteins interacting with DNA repair enzymes [3]. How ubiquitination can regulate the activity of target proteins is still unclear: ubiquitination could change the conformation of target proteins, otherwise a monoubiquitin moiety or a distinct polyubiquitin chain could serve as specific protein-protein interaction domain to recruit proteins carrying various ubiquitin-binding domains.

The ubiquitin attachment to target proteins is mediated by a three-steps enzymatic cascade. In the first step a thioester bond between ubiquitin and the ubiquitin-activating enzyme E1, is formed in an ATP-dependent reaction. Subsequently, ubiquitin is transferred to the ubiquitin-conjugating enzyme E2. Then, the transfer of ubiquitin to the target protein occurs in presence of the ubiquitin ligase enzyme E3, which specifically recognises the target proteins. An isopeptide bond is formed between the carboxyl terminus of ubiquitin and the ε-amino group of a lysine residue on the target protein. The efficient polyubiquitination is facilitated by multiubiquitin chain assembly factors (E4) which transfer additional ubiquitin moieties [4].

Many genomic studies have been carried out for E3 ubiquitin ligases in plants, particularly in species whose genome has been completely sequenced as *Arabidopsis* and rice. More than one thousand E3 ubiquitin ligase members have been predicted in *Arabidopsis* (Table **1**). The huge size of the E3 ubiquitin ligase gene family suggests its role giving specificity to the system, each E3 ubiquitin ligase acting for the ubiquitination of a particular target protein. This review will present the current status on functional genomic studies aimed to characterize the large family of E3 ubiquitin ligases in relation to the main biological processes where they are involved.

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Table 1. Genomic Organization of E3 Ubiquitin Ligase Gene Family in *Arabidopsis*

E3 sub group	Abbreviation	Number of genes	References
Homology to E6-AP C-Terminus	HECT	7	[17]
Really Interesting New Gene	RING	499	[23-26]
Plant U-box	PUB	49	[39-41]
CULLIN	CUL	11	[42-44]
Arabidopsis Skp1-related	ASK	21	[47-49]
Cyclin F proteins	F-box	724	[43, 50, 51, 66]
Bric a brac, Tramtrack and Broad complex	BTB	81	[44, 54]
CULLIN4-Damaged DNA-Binding Protein	CUL4-DDB	5	[28, 57]
Anaphase Promoting Complex	APC	18	[35]
	Total	1415	

E3 LIGASE ORGANIZATION

E3 ubiquitin ligases are classified into two groups depending on the presence of a HECT (Homology to E6-AP C-Terminus – Fig. **1a**) or a RING (Really Interesting New Gene)/U-box domain. RING proteins can act as single components containing both the active site and the binding pocket for the E2-ubiquitin intermediate (Fig. **1b**), or as components of multisubunit complexes which in plants include SCF (SKP1-CULLIN-F-box), CUL3 (CULLIN 3)-BTB/POZ (Bric a brac, Tramtrack and Broad complex/Pox virus and Zinc finger), CUL4-DDB1 (UV-Damaged DNA-Binding Protein 1) and APC (Anaphase Promoting Complex) complexes, as shown in Fig. (**1c**, **d**, **e** and **f**). While RING/U-box E3s generally act as molecular adapters between E2 and target proteins, the HECT E3s form a covalent bond with ubiquitin before transferring it to the protein substrate, using a conserved Cys of the HECT domain to form a ubiquitin-E3 thiole-ester intermediate [5].

The multi-subunit E3 ligases are CULLIN and RING finger-based protein complexes. These enzymes are constituted by two functional modules. The catalytic module is composed by a RING-finger subunit (RBX1- RING Box protein 1 - or APC11) interacting with the E2 enzyme. The second module (adapter) specifically recognises target substrates for ubiquitination. The same catalytic module can be associated to many different adapters, therefore many different E3 complexes are formed, which in turn catalyse the ubiquitination of different substrates. The two modules are brought together by a CULLIN (or CULLIN-like, APC2) protein which acts as a molecular scaffold and also defines the E3 class. The E3 SCF complexes contain four core components: SKP1 (S-phase Kinase associated Protein 1, named ASK1, for Arabidopsis Skp1-related in plants), CUL1, a F-box protein and RBX1. SCF structure has been resolved by Zheng *et al*. [6] in yeast and mammals. RBX1 catalyses the synthesys of polyubiquitin chains and, together with CUL1, forms a catalytic core complex recruiting a cognate E2. The CULLIN subunit acts as a large scaffold protein that ensures optimal presentation of the substrate to the E2 enzyme. It binds both RBX1 and the linker protein SKP1. The SKP1 protein serves as an adapter between CUL1 and the variable F-box protein, which binds the substrate [7-9]. The second family of the multisubunit E3 complexes is defined by the association among the highly conserved CULLIN family member, CUL3, a BTB/POZ domain protein, and the RBX1 protein [10]. In the current animal model for CUL3-BTB E3 ligases, BTB proteins function as substrate-specific adapters, which bind CUL3 through its BTB-domain and interact with the substrate through an associated protein-protein interaction domain [11]. RBX1 binds the E2 enzyme probably leading to its allosteric activation [12]. The BTB/POZ domain appears to assume a three-dimensional structure similar to the CUL1 interaction domain of the SKP1 adapter proteins [9], therefore it has been suggested that BTB proteins function as SKP1/F-box hybrid proteins that deliver the targets to CUL3.

CUL4 protein is a core component of a new class of E3 ubiquitin ligases that regulate replication and transcription in mammals. These E3 ubiquitin ligases also contain the DDB1 and RBX1 proteins. CUL4-DDB1 may recruit substrates directly, or through additional factors, such as the complex formed by De-Etiolated-1 (DET1) and Constitutively Photomorphogenic-1 (COP1) proteins [13].

The APC is a multiprotein complex, constituted by eleven subunits conserved in all eukaryotes, involved in the regulation of the cell cycle progression by degrading cyclins. Two APC proteins, APC2, a distant member of the CULLIN family, and the RING-finger protein APC11 form the minimal ubiquitin ligase module of the APC. The two proteins interact with each other and with E2 ubiquitin conjugating enzymes, and together are able to catalyse ubiquitination of proteins *in vitro*, although without substrate specificity [14]. Stage-specific activation of APC as well as selection and binding of the substrates depend on a WD40 protein able to interact with the substrate. Two types of WD40 proteins have been identified, Cdc20 (Cell division cycle) and Cdh1

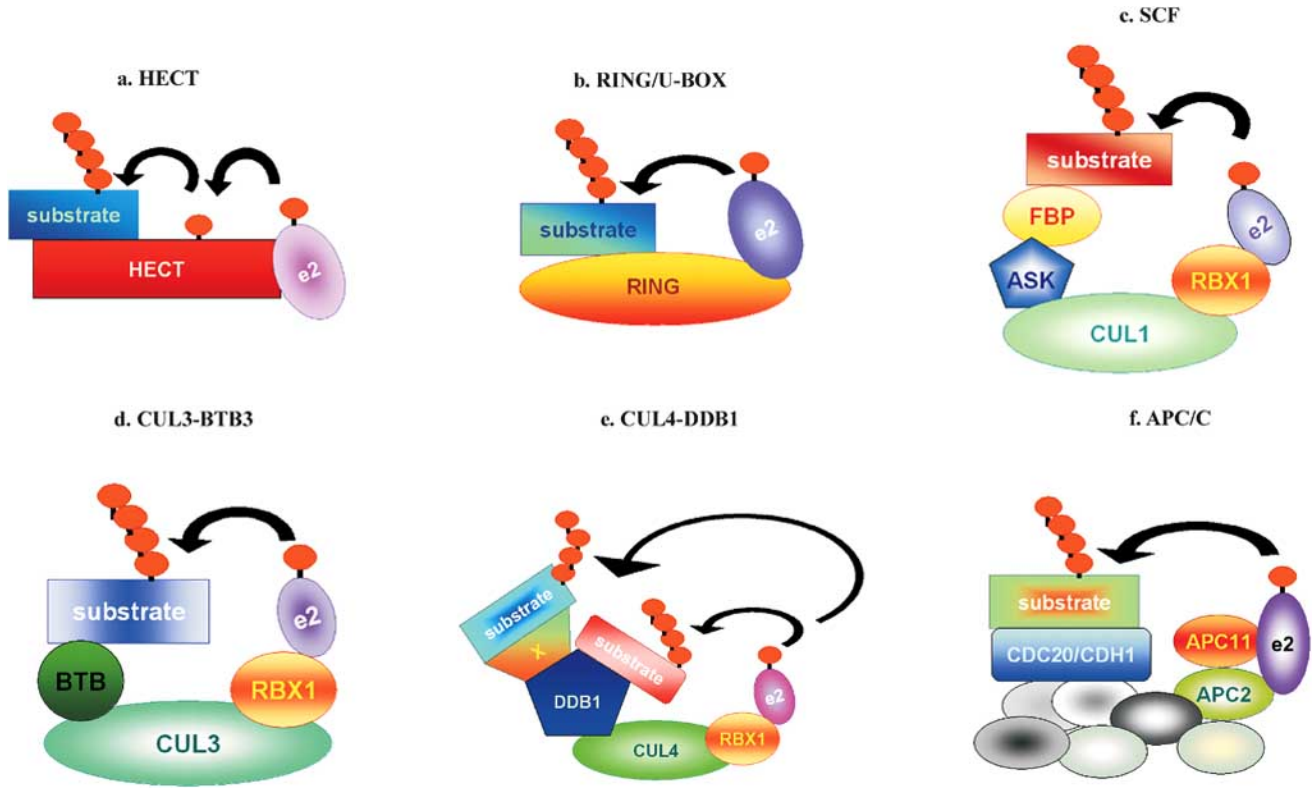

Fig. (1). Organization of E3 ubiquitin ligases. CULLIN and CULLIN-like proteins are in green and RING proteins are in red/orange; small linked circles represent polyubiquitin chains. (**a**) and (**b**): E3 single components. (**c**)-(**f**): E3 multi subunit complexes.

(Cadherin 1), known as Ccs52 (Cell cycle switch 52) in plants, and two different complexes can be formed depending on which WD40 protein interacts with the other APC subunits. In human APC, the interaction between the WD40 proteins and the APC core is mediated by two additional subunits (APC3 and APC7) [15].

E3 LIGASE COMPONENTS

HECT E3 Ubiquitin Ligases

The HECT E3 family contains proteins characterised by the presence of a conserved 350 amino-acid C terminal domain, called HECT from the first described protein, the human *E*6-*A*ssociated *P*rotein (E6-AP), involved in the ubiquitination and degradation of p53 upon its association with the papillomavirus protein E6 [16].

Whereas mammals have greatly expanded the use of HECT E3 type, having a gene family of about 50 elements, no comparable expansion is evident in *Arabidopsis*, where according to a consensus HECT sequence-based search, only seven *HECT* genes, named *UPL1-UPL7* are present [17] (Supplementary Table **S1**). *UPL* genes have been grouped in four subfamilies based on intron/exon positions, length of the corresponding protein and presence of additional protein motifs for subcellular localization (i.e. transmembrane domains) or interaction with other components of ubiquitination pathway or with glycosylated proteins (i.e. C-type lectin-binding domain) [17]. These domains, potentially important for target specificity are upstream to the C-terminal HECT domain and appear co-evoluted with the HECT domain.

Members belonging to different *Arabidopsis* UPL subfamilies often appear more similar to HECT E3 ubiquitin ligases from other species than to each other, suggesting that these subfamilies arose before the split of the animal, fungal and plant kingdoms and that they have catalytic activities that cannot be replaced by other E3 types. By means of an *in vitro* polyubiquitination assay it has been showed that the C-terminal HECT domain of UPL1 is necessary and sufficient to conjugate ubiquitin in a reaction that requires the positionally conserved cysteine within the HECT domain, an E1, and an E2 enzyme specifically belonging to the UBC8 family [18].

RING-Finger E3 Ubiquitin Ligases

RING containing proteins represent, together with F-box proteins, the most abundant E3 ubiquitin ligase gene families. The RING finger motif was first identified in the protein product of the human gene *RING1 — Really Interesting New Gene 1 —* [19].

The RING domain contains fours pairs of zinc ligands binding co-ordinately two zinc ions [20, 21]. The zinc ligands are formed by cysteine and hystidine residues placed

at proper distance in a typical cross-brace structure. The two main RING domains, C3HC4 and C3H2C3, contain a conserved His at metal ligand position 4, but differ for the presence of either a Cys or His residue, respectively, at metal ligand position 5. The RING domains were shown to be essential for E3 ubiquitin ligase activity of human RING-containing proteins [22].

Only a limited number of RING proteins with a predicted or known biological function have been characterised in plants (Supplementary Table **S2**; Table **2**). The complete sequence of the *Arabidopsis* and rice genomes gave new chances for large gene families characterisation in plants. Systematic searches for RING-finger domain containing proteins yielded about 500 distinct sequences in *Arabidopsis*, which should represent an exhaustive list of RING genes in this species [23-26].

Kosarev *et al*. [23] identified 365 genes grouped into six clusters based on sequences similarity and RING domain features. A more extended search, performed by Stone *et al*. [25] led to the identification of 469 genes, characterised by 3 RING-type (RING-H2, RING-Hca and RING-HCb) and five modified RING-type domains (RING-C2, RING-v, RING-D, RING-S/T and RING-G) based on the nature and on the distance between the metal ligand residues. The majority of analysed proteins contained only the RING-finger domain (about 150 sequences) or a RING motif associated with one or more transmembrane domains (about 120 sequences). In the remaining sequences, the RING motif was found associated to a number of known domains which may interact with the target protein, or act as regulatory components. Many RING-associated domains, such as coiled-coil or zinc finger Cx2Cx5Cx2C motifs and Ankyrin repeats, are potentially involved in the protein-protein interaction mechanisms. Furthermore, some domains are supposed to interact with specific classes of proteins, i.e. the WD40 repeats show specificity for ser/thr phosphoproteins [27].

A well characterised RING finger E3 ubiquitin ligase is COP1, which functions as central switch in light control of *Arabidopsis* seedling development. COP1 protein contains three structural domains: a RING finger, followed by a coiled-coil domain and seven WD40 repeats at the C-terminus. A large set of proteins interacting with COP1 have been identified. In particular, it has been proven that the RING domain mediates the interaction with COP10, a protein functioning as E2 ubiquitin conjugating enzyme, and CIP8 (COP1 Interacting Protein 8), another E3 ligase also involved, together with COP1, in the ubiquitination of the transcription factor HY5 (long hypocotyl phenotype 5). The coiled coil domain allows the dimerisation of COP1 and its interaction with a number of polypeptides, such as SPA (Supressor of phyA) proteins, involved in the modulation of COP1 activity. Finally, the C-terminal WD40 repeats are involved in the recognition of the ubiquitination targets: the transcription factors HY5, HYH (HY5-like protein H) and HFR1 (long Hypocotyl in Far Red 1) and the photoreceptors cry2 and phyA [28].

In some cases the RING domain was found associated to putative nucleic acid binding motifs such as C2H2 and C3H1 (DNA-binding zinc finger domains), or KH (K Homology) and RRM (RNA Recognition Motif). Notably, one RING finger protein (At3g54460) is predicted to contain an F-box domain [25].

ARIADNE proteins are characterized by the IBR (In Between Ring) domain, a motif with the pattern C6HC, usually located between two RING domains. The *Arabidopsis* ARIADNE family is formed by 16 genes [24]; twelve of them clustered together by Stone *et al*. [25].

ATL gene family members were originally isolated as genes rapidly induced in response to elicitors [29]. They contain a RING-H2 type domain with a particular signature: a highly conserved proline spaced out a residue upstream from the third zinc ligand, and a highly conserved tryptophan spaced out three residues downstream from the sixth zinc ligand. The analysis of Serrano *et al*. [26] led to the identification of 80 ATL proteins in *Arabidopsis*, most of them clustered together according to Kosarev *et al*. [23] and to Stone *et al*. [25].

For this review, we have listed 499 RING finger proteins (Supplementary Table **S1**), by considering, besides those collected by Stone *et al*. [25], 27 sequences annotated as RING proteins by Kosarev *et al*. [23] and Mladek *et al*. [24], and three individually characterised RING proteins: CER3 [30], HOS1 (High Expression of Osmotically Responsive Gene 1) [31], and PEX12 (Peroxisomal biogenesis protein 12) [32]. These latter sequences, although not perfectly satisfying the criteria imposed by Stone *et al*. [25], could represent potentially active E3 ubiquitin ligases. HOS1 has been recently shown to be active in the ubiquitination of the cold-related transcription factor ICE1 (Inducer of *CBF* Expression) [33].

Despite extensive studies on the RING protein family have been performed only in *Arabidopsis*, some evidences exist that the organization of this family can vary in different plant species. The modified RING domain RING-D appears to be characteristic of *Arabidopsis* (or dicots) species, since a similarity search carried out in rice genome failed to find proteins with the same domain [25].

The *Arabidopsis RING* gene collection also comprises three genes coding for proteins acting as subunits of large complexes: *AtRbx1;1* and *AtRbx1;*2 whose protein products participate in assembling of SCF, CUL3-BTB and CUL4-DDB complexes [34] (Supplementary Table **S1**), and the *APC11* gene, involved in the formation of the APC complex [35, 36].

U-Box E3 Ubiquitin Ligases

The U-box motif, originally identified in the yeast UFD2 (Ubiquitin Fusion Degradation 2) protein [37], is a RING finger-related domain lacking the zinc-chelating residues. One of best known plant U-box containing proteins is the *Brassica* ARC1 (ARM Repeat Containing protein 1) protein, an E3 ubiquitin ligase, acting downstream of S receptor kinase to promote ubiquitination and protein degradation during the rejection of self-incompatible *Brassica* sp. pollen [38]. Besides the U-box domain, ARC1 also contains several ARM (Armadillo) repeats. The association between U-box domain and ARM repeats is frequent in *Arabidopsis*, where 49 U-box genes have been identified, with 41 of them also showing ARM repeats (AtPUB-ARM genes - Supplementary

Table 2. E3 Ubiquitin Ligases Involved in Plant Growth and Development and Known Targets

E3 ubiquitin ligase	E3 type	Biological processes	Interactions with other complex components	Demonstrated E3 ligase activity *in vitro* and E2 partners	Target proteins	References
FIP2 (*Arabidopsis thaliana*)	BTB	pollen tube extension, actin cytoskeleton			AFH1	[95]
UFO (*Arabidopsis thaliana*)	F-box	floral development			APETALA3	[81, 91, 104]
FKF1 (*Arabidopsis thaliana*)	F-box	flowering in response to photoperiod	ASK1,2,11,14		CDF1/2	[105-107]
LKP2 (*Arabidopsis thaliana*)	F-box	flowering time, circadian clocks	ASK1,2,3,4,5, 11,14,20		CDF3, TOC	[87, 106]
ETO1/EOL1/EOL2 (*Arabidopsis thaliana*)	BTB	ethylene biosynthesis	CUL3a		ACS5	[67]
BT4 (*Arabidopsis thaliana*)	BTB	SA and H2O2 response			AtBET10	[94]
BT2 (*Arabidopsis thaliana*)	BTB	SA and H2O2 response			AtBET10	[94]
BT1 (*Arabidopsis thaliana*)	BTB	SA and H2O2 response	CUL3a		AtBET10, AtBET9	[94]
ARIA (*Arabidopsis thaliana*)	BTB	ABA response			ABF2	[108]
TIR1 (*Arabidopsis thaliana*)	F-box	auxin receptor	ASK1/2, AtCUL1		IAA1/AXR5	[46, 109-112]
SLY1/SNE (*Arabidopsis thaliana*)	F-box	GA signaling			RGA, GAI, RGL2 (DELLA proteins)	[84, 113]
GID2 *(Oryza sativa)*	F-box	GA signaling	OsSkp15(ASK1)		OsSLR1	[114, 115]
EBF1/2 (*Arabidopsis thaliana*)	F-box	ethylene signaling	ASK1/2		EIN3/EIL; ERF	[85, 116, 117]
AIP2 (*Arabidopsis thaliana*)	RING-H2	ABA signaling		UBC8, 10, 11, 28, 29, 30, UbcH5B	ABI3	[118]
SINAT5 (*Arabidopsis thaliana*)	RING-HCa	auxin signaling		AtUBC9a	NAC1	[119]
XBAT32 (*Arabidopsis thaliana*)	RING	auxin transport; root initiation		UbcH5B	XBAT32	[120]
COP1 (*Arabidopsis thaliana*)	RING-HCa	photomorphogenesis		UBC9	LAF1, HY5, HYH, cry2, phyA, COL3; HFR1; AtMYB21	[28, 98]
CIP8 (*Arabidopsis thaliana*)	RING-H2	photomorphogenesis		UBC8, 10, 11, 28, 29, 30	HY5 HYH	[28]
DET1 (*Arabidopsis thaliana*)	CUL4 complex	photomorphogenesis			LHY	[121]
NPH3 (*Arabidopsis thaliana*)	BTB	phototropism (hypocotil)			RPT2	[122]

(Table 2) contd….

E3 ubiquitin ligase	E3 type	Biological processes	Interactions with other complex components	Demonstrated E3 ligase activity *in vitro* and E2 partners	Target proteins	References
RPT2 (*Arabidopsis thaliana*)	BTB	phototropism (root), stomatal opening			PHOT1; NPH3	[69, 123]
DET1 (*Lycopersicum esculentum*)	CUL4 complex	photomorphogenesis			H2B	[124]
HOS1 (*Arabidopsis thaliana*)	incomplete RING	cold stress		UBC8	ICE1	[31, 33]
CHIP (*Arabidopsis thaliana*)	U-BOX	temperature stress		UbcH5a	denatured proteins; PP2A	[40]
NPR1 (*Arabidopsis thaliana*)	BTB	salicilic and jasmonic acid response			TGA	[79, 125-129]
PRT1 (*Arabidopsis thaliana*)	RING	N-end rule pathway degradation			N-end rule substrates (aromatic termini)	[130]
APC2 (*Arabidopsis thaliana*)	Cullin-related	cell cycle regulation	APC11; CDC23		D-box proteins	[36]
SKP2 (*Arabidopsis thaliana*)	F-box	cell cycle regularion			E2Fc	[131]
AhSLF-S2 (*Oryza sativa*)	F-box	self-incompatibility		ASK1; CULLIN1-like	S-Rnases	[61]
SFB (*Prunus dulcis*)	F-box	self-incompatibility			S-Rnases	[132]

Table **S1**) [39-41]. Although the U-box lacks the zinc-chelating residues, molecular modelling predicts that a system of salt bridges and hydrogen bonds may stabilize the U-box scaffold into a structure similar to the RING finger without the association of ions [39]. In fact, when six At-PUB-ARM proteins were tested in an *in vitro* polyubiquitination assay, all proteins proved to be active as E3 ubiquitin ligases [41].

The CULLIN Family

The *CULLIN* family encompasses at least six genes in humans and in *Caenorhabditis elegans*. A sequence similarity search in the *Arabidopsis* genome revealed the presence of at least 11 predicted *CULLIN*-related genes [42-44] (Supplementary Table **S1**). However, intact canonical C- and N-terminal domains were retrieved only for six CULLIN proteins, whereas four conserve only the N-terminal domain. EST-based evidences for gene expression have been reported only for five of the complete *CULLIN* genes, while the sixth CULLIN exhibited an apparent frame-shift in the coding region when compared to the most related *CUL2*, and may therefore not be functional. The putative functional *CULLIN* genes have been named *CUL1*, *CUL2*, *CUL4*, and *CUL3A/CUL3B*. The two closely related *Arabidopsis* CUL3 are subunits of CUL3/BTB complexes [45], whereas the *Arabidopsis* CUL1 and the related CUL2 protein showed association with the SCF class of E3 ubiquitin ligases [46]. In *Arabidopsis*, no ortholog for animal CUL2 has been identified. Five remaining truncated *Arabidopsis* CULLIN proteins await assignment, furthermore the unusual organization of these shorter forms raises the intriguing possibility that plants contain other CUL-based E3 ubiquitin ligases that are kingdom-specific [44].

SKP1-Like Family

The *Arabidopsis* genome contains 21 *Arabidopsis SKP1 (ASK)* genes [47-49] (Supplementary Table **S1**). Based on sequence similarity and phylogenetic analysis, they have been grouped in eight classes, and, with the exception of *ASK20* and *ASK21*, the *ASK* genes appear to descend from a common ancestor [43, 48, 49].

F-Box Family

F-box proteins are characterized by a N-terminal 60-residue F-box conserved domain, originally described in the Cyclin F protein [7]. The F-box domain interacts with the SKP subunit of the SCF complex. The first 40 residues represent the core of the SKP-binding site, while the other 20 residues represent a variable domain conferring binding preferences toward SKP proteins. The C-terminal portion of the F-box proteins typically contains a variable protein–protein interaction module that presumibly participates in

substrate recognition and binding, thus conferring specificity to the SCF complex.

Blast searches on the complete *Arabidopsis* genome for genes predicted to encode the F-box consensus sequence led to the identification of about seven hundred genes (703 according to Risseeuw *et al*. [43], 694 according to Gagne *et al*. [50] and 568 according to Kuroda *et al*. [51]), a number that could allow for the specific ubiquitination of many different substrates. A complete list of all F-box genes of *Arabidopsis* is presented in Supplementary Table **S1**. A phylogenetic analysis based on the F-box domain sequences, grouped the F-box protein genes in five families (A-E), whose A-C families are further divided into 18 subfamilies, giving 20 distinct classes. Residues conserved among the 20 families correspond with those implicated in the SKP association, while the additional positions conserved within the subfamilies could be important for preferential binding of different ASK proteins [50].

The analysis of the C-terminal region revealed an array of potential protein-protein interacting domains, including Leucine-Rich Repeat (LRR), Kelch, WD-40, ARM repeats, ThetracoPeptide Repeats (TPR), TUBBY, actin-related motif, DEXDc (DEAD-like helicase), and the metal-binding site Jmj-C (jumonji) domains [50]. The LRR and Kelch repeats are the most widely represented. Nevertheless, a large number of the *Arabidpsis* F-box proteins had C-terminal regions with no obvious similarity to known motifs. The output of the F-box phylogenetic tree suggests a general co-evolution of the F-box motif with the target interaction domain [50]. A survey of genomic arrangements revealed that events of tandem duplications of chromosomal regions should have played a major role in creating this sheer size family. However, sequence comparisons and genetic analyses suggest that most of the proteins do not have functional paralogs, that is F-box proteins, although with similar F-box motifs and C-terminal domains, have unique functions and so distinct targets [50].

The high number of *F-box* genes (significantly higher in plants than in other eukaryotes) and some associations between F-box and protein-protein interacting domains unique to plants may indicate that plants exploited the F-box family to broad their ability for specific target recognition.

BTB Family

The BTB/POZ domain was originally identified as a conserved motif present in *Drosophila melanogaster ZID* (Zinc finger protein with Interaction Domain) gene [52]. A variety of functional roles have been proved for this domain, including transcriptional regulation, cytoskeleton dynamics, ion channel assembly and gating and targeting proteins for ubiquitination. In most of these functional classes, the BTB domain acts as a protein-protein interacting module able both to self-associate and to interact with other proteins [53].

A search on *Arabidopsis* genome identified about 80 loci encoding proteins with one or more BTB motifs. We compared the two published lists [44, 54] to obtain an updated collection with 81 loci (Supplementary Table **S1**). Nearly all genes appear singletons, with just one example of apparent tandem duplication. Most of the BTB proteins harbour additional protein domains, either upstream or downstream of the BTB domain, acting as protein interactors (Ankyrin repeats, Meprin And TRAF Homologous domain – MATH -, ARM and TPR repeats), DNA binding motifs (Myb repeats, Transcriptional Adapter putative Zinc finger - TAZ), as well as the plant specific Nonphototropic Hypocotil domain (NPH3) and the potassium channel tetramerisation domain. The different plant BTB-domain proteins have been phylogenetically classified in various subgroups, and a general co-evolution between the BTB domain and the other associated protein domains have been evidenced [44, 54].

CUL4-DDB1 Subunits

Although the CUL4 protein has been conserved during evolution in fission yeast, plants, worms, flies and mammals, only recently evidences have shown a CUL4 dependent E3 ligases activity in *Arabidopsis*. Currently, knowledge from human allowed identifying plant orthologs for all the subunits of the human CUL4-DDB1 complexes (Supplementary Table **S1**). Two highly related *DDB1* genes, named *DDB1a* and *DDB1b* were found in *Arabidopsis* [55]; other subunits are DET1 [55], COP10, COP1 [56] and DDB2 [57].

APC Complex Subunits

Hortologous genes have been individuated in *Arabidopsis* for all known vertebrates APC subunits, in some cases with gene duplication events [35]. The functional APC complex is constituted by eleven subunits. Among them, *Arabidopsis* APC2 and APC11 form the catalitic core [36]; the *Cdc27* gene, homologous to the human *APC3* gene, mediates, together with other Cdc subunits, the interaction between the APC core and the WD40 protein, Cdc20 or Ccs52. Many different APC complexes can be assembled, considering the existence in *Arabidopsis* of two *Cdc27* genes (*Cdc*27A and *Cdc27B/HOBBIT*), three *Ccs52* (*Ccs52A1*, *Ccs52A2* and *Ccs52B*) and six *Cdc20* genes, all shown to be part of APC complex *in vivo* in *Arabidopsis* [35, 58].

E3 UBIQUITIN LIGASES: EXPRESSION PATTERNS AND MOLECULAR INTERACTIONS

Genomic studies indicate that plant E3 ubiquitin ligase gene family comprises about 1500 members (Table **1**). A further complexity derives from the possibility of different functional combinations among the different subunits within E3 complexes as well as among the E3 ubiquitin ligases and different E2 conjugation enzymes. Indeed, the E2 gene family, characterised by the UBC domain, comprises 37 genes in *Arabidopsis* [59].

Many studies have supplied evidences that the most of the genes coding for E3 ubiquitin ligases are indeed active in a number of plant tissues: nearly all RING, U-box, ASK and BTB proteins are expressed [26, 44, 48, 49, 60], while only for less than half *F-box* genes there is an evidence of expression [50, 61].

Some E3 ubiquitin ligases or E3 components are ubiquitously expressed in all analysed tissues, while others show more restricted expression patterns which could be related to a particular function in plant development.

A wide expression analysis carried out on all E3 ubiquitin ligases annotated in this work (Supplementary Table **S1**)

in different physiological conditions may help to understand and quantify the key role of the ubiquitination pathway in plant life. The analysis was performed *in silico* by screening all *Arabidopsis* 22K microarray data available at the GENEVESTIGATOR site (https://www.genevestigator.ethz.ch – Metanalyser - Supplementary Table **S3**; Fig. **2**) [62]. A gene was considered up- or down-regulated when transcript amount increased or decreased of at least two times in treated samples respect to control samples. The physiological conditions tested included biotic, abiotic and light stresses, various chemical treatments (i.e. hormone inhibitors), hormone response, senescence, sugars supply and nutrient starvation. About the 70% of E3 genes were present as probeset in the GENEVESTIGATOR dataset, but probeset for only 43% of F-box genes were found, according to EST-based expression data reported by Gagne *et al.* [50]. The expression level of only 128 E3 genes was not found affected by any of the considered treatment conditions. Genes not yet reported as expressed may be characterised by a very low or spatially/temporally restricted expression profile; alternatively, some of them could be pseudogenes.

Alternative splicing is one possibility to increase the number of proteins and their translation efficiency. An alternative splicing event was detected for *ARI15* due to the existence of two different mRNA forms with or without the last intron within the 3'UTR, whose relative amount depended to some extent on the analysed tissue [24]. A similar intron retention event was described for other two *Arabidopsis* RING genes (At4g39140 and At2g21500) and for the homologous RING gene of durum wheat, *6G2*, whose mRNA retained the last 3'UTR-located intron following exposure to abiotic stresses [63]. For several BTB *Arabidopsis* genes, available ESTs predicted the presence of multiple splice variants, although only for two genes, the protein coding region was predicted to be altered [44].

The expression analysis may supply important indications on the function of E3 ligases. *AtRbx1* genes, participating to the assembly of CUL1-SCF and CUL3-BTB complexes, were found expressed in all plant organs, above all in tissues containing actively dividing cells, suggesting that ubiquitination may be involved in the turnover of key cell cycle regulatory plants proteins [34].

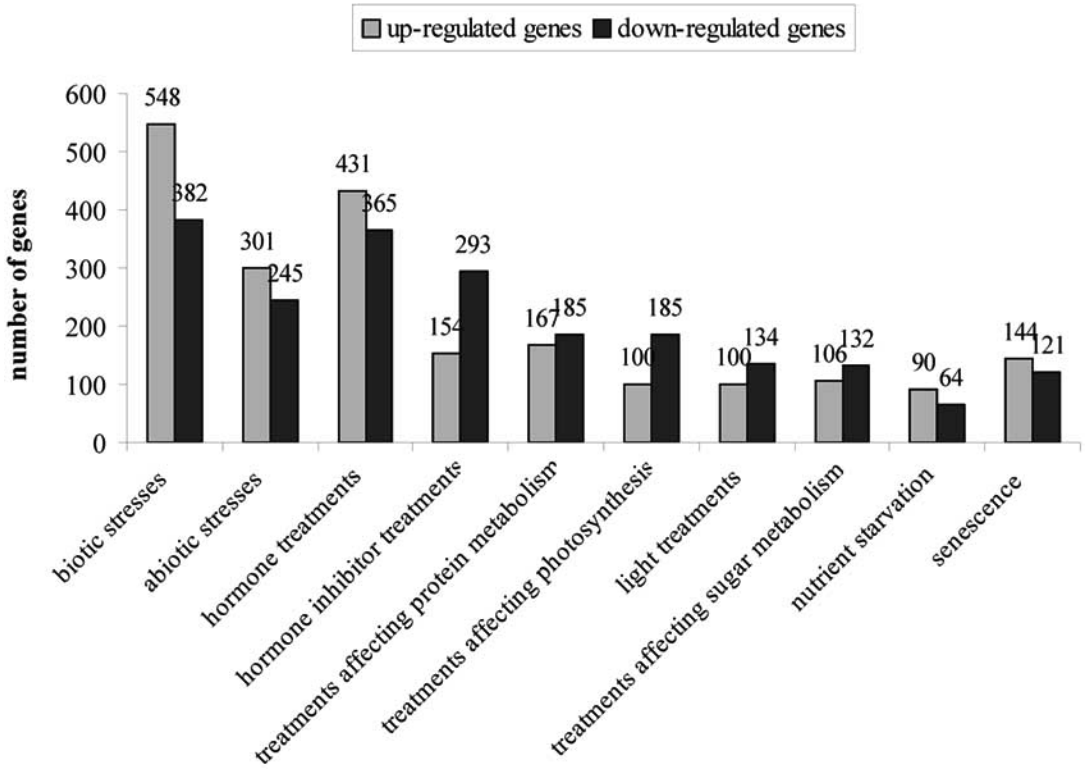

Fig. (2). Changes in genes expression of E3 ubiquitin ligase genes following different conditions. A gene was considered up- or down-regulated when transcript amount increased or decreased of at least two times in treated samples respect to control samples. Up-regulated genes are reported in grey, down-regulated genes in black. Number of up- and down-regulated genes is indicated for each condition. The conditions considered are: biotic stresses (bacteria, fungi, insects, mycorrhiza, nematodes, syringolin, chitin); abiotic stresses (anoxia/ipoxia, cold, drought, heat, wounding, ozone, H_2O_2, osmotic, oxidative and salt stresses); hormone treatments (cytokinin, salicylic acid, jasmonate, auxin, gibberellin 3, ethylene, brassinosteroids, 1-aminocyclopropane-1-carboxylic acid (ACC) and ABA), hormone inhibitors (inhibitors of auxin, gibberellin, ethylene and brassinosteroids), treatments affecting protein metabolism (cycloheximide as inhibitor of protein synthesis, MG13 as inhibitor of proteasome), treatments affecting photosynthesis (high CO2, PNO8 as photosynthesis inhibitor, norflurazon as inhibitor of carotenoids synthesis), light treatments (dark, blue, far red, red, UV), treatments affecting sugar metabolism (glucose and sucrose supply), nutrient starvation (potassium, nitrogen, sulphur) and senescence. A gene was considered up- or down-regulated by a given condition when its expression changed following at least one of the treatments grouped in the considered condition. Expression data were obtained from GENEVESTIGATOR (https://www.genevestigator.ethz.ch – Metanalyser) [62].

The three *Ccs52* genes, which can form three different APC complexes, are expressed in different times during the progression cell cycle, furthermore Ccs52 proteins showed different specificity for different *Arabidopsis* cyclins, strongly indicating that APC complex composition and activity could be tightly regulated to control cell cycle progression by destroying different cyclins at the proper phase of the cell cycle [58].

Many *ASK* genes are expressed in the *Arabidopsis* gametophytes, mostly in the male one, suggesting some functional redundancy of the *ASK* genes and/or formation of different SCF complexes, based on the specificity of interaction between ASKs and F-box proteins during pollen grain development [49, 48].

Co-expression or interaction data on different E3 ubiquitin ligases and E2 conjugating enzymes, or different subunits of larger complexes, could implicate their functional association to control a specific biological process. Forty-five RING proteins representative of most RING and modified RING domains resulted to be active in an *in vitro* polyubiquitination assay carried out by using AtUBC8 as E2. The mutation of an amino acid at metal ligand positions C3, H4 and H/C5 of two RING-H2 proteins and the treatment of RING proteins with zinc chelators disrupted the ubiquitin ligase activity of the RING domain, demonstrating that also in plants RING proteins require an intact zinc-coordinating RING domain to mediate E2-dependent protein ubiquitination [25]. When the same set of RING proteins was assayed in combination with 15 different *Arabidopsis* UBC genes coding for E2 conjugating enzymes, a variable degree of specificity between E3 and E2 enzymes was observed. The UBC34 showed the highest specificity, being active in combination with only two E3 ubiquitin ligases. On the other hand, the most generic E2 conjugating enzymes were members of the UBC8 group, active with both canonical and modified RING types [59]. This experiment also showed that the same E3 ubiquitin ligase could act in association with different E2 conjugating enzymes suggesting that the activity of a single E3 component could depend not only on its own expression, but also on availability of a number of other proteins, E2, target and regulatory factors. The AIP2 (ABI3 interacting protein) E3 ligase mediates the ubiquitination of the transcription factor ABI3 involved in ABA (Abscisic Acid) signaling. However, whereas AIP2 transcripts were found ubiquitously in all plant tissues, ABI3 was exclusively expressed in developing and mature siliques and seeds. Therefore the same E3 ubiquitin ligase could act in different tissues and have different functions depending on the availability of the target protein [64].

The function of large complexes E3 ligases is a much more complicated task, because the E3 activity depends on the presence of a number of different subunits. Many E3 complexes have been characterised giving evidences supporting their critical role in plant life. Detailed studies have shown that the correct expression of some subunits can regulate the accumulation of the interacting proteins, or stabilise their interaction. For instance, *RBX1* dsRNA lines contained a significant reduction of CUL1 protein level, suggesting that RBX1 protein is necessary to accumulate a stable SCF complex [34]. Furthermore, the presence of the F-box protein may stabilise interaction between CULLIN and the respective ASK adapter in SCF complexes. Indeed only weak interactions were observed by means of yeast two hybrid analysis for nearly all *Arabidopsis* SKP-1 related proteins (ASK1/2/3/4, ASK13/14/15/16 and ASK18/19), but a strong interaction between some ASK proteins and CUL1 was observed in presence of EID1 (*Empfindlicher im Dunkelroten Licht 1* – i.e. more sensitive in far-red light) F-box [65]. Therefore, SCF assembly could be a two-step association process, in which the F-box protein first associates with the ASK adapter to form a unit that afterwards binds to the CULLIN core complex.

Despite the combinatorial possibilities for SCF complexes are very extensive, resulting from alternative association of 21 ASK proteins with 700 F-box proteins, a relatively limited number of functional SCF complexes with different ASK proteins and F-box combinations can be formed [43, 50]. For some F-box proteins no physical interactions were detected suggesting other functions rather than polyubiquitination [66], or requirement of the target or additional factors to associate with the rest of the SCF complex [50]. This possibility has been verified for some F-box proteins, which require another factor besides ASK/SKP1 for the interaction with CUL1 [43]. Additional evidences also suggest that higher order SCF complexes could be formed, involving more than one F-box protein. F-box dimerisation would allow more possibilities for regulation of the process, as well as higher level of potential combinatorial diversity.

Various interaction studies have been performed to support the presence of CUL3-BTB E3 complexes in *Arabidopsis*. Both CUL3A and CUL3B proteins interact with RBX1 and with a limited number of BTB protein [54, 67, 68]. However, the lack of interaction of CUL3 with some BTB-domain proteins indicates that BTB-proteins could function also in a CUL3-independent context. A more detailed analysis has been accomplished on the six members of the *Arabidopsis* BTB/POZ-MATH family (*AtBPM1-6*) [68], testing different interactions between AtBPM and CUL3 subunits. According to Dieterle *et al.* [54], only few AtBPM proteins were able to assemble with CUL3A and CUL3B. Interestingly, each AtBPM protein was able to form homodimers and heterodimers with all other members of the family, as reported also for some other BTB proteins, as hypothesised also for F-box proteins in SCF complexes [45, 69]. If verified *in planta*, this finding could have a potent consequence. Indeed, although limited interactions between CUL3 and BTB proteins reduce the combinatorial possibilities for BTB/POZ-dependent E3 ligases, the possibility of BTB/POZ homodimerization and heterodimerization supports a potentially very large number of distinct E3 ubiquitin ligases, rivalling the number of SCFs-type E3s.

Interaction studies allowed to verify the presence of the CUL4-DDB1 complexes in plants. *Arabidopsis* CUL4 forms complexes with DDB1 proteins, RBX1, DET1 and DDB2 *in vitro* and *in planta* [70]. DDB1a associates with DET1 and COP10, forming a stable protein complex, called CDD [56]. Furthermore, in dark conditions, the CDD complex directly interacts with COP1 and with the COP9 signalosome, a complex with regulatory function, able to post translationally modify the CULLIN subunit of E3 complexes [71]. In this

case COP1, whose intrinsic ubiquitin ligase activity has been proved *in vitro* [72], could function as an adapter subunit of a larger E3 complex, maintaining the capability to bind both the E2 enzyme and substrates [56]. The COP1 interactors, including transcription factors and photoreceptors [28], suggest a strong involvement of the CUL4-DDB1 dependent ubiquitination mechanism in photomorphogenesis process.

UBIQUITIN-MEDIATED DEGRADATION: A RECURRENT THEME IN PLANT LIFE CYCLE

Most aspects of plant life cycle are somehow regulated by ubiquitin-mediated degradation of key proteins. This feature gets critical when target proteins are short-lived regulatory components, like enzymes directing rate-limiting steps, signaling receptors and transcriptional factors [73].

Most of the E3 ubiquitin ligases characterised up-to-date act in regulating plant development and hormone signaling [74, 75] (Table **2**). Notably, an effect frequently observed in E3 ligase *Arabidopsis* insertional mutants is the arrest of embryo development. For four T-DNA insertional lines for *ATL-RING* genes only hemizygous lines were recovered, suggesting that the mutated genes could be essential for plant viability [26]. The embryo lethality associated to gene disruption was also described for other RING E3 ubiquitin ligases, as the *Arabidopsis* RIE1 [76] and PEX12. The analysis of RNA interference plants with partial reduction of the *PEX12* transcript, exhibiting impaired peroxisome biogenesis and function, addressed the PEX12 function to the regulation of the peroxisome development [77].

Since RBX1, ASK and CUL proteins participate to form several E3 complexes, they could be involved in the control of many different processes, therefore, in absence of functional redundancy, many loss of function mutants may have lethal phenotype. Indeed, *Arabidopsis* mutants containing T-DNA insertions in the *CUL1* gene displayed an arrest in early embryogenesis, before the first cell division of both embryo and endosperm cells [42]. Consistently, both the transcript and the product of the *CUL1* gene were found to accumulate in embryos. The CUL1 protein was localized mainly in the nucleus, weakly present in the cytoplasm during interphase and co-localized with the mitotic spindle in metaphase. *CUL3A* and *CUL3B* appear functional redundant, since *cul3a* and *cul3b* mutants are viable and, consistently, they have largely overlapping expression patterns. However, the disruption of both the *CUL3A* and *CUL3B* genes affected both embryo pattern formation and endosperm development, causing embryo lethality [44, 45, 78]. Although arrest at the heart stage was predominant, block of embryogenesis occurring at multiple stages of embryo development indicated a general growth inhibition of the embryo. CUL4-DDB1 complex also appears essential, being the *ddb1b* mutation embryo lethal, although *Arabidopsis* contains two highly related DDB1 proteins [55]. According to RBX1 involvement in the formation of SCF, CUL3-BTB and CUL4-DDB1 complexes, severe phenotypes were observed for *Arabidopsis RBX1* dsRNA lines, consistently with the suppression level [34], including the block of seedling development, death of young seedlings, or severe dwarf phenotypes. These findings strongly supported the essential role of E3 complexes in many plant biological processes activated during plant embryogenesis and post-embryonic development.

Deregulation of RBX1 protein level in dsRNA lines and mutations in *ASK1* gene led also to phenotypes with reduced auxin response and decreased jasmonate response, similar to those observed in the *axr1*, *tir1*, and *coi1* mutants, substantiating the role of RBX1 and ASK1 as components the SCF^{Tir1} and SCF^{Coi1} complexes, known to be involved in auxin and jasmonate signaling respectively [46, 79, 80].

Ask1 mutants and RNAi lines showed also severe flower defects [49], in accordance to previous evidences of association between ASK1 and the F-box protein UFO, involved in flower development and male sterility [81-83]. Although a role of ASK1 in GA-signal transduction is not yet demonstrated, the SCF-complex SLY (SLEEPY) regulates GA response [84] and the RNAi line showed also features similar to those found in GA-deficient or insensitive mutants [49]. ASK1 was found to interact also with EID1, a F-box involved in phytocrome-A specific light signaling [65]. The F-box proteins EBF1/2 (EIN3-Binding F-box) [85] known to belong to the ethylene signaling pathway, and ORE9 (ORESARA9 - "long living"), are involved in the regulation of plant leaf senescence [86], whereas the F-box protein LKP2 (LOV KELCH Protein 2) and ZTL (ZEITLUPE) are involved in the regulation of circadian timings [87, 88]. Very recently the proteomes of the *Arabidopsis* wild type and *ask1* mutant flower buds have been compared evidencing ten proteins involved in photomorphogenesis, circadian oscillation, post-translation process, stress-responses and cell expansion or elongation, all processes being misregulated in the mutant [89].

Although the implication of ASK1 in various cell processes, *ask1* mutants are viable. In a similar manner, although the ASK2 protein was found to be associated with several F-box proteins, such as TIR1 (Transport-Inhibitor Response 1) [46], COI1 (Coronatine-insensitive) [90], UFO (Unusual Floral Organ) [91], and EID1 [92], *ask2* mutant is morphologically similar to wild type. Therefore partially functional redundancy has been evocated to explain the viability of the two mutants. Indeed, the *ask1-ask2* double mutation affects cell division and cell expansion/elongation, resulting in delayed embryogenesis and seedlings lethality, and demonstrating a vital role for ASK1 and ASK2 in embryogenesis and postembryonic development [93].

Functional analyses have been accomplished for some BTB proteins linking individual BTB proteins to several processes in plants. The best characterised BTB protein is ETO1 (Ethylene-Overproducer 1), a protein involved in the control of ethylene biosynthesis. As reported above, it is able to interact with CUL3A, likely forming a BTB^{ETO1}. Moreover it was found to target for degradation of the ACC synthase ACS5, the rate limiting enzyme of the ethylene biosynthesis and the first reported substrate for a plant CUL3-based E3 [67].

For other *Arabidopsis* BTB proteins, possible functions have been inferred biochemically, as the transcriptional regulation activity of some members of BTB family, characterised by a calmodulin-binding domain, in response to H_2O_2 and salicylic acid treatments [94]. FIP2 (Rab11-Family of Interacting Protein 2) protein was found to interact with *Arabidopsis* Formin Homology 1, AFH1 [95], a protein required for polar pollen tube extension [96]. The involvement

of E3 ubiquitin ligases in signaling of hormones as ABA, jasmonic and salicilic acids indicates a strong functional role of ubiquitination pathway in plant response to environmental clues. The most studied system is represented by COP1/CIP8 and their role in regulation of photomorphogenesis [28, 97, 98]. The role of E3 ubiquitin ligases in response to plant pathogen has also been widely described [99-102]. Regarding response to abiotic stresses, some E3 ubiquitin ligases regulate stress response acting also in hormone independent pathways, as HOS1 ubiquitinating the key regulator of cold stress response ICE1 [33].

A recent work [70] has proven that *Arabidopsis* CUL4 participates in important processes such as the cell cycle, light-dependent growth control, modulation of chromosomal structure and DNA repair. *Cul4* mutants are also severely affected in different aspects of the development: a reduced level of *CUL4* expression leads to a reduced number of lateral roots, to abnormal vascular tissue and stomatal development, and to weakly altered response to photomorphogenic stimuli.

In silico expression analysis indicated that the E3 ubiquitin ligases gene family could be involved in much more biological processes than those known until now since changes in E3 gene expression were found in response to a wide range of growing conditions (Table **2**, Fig. **2**, Supplementary Table **S3**). A role of E3 ubiquitin ligases has been widely described in auxin, gibberellins, ethylene and ABA responses, nevertheless the expression data also indicate a strong involvement of E3 ubiquitin ligases in biological processes mediated by brassinosteroids (231 and 70 genes up- and down regulated respectively, Supplementary Table **S3**). Genes belonging to all E3 ubiquitin ligase sub families were reported as regulated by these hormones, including one HECT gene, suggesting a new role for this group whose function is very poorly understood in plants.

Particularly interesting is the case of APC complexes. They have been originally described as involved in the cell cycle progression [35]. Recently, loss of function mutants for the *HOBBIT/Cdc27B* gene, homologous of *APC3* human subunit, were found unable to degrade the AXR3/IAA17 repressor of auxin response, indicating that the APC complex is also involved in hormone response [103]. Expression analyses suggest a role for these complexes in pathways controlled by many other hormones (some APC subunits are up-regulated by ABA, ACC, brassinosteroids, gibberellins and auxin and down-regulated by ethylene, salicylic acid and cytokinins). Furthermore, APC subunits were found up-regulated by light treatments, and down-regulated by all abiotic stresses except anoxia and cold (Supplementary Table **S3**).

A lower number of E3 ubiquitin ligase genes are also regulated by abiotic stresses, light and photosynthetic activity. Furthermore, the *in silico* expression analysis has provided evidences for the involvement of E3 ubiquitin ligases in other processes not previously described as potentially regulated by ubiquitination such as senescence, ipoxia/anoxia, sugar regulated pathways and nutrient starvation (Supplementary Table **S3**; Fig. **2**).

Unfortunately, despite the huge knowledge obtained on large E3 ligase family in plants from a genomic point of view, wide information are still lacking about individuation of target proteins, that remains the most intriguing, and poorly understood aspect of the ubiquitination pathway, which, by specifically affecting many components of cellular regulation, can participate to the fine tuning of cellular response to the variable life conditions.

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