Molecular and functional characterization of Arabidopsis Cullin 3A

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Summary

Cullin proteins, which belong to multigenic families in all eukaryotes, associate with other proteins to form ubiquitin protein ligases (E3s) that target substrates for proteolysis by the 26S proteasome. Here, we present the molecular and genetic characterization of a plant Cullin3. In contrast to fungi and animals, the genome of the model plant Arabidopsis thaliana contains two related CUL3 genes, called CUL3A and CUL3B. We found that CUL3A is ubiquitously expressed in plants and is able to interact with the ring-finger protein RBX1. A genomic search revealed the existence of at least 76 BTB-domain proteins in Arabidopsis belonging to 11 major families. Yeast two-hybrid experiments indicate that representative members of certain families are able to physically interact with both CUL3A and CUL3B, suggesting that Arabidopsis CUL3 forms E3 protein complexes with certain BTB domain proteins. In order to determine the function of CUL3A, we used a reverse genetic approach. The cul3a null mutant flowers slightly later than the control plants. Furthermore, this mutant exhibits a reduced sensitivity of the inhibition of hypocotyl growth in far-red light and miss-expresses COP1. The viability of the mutant plants suggests functional redundancy between the two CUL3 genes in Arabidopsis.

Keywords: ubiquitin-dependent proteolysis, BTB proteins, E3 ubiquitin ligase, light signalling.

Introduction

In recent years, the ubiquitin/26S proteasome pathway was discovered to be a central player for rapid and selective degradation of key short-lived regulatory proteins that play important roles in a variety of cellular processes (Ciechanover et al., 2000). In particular, cell cycle progression and development are tightly controlled by ubiquitin-dependent protein degradation. Degradation via this pathway is a two-step process: the protein is first tagged by covalent attachment of ubiquitin, and subsequently degraded by a multicatalytic protease complex called the 26S proteasome. Conjugation of ubiquitin to the protein involves a cascade of three enzymes: E1, E2 and E3. Ubiquitin-activating enzyme (E1) forms a high-energy bond with ubiquitin, which is then trans-esterified to a ubiquitin conjugating enzyme (E2). The transfer of ubiquitin to the target protein substrate usually requires a ubiquitin protein-ligase (E3). Polyubiquitylation of the protein substrate is sufficient to target it for degradation by a large (26S) ATP-dependent multicatalytic protease, the proteasome.

To date several classes of E3 have been reported. Among the major types of E3 are the SCF (SKP1-CUL1/CDC53-F-box protein) complexes, which are composed of four primary subunits: CULLIN1 (CUL1 or CDC53 in budding yeast), RBX1, SKP1 and an F-box protein. The CUL1 protein serves as a scaffold to assemble the components of the complex. Its N-terminal domain interacts with SKP1 and its C-terminal half forms a globular domain, which binds RBX1. F-box proteins contain a rather variable interaction domain known as the F-box that makes contact with the SKP1 subunit of the SCF complex. Additionally, F-box proteins carry a great variety of typical protein–protein interaction domains that confer substrate specificity for ubiquitylation. Thus, the F-box proteins are the adaptorsubunits that specifically recruit substrates to the core ubiquitylation
complex through a physical interaction between the F-box domain and the SKP1 subunit.

In addition to CUL1, all eukaryotes contain other cullin genes (Gieffers et al., 2000; Pintard et al., 2004), including members of the cullin3 family. Whereas deletion of the CUL3 gene has no obvious phenotype in budding yeast (Michel et al., 2003), CUL3 knockout mice are not viable and their early arrested embryos accumulated cyclin E (Singer et al., 1999). In Drosophila melanogaster, CUL3 may be involved in the degradation of Cubitus interruptus (Ci) in the posterior cells of the eye disc (Ou et al., 2002). Using RNA interference in Caenorhabditis elegans (Kurz et al., 2002), it was shown that knockdown of CUL3 impaired microtubule dynamics, most probably due to a failure to degrade the catanin-like protein MEI-1 (Pintard et al., 2003). Despite these results, our knowledge concerning CUL3 functions is still very limited. A major breakthrough was the recent finding that CUL3 proteins interact with ‘Bric a brac, Tramtrack and Broad Complex/Pox virus and Zinc finger’ (BTB/POZ) domain proteins and thus form a novel class of E3-based ubiquitin protein-ligase complexes (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Xu et al., 2003). In the current model, the BTB proteins combine the properties of the SKP1/F-box proteins and may thus function as substrate-specific adaptors. Consistent with this model the nematode MEL-26 protein binds to CUL3 through its BTB-domain and recruits the substrate MEI-1 through a MATH protein-protein interaction domain, thus promoting its ubiquitylation (Furukawa et al., 2003).

In contrast to the situation in animals, the genome of the model plant Arabidopsis thaliana contains two related CUL3 genes, called CUL3A and CUL3B. The results reported here focus on CUL3A, which is ubiquitously expressed in various plant organs. We used a phylogenetic approach to classify all Arabidopsis BTB-domain proteins into 11 major gene families. Representative members for seven of the identified families were tested for interaction with both CUL3 proteins in yeast two-hybrid experiments. The interaction of Arabidopsis CUL3 proteins with both RBX1 and different BTB-domain proteins suggests the existence of multiple CUL3-based E3 protein complexes in plants. To investigate the role of CUL3A in Arabidopsis, we identified and analysed T-DNA insertion mutants. Interestingly, CUL3A loss-of-function leads to a late-flowering phenotype and a reduced sensitivity of the inhibition of hypocotyl elongation under continuous far-red light.

Results

The Arabidopsis CUL3A gene is ubiquitously expressed in various plant organs

The Arabidopsis genome and EST sequences revealed the presence of five expressed related cullin genes, called CUL1, CUL2, CUL3A, CUL3B and CUL4 (Risseeuw et al., 2003). CUL3A (At1g26830) and CUL3B (At1g69670) map to the same phylogenetic clade as fission yeast, worm, fly and human CUL3, indicating a high conservation of this cullin family throughout evolution. The plant CUL3-type proteins share 88% of identity at the amino acid sequence level and may thus play similar functions. Northern analysis with a CUL3A-specific probe indicates that the CUL3A gene is expressed in all plant organs analysed (Figure 1a), suggesting its involvement in basic cellular functions. To gain more insight into CUL3A gene expression patterns, we created a promoter-GUS fusion construct and introduced it into wild-type plants. Histochemical staining of seedlings and adult plants revealed that the CUL3A-GUS construct is expressed at all stages of plant development analysed, starting from young seedlings to flowering plants (Figure 1b–d and data not shown). In the stamens, strong GUS activity was detected in sporogenous cells (Figure 1e). In the root strong staining was observed in the root tip (Figure 1f) and in the zones of secondary root outgrowth (Figure 1g).

To investigate CUL3 expression at the protein level, a 15 amino acid peptide was designed in a region of low conservation of CUL3A (Figure 1h). Antibodies were produced in rabbits and affinity-purified against the antigen. This serum specifically recognized the CUL3A protein, which is detected as two bands migrating close together (Figure 1). It is known that cullins are covalently modified by a small peptide called NEDD8 (RB1 in budding yeast) (Hori et al., 1999), and the two protein bands most probably correspond to the free and neddylated forms of the plant CUL3A. Consistent with the transcript and promoter-GUS fusion analyses, the CUL3A protein was detected in all the Arabidopsis organs analysed (Figure 1j).

CUL3A interacts with Arabidopsis RBX1 and different BTB-domain proteins

All mammalian cullin family members interact with the RING-H2 finger protein RBX1 (also called ROC1 or HRT1) (Ohta et al., 1999). RBX1 binds the E2 enzyme and is thought to allosterically activate it (Furukawa et al., 2002). Thus we first tested whether CUL3 proteins interact with the previously described Arabidopsis RBX1 (Lechner et al., 2002). CUL3A and CUL3B were used as bait in a yeast two-hybrid assay with RBX1. Both CUL3 proteins, as well as the positive control CUL1, interacted with RBX1 (Figure 2). We next determined whether the CUL3A or CUL3B are also able to interact with ASK1, the Arabidopsis protein most similar to SKP1. In contrast to CUL1, no interaction between ASK1 and the two CUL3 proteins was observed in the yeast two-hybrid assay (Figure 2), suggesting that CUL3 proteins are not involved in SCF protein complexes. This interpretation is further supported

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by the fact that CUL3A, in contrast to Arabidopsis CUL1, is unable to complement yeast cdc53 mutants (data not shown).

Only recently it has been found that CUL3 proteins from yeast and animals interact with various BTB-domain proteins and might thus form a novel class of cullin-based
finger. The biggest clade is defined by the protein family followed by a transcriptional adaptor putative zinc (TAZ) plant-specific combination, which consists of a BTB domain 26 (Pintard sequence-related to the MATH domain upstream of the BTB domain and are also predicted to have additional protein domains, either upstream or downstream of the BTB-domain (Table 1). Six of the predicted proteins contain two BTB domains (indicated as A and B after the AGI number), resulting in a total number of 78 BTB domains in 76 proteins in Arabidopsis. It is noteworthy that no major deviation was observed when the complete protein sequences were used to construct the tree (data not shown).

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ubiquitin protein-ligases (for review see Pintard et al., 2004). In the proposed model, the BTB-domain protein plays the function of a substrate-specific adaptor, which binds CUL3 through its BTB-domain and interacts with the substrate through an associated protein–protein interaction domain. In Arabidopsis, 76 annotated sequences were predicted to encode BTB-domain proteins in the smart, pfam interpro and tairn databases. Among them, At2g30600 and At2g04740 even contain two BTB domains. Most of the BTB proteins are also predicted to have additional protein domains, either upstream or downstream of the BTB-domain (Table 1 and Figure 4a). Some of them are known as protein interaction domains, such as the meprin and TRAF homology (MATH) domain, the Armadillo repeats (ARM) and the tetratricopeptide repeats (TPR). We performed a phylogenetic analysis using the BTB domains for the alignment (Figure 3). In addition, we also grouped the different proteins in several classes according to their predicted domain architecture (Figure 3 and Table 1). Six of the predicted proteins contain an MATH domain upstream of the BTB domain and are sequence-related to the C. elegans BTB domain protein MEL-26 (Pintard et al., 2003). The members of family II represent a plant-specific combination, which consists of a BTB domain followed by a transcriptional adaptor putative zinc (TAZ) finger. The biggest clade is defined by the protein family containing the plant-specific NPH3 (nonphototropic hypocotyl) domain and represents most of the Arabidopsis NPH3 domain proteins. NPH3 and RPT2, the founder members of this family, are involved in phototropic responses (Motchoulski and Liscum, 1999; Sakai et al., 2000). NPR1 is known for its role in systemic acquired resistance (Cao et al., 1997) and belongs to the class IV ankyrin repeat containing proteins. Other clades are formed by the ARM and by the third plant-specific family defined by the tetratricopeptide repeat, two motifs that are predicted to be involved in protein–protein interactions, and the Myb DNA-binding domain repeat signature. The potassium channel tetramerization domain, a child of the BTB domain, defines another subfamily. Both classification methods result in the formation of 11 major BTB domain protein clusters (Figure 3 and Table 1). Overall, our analyses suggest a co-evolution of the BTB domain with other associated protein domains, although there are a few exceptions, like the TPR protein At4g02680.

Representatives of seven Arabidopsis BTB protein classes were tested for interaction with the CUL3 proteins (Figure 4). Expression of the fusion proteins in yeast was verified by Western blot analysis (Figure 4b). CUL3A, as well as CUL3B, interacted with the MEL-26 like protein At2g39760. MEL-26 is thought to form a CUL3-based E3 (Pintard et al., 2003). Also

Table 1 The BTB domain containing proteins of Arabidopsis classified according to their protein domain architecture

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the ARM domain protein At5g13060 and At1g21780, representatives of class V and VII, showed an interaction specifically with both CUL3 proteins. These data indicate that CUL3A and CUL3B may form complexes with several, but not all, BTB domain proteins. Surprisingly CUL1, but not the CUL3 proteins, interacted with the small BTB domain protein.

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At1g01640. This result was rather unexpected as no BTB domain proteins are known to form protein complexes with CUL1. Additional experiments will tell us whether this interaction also exists in planta.

**Mutation of the CUL3A gene renders plant slightly late flowering and hyposensitive to far-red light**

To gain insights into the function of the CUL3A gene in plants, we searched for T-DNA insertion mutants. The cul3a-1 mutant was identified by PCR screening the INRA-Versailles T-DNA collection. A second mutant in the same gene was obtained from the Salk collection. In both mutants, T-DNAs are inserted in the coding region of the CUL3A gene (Figure 5a). In the cul3a-1 mutant, the T-DNA interrupts the cullin domain and thus is expected to be non-functional for CUL3A. Furthermore, although the cul3a-1 mutant line produces low abundance truncated transcripts (Figure 5b), no CUL3A protein band could be detected with the CUL3A-specific antibody (Figure 1d). In the cul3a-2 mutant, the T-DNA is inserted after the CUL1N domain. In this mutant, a CUL3A-truncated mRNA of slightly increased size was detected (Figure 5b).

Homozygous cul3a mutant plants were analysed under different growth conditions and at different developmental stages. The cul3a-1 mutant line exhibited a slightly late-flowering phenotype (Figure 5c,d). Mutant plants produced more leaves until the onset of flowering and they flowered with a delay of 2 days. This phenotype was also observed in the homozygous cul3a-2 line, although the phenotype was less severe (data not shown). To confirm that the mutation of CUL3A is responsible for the phenotype, genetic complementation was carried out. A genomic fragment, spanning a region from 2.2 kbp upstream of the ATG start to 220 bp downstream of the stop codon, was transformed into the cul3a-1 mutant. Twenty resistant plants were recovered and their progeny analysed. Partial to total rescue of the phenotype was observed in most of the lines (Figure 5d).

Timing of the transition from vegetative to reproductive development is under the control of various endogenous and exogenous factors (Koomen et al., 1998). Among the exogenous factors, light plays a central role. To analyse the effects of light on the mutant, fluence rate response curves for inhibition of hypocotyl elongation were measured under different light conditions (Figure 6a–c). The cul3a-1 mutant exhibited clearly reduced sensitivity to far-red light (Figure 6c,e). This reduced light sensitivity was specific to far-red light because it was not observed under red or blue light. The response towards far-red light is mediated by the photoreceptor phytochrome A. However, the promotion of root growth under continuous far-red light, another phytochrome A-dependent response, was not altered in the mutant (Figure 6d).

In contrast to the cul3a-1 mutant, hypocotyl elongation of the cul3a-2 line was not hyposensitive to far-red light (data not shown). This discrepancy might be a result of differences in light regulation between different ecotypes (WS for cul3a-1 and Col-0 for cul3a-2), because it is well known that phyA-dependent very low fluence responses are absent in Col-0 (Yanovsky et al., 1997). However, it is also possible that cul3a-2 is a weak mutant allele as the T-DNA is inserted at the end of the coding sequence. To prove that the hyposensitivity to far-red light is linked to the mutation, we measured the hypocotyl length in the cul3a-1 lines transformed with the genomic fragment covering the CUL3A gene. Partial to totally normal hypocotyl sensitivity to far-red light was restored in several of these lines (Figure 6f).
25S rRNA encodes an F-box protein (Dieterle et al., 2001), which may be involved in ubiquitin-dependent proteolysis. We thus investigated whether there is a genetic interaction between the spa1 and eid1 mutants, show enhanced phyA-specific light responses and are thus hyper-sensitive to far-red light. Interestingly, the EID1 gene encodes an F-box protein (Dieterle et al., 2001), which may be involved in ubiquitin-dependent proteinolysis. We thus investigated whether there is a genetic interaction between the spa1 and eid1 mutants. However, none of the mutations was epistatic to the other and the eid1-3 cul3a-1 double mutant exhibited an intermediary phenotype with respect to the hypocotyl elongation under different fluence rates of far-red light (Figure 6h). This suggests that the two genes act in independent pathways.

Mutations in CUL3A lead to alterations of gene expression patterns under continuous far-red light

To further study the role of CUL3a in far-red light-dependent signalling, microarray gene expression profiles were analysed for the cul3a-1 mutant and the corresponding wild type under selective far-red light conditions. We thought that our material is particularly appropriate for this approach, as the phenotype is rather weak, mainly affecting the hypocotyl, and is thus not expected to lead to massive differential gene expression. We used The Complete Arabidopsis Transcriptome MicroArray (CATMA) that contains 24 k genes of the Arabidopsis genome (http://www.catma.org). The genome expression profiles were compared in 4-day-old cul3-1 and wild-type WS seedlings irradiated with continuous weak far-red light. Fluorescent cDNA probes were synthesized from total RNA harvested from three independent biological samples of wild-type and mutant seedlings in two separate experiments permitting two independent pair-wise comparisons of mutant and wild-type seedlings. Based on the statistical test (see Experimental procedures) 1051 genes were found differentially expressed between cul3a-1 mutant and the wild-type plants (P < 0.05). The complete set of microarray data from these experiments has been submitted to ArrayExpress (Accession number E-MEXP-175) and the list of the 1051 differentially regulated genes is available in Supplementary Material. Ninety-five genes were upregulated and 271 genes were downregulated with a twofold or greater differential expression in the cul3a-1 mutant compared with the WS seedling (Supplementary Material). Among those genes, several encode components of the ubiquitin 26S proteasome pathway (Table 2). Interestingly, among the upregulated genes, we identified COP1. COP1 is a RING-finger protein, which has been proposed to function as an E3 ubiquitin ligase, that targets different transcription factors positively regulating photomorphogenesis (Holm et al., 2002; Osterlund et al., 2000) including LAF1, which is specific to far-red light (Seo et al., 2003). Consistent with
the overexpression of COP1, several genes, which may be involved in photomorphogenesis, were found to undergo a twofold or more differential downregulation in the mutant (Table 2). These genes include photosystem I reaction centre subunits (At1g31330 and At5g64040) and several components of the light-harvesting chlorophyll a/b-protein complexes (At5g54270, At1g61520, At1g19150 and At3g61470). Two genes, COP1 (At2g32950) and a light-harvesting chlorophyll a/b-binding protein (At1g19150), differentially expressed between the mutant versus wild type, according to the array data, were verified by real-time PCR. Both displayed delta CT over 1 and –1 thus confirming the up- and down-regulation of the two genes in the mutant (Table 3).

Figure 6. cul3a mutation reduces the responsiveness of hypocotyls to far-red light.

(a–c) Hypocotyl length of wild type and cul3a-1 grown under continuous blue light (a), red light (b) and far-red light (c) of various fluence rates.

(d) Root length of wild type and cul3a-1 mutant seedlings grown under continuous far-red light of various fluence rates.

(e) Typical phenotypes of wild type and cul3a-1 mutant seedlings grown under far-red light of various fluence rates.

(f) Hypocotyl length of wild type, cul3a-1 mutant seedlings and cul3a-1 lines transformed with a CUL3A genomic fragment grown in 0.4 μmol m^{-2} sec^{-1} far-red light.

(g) Western blot of PHYA protein extracted from wild type and mutant seedlings after 3 days of far-red light irradiation. Numbers refer to the far-red light intensity used for the irradiation (see Figure 6c).

(h) Fluence rate response curves for the inhibition of hypocotyl growth in continuous far-red light for wild type, cul3a-1, eid1-3 and cul3a-1 eid1-3 double mutant seedlings.

All measurements were performed 3 days after the induction of germination. Each point represents the mean ± standard deviation. Each measurement was repeated at least two times.

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*a Log I: log2 average signal intensity, the range is from 5 to 16.

*b Log Rat: log2 ratio, a positive ratio indicates that the gene is overexpressed in the mutant, whereas a negative ratio indicates that the gene is underexpressed in the mutant compared with the wild type.

*c Bonferroni-corrected.
Table 3 Quantitative RT-PCR

<table>
<thead>
<tr>
<th>ID AGI</th>
<th>TIGR function</th>
<th>Q-RT-PCR</th>
<th>Microarray</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g04550</td>
<td>IAA12</td>
<td>32.65</td>
<td>31.70</td>
</tr>
<tr>
<td>At1g30230</td>
<td>Elongation factor-1</td>
<td>26.20</td>
<td>27.10</td>
</tr>
<tr>
<td>At1g19150</td>
<td>Light-harvesting chlorophyll a/b binding protein</td>
<td>35.15</td>
<td>37.2</td>
</tr>
<tr>
<td>At2g32950</td>
<td>COP1</td>
<td>32.30</td>
<td>29.95</td>
</tr>
</tbody>
</table>

\( ^a CT, \) competitive templates.
\( ^b \)Norm \( \Delta CT = -(CT \text{ WT} - CT \text{ cul3a})/(\Delta CT \text{ EF1} + \Delta CT \text{ IAA}/2). \)

**Discussion**

Recent findings have identified CUL3 from *C. elegans* and its homologue Pcu3 from fission yeast as part of a novel class of cullin-based E3 ligases (reviewed in Pintard et al., 2004). In such complexes, the CUL3 proteins physically interact with BTB-domain-containing proteins that are supposed to bridge the cullin to the substrates for subsequent ubiquitylation. Thus, the MEI1 protein from *C. elegans*, which acts to maintain short microtubules during meiosis, is recruited for CUL3-dependent ubiquitylation by the BTB-domain adaptor protein MEL26 (Furukawa et al., 2003; Pintard et al., 2003). Moreover, the BTB-domain proteins might themselves be substrates of CUL3-dependent ubiquitylation and degradation (Geyer et al., 2003; Wilkins et al., 2004).

We explored the function of CUL3 in the model plant *A. thaliana*. In contrast to the other organisms for which genomic sequences are available, the Arabidopsis genome encodes two CUL3 proteins. The present work focuses on one of them, CUL3A. This gene is expressed in seedlings, as well as in various organs of the adult plants, suggesting that it is involved in basic cellular functions.

Whereas fission yeast only possesses 3 BTB-domain proteins, 105, 141 and 208, such proteins have been predicted in *C. elegans*, *D. melanogaster* and human respectively (Geyer et al., 2003). Using database searches 76 BTB-domain proteins were identified in Arabidopsis. These genes include NPR1, which is an essential regulator of plant systemic acquired resistance (Cao et al., 1997), and RPT2 (Sakai et al., 2000) and NPH3 (Motchoulski and Liscum, 1999), which are involved in the regulation of phototropism response. However, it has not yet been determined whether these BTB proteins can interact with CUL3 proteins. We grouped the different plant BTB-domain proteins into 11 families, based on phylogenetic analyses and on the protein motif(s) associated with the BTB-domain. At least in yeast two hybrid assays, we could demonstrate that both Arabidopsis CUL3 proteins interact with three of the seven BTB-domain proteins tested: those containing an MATH domain (class I), an ARM domain (class V), or a short N-terminal extension of unknown function (class VII). However, no specificity of interaction between the BTB domain proteins and the two CUL3 proteins was detected. In addition, it was recently found that ETO1, an Arabidopsis protein containing a BTB-domain and six TPR motifs (belonging to class VIII), is also able to physically interact with CUL3A (Wang et al., 2004). Moreover, ETO1 also interacts with ACS5, which is a rate-limiting enzyme in ethylene biosynthesis and might thus be involved in its turnover (Wang et al., 2004). Thus, the interaction of CUL3A and CUL3B with several BTB-domain protein family members suggests the existence of multiple CUL3-based E3s in plants, whose functions and substrates now await elucidation.

The lack of interaction with BTB-domain proteins of the other families that have been tested (classes II, III, IV and VI) may simply reflect the limits of the yeast two-hybrid system. Alternatively, the BTB domain itself may be used in a CUL3-independent context as a protein interaction and dimerization motif.

In contrast to budding yeast (Michel et al., 2003) and fission yeast (Geyer et al., 2003), CUL3 function seems essential in higher eukaryotes. Thus, CUL3 knockout in mouse caused an embryonic lethal phenotype (Singer et al., 1999), whereas in *C. elegans*, loss of CUL3 leads to a failure of cytokinesis in single-cell embryos (Kurz et al., 2002). The Arabidopsis genome encodes two related CUL3 genes and it remains to be determined whether cul3a and cul3b double loss-of-function mutant plants can be obtained. However, disruption of the CUL3A gene does not affect plant viability or fertility. Nevertheless, we found that this mutation in Arabidopsis (ecotype WS) affects the response of hypocotyl growth to specific light conditions, resulting in hyposensitivity to far-red light. In Arabidopsis, phyA is the primary photoreceptor that perceives and mediates responses to far-red light. The ubiquitin pathway plays a function in phyA-dependent signalling (reviewed in Wang and Deng, 2003). In particular, two F-box proteins are involved in this process. The F-box protein EID1 is a negative regulator of phyA-
mediated responses and its mutation leads to enhanced phyA-dependant responses (Dieterle et al., 2001). In contrast to EID1, the F-box protein AFR1 promotes phyA-mediated responses (Harmon and Kay, 2003). Although EID1 and AFR1 both interact with ASK1 and are believed to form CUL1-based E3s, their protein targets have not yet been identified. Our work suggests that a CUL3-based E3 is also involved in this process and that its activity is required for normal phyA-mediated responses. Overexpression of COP1 in Arabidopsis is known to result in partial suppression of light-mediated development (McNellis et al., 1994). Thus, higher COP1 expression may account for the cul3a-1 mutant phenotype. Whether COP1 overexpression is a direct or an indirect consequence of CUL3A mutation is unknown. It is noteworthy however, that other factors also impinge on the response to light, such as the phytohormones (reviewed in Nemhauser and Chory, 2002). A major challenge for the future will now be the identification of the signalling pathways involving CUL3-based E3s.

Experimental procedures

Plant material and transgenic plants

The cul3a-1 insertion line was identified by PCR screening of 37 000 independent transgenic lines from the INRA-Versailles T-DNA collection (Bechtold et al., 1993; Bouchez et al., 1993, ecotype WS). The cul3a-2 line was identified in the SIGNAL T-DNA collection (Alonso et al., 2003; SALK 050756, ecotype Col). Arabidopsis plants were transformed by floral dip as described by Clough and Bent (1998).

For complementation of the cul3a-1 mutant the CUL3A gene was amplified from genomic DNA by using the primers Cul3a5'-'GGGGTACCGAGAGACGTGACGTGTGATTGGTA-3' and Cul3a3 5'-'ATGGGCTTAAAAAGCCCTCGAGC-3' and cloned into a modified pBIN vector containing the hygromycin selection marker. For promoter-GUS analysis, 2.2 kbp of the CUL3A promoter, including the 5'-UTR sequence, was cloned into pBGWFS7 (Karimi et al., 2002). More than 20 transgenic Arabidopsis lines were obtained and analysed in T2 and T3 generations.

Plant growth and phenotypic analysis

For light experiments, seeds were sown on four layers of Schleicher & Schüll 595 filter paper circles (Schleicher & Schüll, Dassel, Germany), which were placed in Greiner 94/16 Petri dishes (Greiner, Kremsmünster, Austria) supplemented with 5 ml distilled water. The standard sowing procedure was followed by 2 days cold treatment at 8°C in the dark and 1 day of red light induction of germination at 25°C before onset of different light treatments for 3 days. Induction of germination was performed with a standard light field \(I_{\text{max}} = 650 \text{ nmol m}^{-2} \text{ sec}^{-1}\); Heim and Schäfer, 1982). For other light treatments modified Leitz Prado 500-W universal projectors (Leitz, Wetzlar, Germany) were used together with Osram Xenophot longlife lamps (Osram, Berlin, Germany). Red light was obtained by passing the light beam through KG65 filters \(I_{\text{max}} = 650 \text{ nmol m}^{-2} \text{ sec}^{-1}\); Balzers, Liechtenstein). Far-red light treatments were performed with 716-nm DAL interference filters (Schott, Mainz, Germany).

To screen for double mutants, the F2 of crosses between efd1-3 and cul3a-1 were pre-selected for an increased light sensitivity under continuous far-red light (Büche et al., 2000; Dieterle et al., 2003). The genotype of hypersensitive plants was further analysed using T-DNA-specific oligonucleotides and an efd1-3-dCAPS marker (Zhou et al., 2002). Three independent double mutant lines were used for further analyses.

Hypocotyl length was measured manually with a ruler. All data represent the mean of at least 40 seedlings analysed in at least two independent experiments. For microarray analysis, seedlings were irradiated with 0.4 \(\mu\text{mol m}^{-2} \text{ sec}^{-1}\) far-red light.

For flowering time determination and GUS assay, seeds were sown on soil and incubated for 2 days at 8°C. Afterwards they were transferred to a growth chamber with a 12 h light/12 h dark cycle (20/16°C). Flowering time was recorded as the number of days and the number of rosette leaves formed from the time when the seeds were transferred to light until the opening of the first flower bud. The experiment was repeated three times using six to 12 plants from each genotype in each experiment.

Histochemical analysis

Seedlings or soil-grown plants were used for GUS assays. The method for histochemical assays for GUS activity is described in Capron et al. (2003).

RNA isolation and Northern blot analysis

RNA was extracted from plant material using the Trizol reagent (Invitrogen, Paisley, UK) for Northern analysis or RNeasy Plant Mini Kits (Qiagen, Hilden, Germany) for Microarray experiments. RNA gel blot analysis was performed with 20 \(\mu\text{g}\) of total RNA per lane. Northern blot procedure is described in Genschik et al. (1998). \(^{32}\text{P}

labelled probes were synthesized with the Prime-a-Gene random prime labelling kit (Promega Corporation, Madison, WI, USA) using a 900-bp CUL3A cDNA fragment amplified with two gene-specific primers \(5'\text{-ATGGATTTGGGTGAATCTGT-3'}\) and \(5'\text{-CTCGGGGTGACTGCCATA-3'}\).

Transcriptome studies

The microarray analysis was performed with the CATMA array containing 24576 gene-specific tags from \(A.\ thaliana\) (Crowe et al., 2003; Hilson et al., in press). The GST amplicons were purified on multiscreen plates (Millipore, Bedford, MA, USA) and resuspended in TE-DMSO at 100 ng \(\mu\text{l}^{-1}\). The purified probes were transferred to 1536-well plates with a Genesis workstation (TECAN, Männedorf, Switzerland) and spotted on UltraGAPS slides (Corning, New York, NY, USA) using a Microgrid II (Genomic Solution, Huntington, UK). The current CATMA version printed at the URGV consists of three metabolics, each composed of 64 blocks of 144 spots. A block is a set of spots printed with the same print-tip. In these arrays, a print-tip is used three times to print a block in each metabolite. For the transcriptome studies, whole seedlings were collected at stage 1.0 according to Boyes et al. (2001) from \(A.\ thaliana\) WS and cul3a-1 seedlings irradiated with weak far-red light. RNA from three independent biological repetitions were pooled and analysed on two different dye swaps. RNA integrity was checked with the Bioanalyser from Agilent (Waldbronn, Germany). cRNAs were produced from 2 \(\mu\text{g}\) of total RNA from each pool with the ‘Message Amp aRNA’ kit (Ambion, Austin, TX, USA). Then 5 \(\mu\text{g}\) of cRNAs was reverse transcribed in the presence of 200 U of SuperScript II
Statistical analysis of microarray data

The statistical analysis was based on two dye-swaps, that is four arrays each containing the 24576 GSTs and 384 controls. The controls were used for assessing the quality of the hybridizations but were not included in the statistical tests. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelength 635 nm (red) and 532 nm (green). No background was subtracted. In the following description, log-ratio refers to the differential expression between the mutant and the control. It is either log₂(red/green) or log₂(green/red) according to the experimental design. An array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered badly formed features by the experimenter. Then we performed a global intensity-dependent normalization using the loess procedure (Yang et al., 2002) to correct the dye bias. Finally, on each block, the log-ratio median is subtracted from each value of the log-ratio of the block to correct any print-tip effect on each metablock. To determine differentially expressed genes, we performed a paired t-test on the log-ratios. The number of observations per spot varied between two and six and is inadequate for calculating a gene-specific variance. For this reason we assume that the variance of the log-ratios is the same for all genes. The raw P-values were adjusted by the Bonferroni method, which controls the family wise error rate.

Quantitative RT-PCR

Primers for RT-PCR were selected using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi, optimal length 21nt, optimal Tm 60°C). BLASTN and Electronic PCR (ftp://ncbi.nlm.nih.gov/pub/schuler/e-PCR/) was used to predict whether the primer pairs amplified a single product of the correct size from the Arabidopsis genome. All primers used for standard RT-PCR and for quantitative PCR were capable of amplifying a unique product. All primer pairs used for quantitative PCR were first tested on a dilution series of genomic DNA (5, 0.5, 0.05, 0.005 ng) to generate a standard curve and assess their PCR efficiency. The primer pairs used following this validation all had an efficiency between 90 and 99%.

Reverse transcription was performed on 500 ng of WT and cu3A mutant total RNA using an oligo(dT) primer (18mer) and the Superscript II reverse transcriptase (Invitrogen), for 1 h at 42°C. The enzyme was then heat-inactivated at 65°C and the samples were treated with RNase H. Quantitative PCR was performed in 15 µl, using 1 µl of RT reaction, 900 nm final concentration of each primer pair and SYBR Green PCR master mix 2X (Eurogentec, Seraing, Belgium). Corresponding minus RT controls were performed with each primer pairs. Conditions were as follows: 95°C 10 min, 40× (95°C 15 sec, 60°C 1 min) and a dissociation step that allows to discriminate between primer dimers and PCR product. All reactions were performed in duplicate with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and data were analysed with the SDS software provided by the manufacturer. The amplified DNA was also visualized on 2.0% agarose gel stained with ethidium bromide, to confirm the PCR product size.

Two primer pairs corresponding to genes not found differentially expressed in the array data were used to normalize the Q-RT-PCR data: EF1 and IAA12. Then normalized ΔCt were calculated as follows: Norm ΔCt = -(ΔCt WT−ΔCt cu3A)−(ΔCt EF1 + ΔCt IAA12)/2.

Antibodies and immunoblotting

A peptide of 16 amino acids located in the N-terminal domain of the CUL3A protein [TWQLERAIHQIYNQ-(C), where C indicates an extra cysteine] was synthesized, linked to KLH carrier protein, and used to immunize rabbits. The antisera was immunoadfinity purified against the same peptide bound to Sepharose matrix. The phyA antibody is directed against an N-terminal 555 amino acid fragment of phyA and was kindly provided by Daniel Kirchenbauer (University of Freiburg, Germany). A denaturing buffer was used to extract proteins from ground plant material (Büche et al., 2000). For immunodetection 20 µg of total protein extracts was separated by SDS gels and blotted onto Immobilon-P membrane (Millipore). For detection of the GAL4-AD-fusion proteins a monoclonal antibody directed against the HA epitope tag was purchased from Sigma (Sigma-Aldrich, Saint Louis, MI, USA) (clone HA-7).

Interaction assay

The coding region of CUL3A and BTB domain proteins was amplified from RIKEN full-length cDNA clone (Seki et al., 2002), pUNI clones (Yamada et al., 2003) or from cDNA with gene-specific primers. The CUL3B cDNA was kindly provided by H. Hellmann. The PCR products were cloned via a BP clonase reaction in pDONR207 and/or pDONR201 (Invitrogen) to obtain the different pENTR vectors. The gateway cassette (Invitrogen) was inserted in the Smal site of vectors pGBK7, pGBT9 and pGAD77 and the pENTR clones were recombined with these vectors. The RBX1 and CULLIN1 clones are described in Lechner et al. (2002) and Shen et al. (2002). Yeast two-hybrid assay and preparation of yeast protein extracts (TCA method) were performed according to the ‘Yeast manual handbook’ (BD Biosciences Clontech, Palo Alto, CA, USA).

Prediction of BTB domain proteins, alignments and phylogenetic analysis

Arabidopsis proteins predicted to carry BTB domains were extracted by using InterPro, SMART, Pfam and ‘The Arabidopsis Information Resource’ databases (http://www.arabidopsis.org/index.jsp). Seventy-six non-redundant sequences were identified. Two of the predicted proteins contain two BTB domains. All Arabidopsis BTB domain protein sequences were downloaded on the NCBI server, in the refseq databank (http://www.ncbi.nlm.nih.gov/RefSeq/). The full-length proteins were aligned using Clustal X 1.83. The conserved core sequences corresponding to the BTB domain were retrieved for the phylogenetic analysis. Phylogenetic analysis was performed with Phylop (http://bioweb.pasteur.fr/seqanal/phylogeny/phylop-uk.html). Three methods with bootstrap (100 replications) were used: Fitch-Margoliash least-square distance method, neighbour-joining method and Parsimony method. The phylogenetic tree was drawn with the R package: ape_1.2.3 at http://cran.r-project.org/src/contrib/Descriptions/ape.html (Paradis et al., 2004). Additional protein domains were identified using the Interpro database (http://www.ebi.ac.uk/InterProScan/).
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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2302/TPJ2302sm.htm

Complete list of differentially expressed genes between mutant and wild-type seedlings under far-red irradiation.

References


Characterization of Arabidopsis Cullin 3A


