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# COI1 links jasmonate signalling and fertility to the SCF ubiquitin–ligase complex in *Arabidopsis*

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**Summary**

**Jasmonates (JAs) regulate *Arabidopsis thaliana* wound and defence responses, pollen development, and stress-related growth inhibition. Significantly, each of these responses requires COI1, an F-box protein. Other F-box proteins interact with SKP1 and cullin proteins to form SCF complexes that selectively recruit regulatory proteins targeted for ubiquitination. To determine whether COI1 also functions in an SCF complex, we have characterized *Arabidopsis* proteins that bind to COI1. An *Arabidopsis* cDNA expression library was screened in yeast for clones that produce proteins which can bind to COI1. We recovered two SKP1 homologues and a histone deacetylase. The *Arabidopsis* F-box protein TIR1 interacted with SKP1 proteins, but not with the histone deacetylase. Mutant COI1 proteins revealed that the F-box is required for interaction with SKP1s, but that sequences in leucine-rich repeat domains are required for interaction with the histone deacetylase. Epitope-tagged COI1 was introduced into *Arabidopsis* plants and cell cultures. Co-immunoprecipitation experiments confirmed the interaction *in planta* of COI1 with SKP1-like proteins and histone deacetylase, and also indicated that COI1 interacted with cullin. These results suggest that COI1 forms an $SCF^{COI1}$ complex *in vivo*. COI1 is therefore expected to form a functional E3-type ubiquitin ligase in plants and to regulate expression of jasmonate responsive genes, possibly by targeted ubiquitination of a histone deacetylase.**

**Keywords: *Arabidopsis*, COI1, jasmonate, histone deacetylase, proteasome, ubiquitination**

## Introduction

Jasmonates (JAs) are cyclopentanone derivatives synthesized from linolenic acid via the octadecanoic pathway. They inhibit plant growth generally, but in addition they promote diverse processes including fruit ripening, senescence, tuber formation, tendril coiling, pollen formation, and defence responses against insect pests and pathogens (reviewed in Creelman and Mullet, 1997). In *Arabidopsis thaliana*, JAs inhibit root elongation (Staswick *et al.*, 1992), and are required for pollen development, anther dehiscence (Feys *et al.*, 1994; McConn and Browse, 1996), and defence against insects (McConn *et al.*, 1997) and necrotrophic pathogens (Staswick *et al.*, 1997; Thomma *et al.*, 1998).

The *coi1*-1 mutant was isolated in a screen for *Arabidopsis* mutants insensitive to growth inhibition by the bacterial toxin coronatine, which is structurally related to jasmonic acid (Feys *et al.*, 1994) and 12-oxo-phytodienoic acid (OPDA) (Weiler *et al.*, 1994). The *coi1* mutants are also unresponsive to growth inhibition by MeJA, are male-sterile, fail to express JA-regulated genes such as *VSP*s (Benedetti *et al.*, 1995), *Thi2.1* (Bohlmann *et al.*, 1998), and *plant defensin 1.2* (*PDF1.2*, Penninckx *et al.*, 1998), and are susceptible to insect herbivory and to pathogens (McConn *et al.*, 1997; Thomma *et al.*, 1998). *coi1*-16 is a recently cloned allele that was isolated in a screen for failure to activate the *VSPA* promoter (Ellis and Turner, 2002). *coi1*-16 exhibits fertility in a temperature-sensitive manner.

The *COI1* gene encodes a 67 kDa protein containing 16 leucine-rich repeats (LRRs) and an N-terminal F-box motif (Xie *et al.*, 1998). F-box proteins occur in the eukaryote kingdom in organisms from yeast to man, and function as receptors that recruit regulatory proteins as substrates for ubiquitin-mediated destruction in the proteasome. F-box

proteins associate with SKP1, cullin and Rbx proteins to form an E3 ubiquitin ligase known as the SCF complex (Bai *et al.*, 1996; Deshaies, 1999).

In *Arabidopsis*, there are at least 10 cullin proteins (Bachmair *et al.*, 2001), 19 Skp1-like proteins, or ASKs (Farrás *et al.*, 2001), and 337 F-box proteins (*Arabidopsis* Genome Initiative, 2000). The F-box proteins provide specificity to the E3 complex. Plant F-box proteins with known function include TIR1, involved in auxin response (Ruegger *et al.*, 1998), UFO/FIM, required for floral development (Ingram *et al.*, 1997), COI1, required for jasmonate response (Xie *et al.*, 1998), and FKF and ZTL, both involved in the control of circadian rhythms (Nelson *et al.*, 2000; Somers *et al.*, 2000). Database searches have identified a large number of F-box proteins in *Arabidopsis*, most of which have unknown functions (*Arabidopsis* Genome Initiative, 2000; Xiao and Jang, 2000).

The yeast and human *SKP1* genes have been found to regulate the mitotic cell cycle (Hoyt, 1997). In *Arabidopsis*, an *SKP1* homologue, *ASK1* has been shown to be highly expressed in dividing cells, such as those in meristems and organ primordia (Porat *et al.*, 1998). A male-sterile mutant *ask1*-1, has been isolated and found to be defective in male meiosis (Yang *et al.*, 1999). Failure to separate homologous chromosomes during male anaphase I has been interpreted as the ability of the ASK1 protein to regulate the degradation and/or modification of a protein required for homologue association.

In yeast two-hybrid screens, F-box proteins interact preferentially with certain SKP1 proteins (Gray *et al.*, 1999; Samach *et al.*, 1999). However, it is not known whether F-box proteins interact with only one SKP1 and cullin protein *in vivo* or with numerous SKP1 and cullin proteins to form a combinatorial variety of SCF complexes. The structure of the Cul1–Rbx–Skp1–F-box$^{Skp2}$ SCF ubiquitin ligase complex has been recently determined (Zheng *et al.*, 2002). Crystallographic data of complexes of human SKP1 with the F-box protein SKP2 suggested that divergent C-terminal sequences of SKP1 proteins may allow preferential binding of subsets of F-box proteins (Schulman *et al.*, 2000). ASK1 has been shown to interact with TIR1 (Gray *et al.*, 1999), UFO (Samach *et al.*, 1999), ORE9 (Woo *et al.*, 2001) and EID1 (Dieterle *et al.*, 2001). Similarly, each of these proteins binds to multiple ASKs (Dieterle *et al.*, 2001; Farrás *et al.*, 2001; Gray *et al.*, 1999; Woo *et al.*, 2001; Zhao *et al.*, 1999). Apparently, ASK1 can interact with several F-box proteins that regulate different plant processes, indicating the possibility of a higher level of cellular control through the combinatorial assembly of SCF complexes. The large number of potential SCF complexes and the diversity of the pathways they affect suggest that the regulation of protein turnover is a control element common to many plant processes (Ellis, Turner and Devoto, 2002). An example of how F-box proteins may regulate defences is revealed by the F-box proteins βTrCP1 and βTrCP2, which regulate NF-κB activity in man. NF-κB is an inducible transcription factor involved in immune, inflammatory, stress and developmental processes. NF-κB is activated following removal by an SCF complex, of ubiquitinated plκBα, in the proteasome (Suzuki *et al.*, 1999; Yaron *et al.*, 1998). In *Arabidopsis*, the proteins AXRZ/IAAZ and AXR3/IAA17 have been identified as substrates for SCF$^{TIR1}$-dependent ubiquitination (Gray *et al.*, 2001).

To address whether COI1 also forms part of an SCF complex, we used a yeast two-hybrid screen and immuno-assay of plant extracts for proteins that co-immunoprecipitate with an intron–epitope-tagged COI1. We show here that COI1 interacts with SKP1 proteins and cullin, indicating the occurrence of an SCF$^{COI1}$ complex in *Arabidopsis*, and present evidence that COI1 also binds a histone deacetylase that regulates some JA-dependent processes in *Arabidopsis*.

## Results

### *COI1 interacts with components of the SCF complex, with a repressor of transcription and with a small subunit of Rubisco in yeast*

An *Arabidopsis* two-hybrid cDNA library in pB42AD (van der Biezen *et al.*, 2000) was introduced into yeast with pLexA-*COI1*, and $5 \times 10^6$ primary transformants were screened for clones in which there was interaction between the fusion proteins. We identified 137 colonies that expressed β-galactosidase activity and were prototrophic for leucine. The corresponding cDNAs were amplified by PCR and the products were digested with *Rsa*I. Eight distinctive digestion band profiles were identified. Representative cDNAs displaying the four most abundant digestion band profiles, named COI1-interacting protein 1 (*CIP1*) to *CIP4*, were sequenced and analysed further. *CIP1* (represented by 49 clones) was the *SKP1* homologue *ASK1* (ATU60981), which codes for a protein of 160 amino acids and a predicted molecular weight of 17.8 kDa. *CIP2* (represented by 13 clones) encoded the *SKP1* homologue *ASK2* (AF059295) and the predicted protein had 171 amino acids and a molecular weight of 19.1 kDa. *CIP3* (represented by 27 clones) corresponded to the *Arabidopsis* histone deacetylase RPD3b (AB008265; Wu *et al.*, 2000; Murfett *et al.*, 2001), encoding a protein of 471 amino acids with a predicted molecular weight of 52.6 kDa. *CIP4* (represented by nine clones) encoded the small subunit chain 1b of ribulose bisphosphate carboxylase (AB005248), a protein of 181 amino acids and a molecular weight of approximately 20.3 kDa. ASK1 and ASK2 also interact with the *Arabidopsis* F-box proteins TIR1 and UFO (Gray *et al.*, 1999; Samach *et al.*, 1999). Alignment of the amino acid sequences of CIP1 and 2 and their homologues in yeast and man revealed

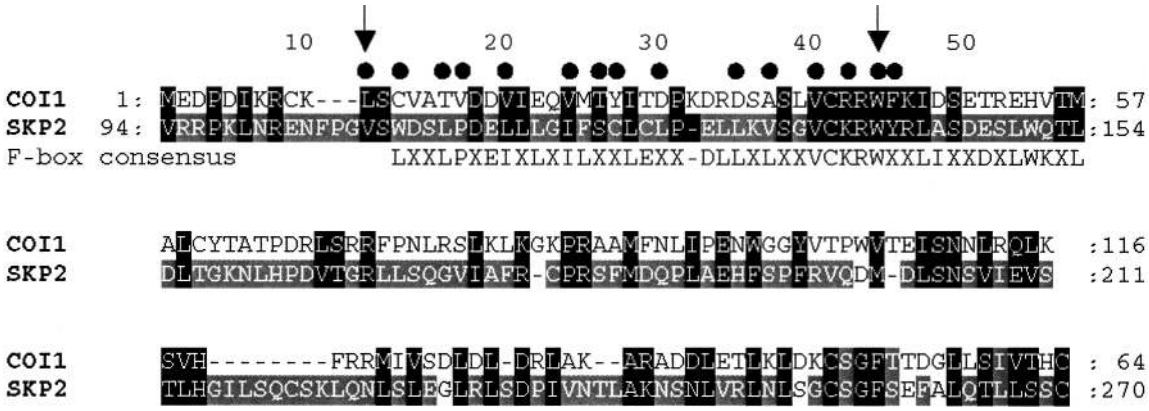


**Figure 1.** Conservation of amino acid contacts required by COI1 to bind SKP1-like proteins.
Multiple alignment of the N-terminal regions of the human SKP2 and *Arabidopsis* COI1. Black dots indicate residues that contact SKP1. Arrows indicate COI1 amino acids subjected to mutagenesis. Identity is indicated in black and decreasing similarity by lighter shades of grey. Positions are relative to the COI1 full-length protein. The alignment was generated using Clustal W and shaded using BoxShade (see Experimental procedures).

conservation of residues required for interaction with the F-box (data not shown, Schulman *et al.*, 2000). We also compared the amino acid sequences of the *Arabidopsis* COI1 and the human SKP2 and found conservation of the amino acid contacts previously identified as essential for SKP2 to bind SKP1 (Figure 1 and Schulman *et al.*, 2000).

### *Integrity of the COI1 F-box, leucine-rich repeats and C-terminus is required for and differentially affects the interaction with CIPs in yeast*

To investigate the role of the F-box domain (amino acids 11–56) of COI1 in binding CIPs, we deleted sequences coding for the F-box of the LexA::COI1 fusion protein, obtaining pΔFbox. Also, in the fusion protein LexA::COI1, we mutated the conserved amino acids in the COI1 F-box sequence, Leu11 (pCOI1$_{L11A}$) and Trp44 (pCOI1$_{W44A}$) to alanine (Figure 2a). The constructs were introduced into yeast and the interactions of the mutant fusion proteins with CIP1–4 were determined in the yeast two-hybrid assay. The mutant proteins LexA::ΔFbox and LexA::COI1$_{W44A}$ did not interact with CIP1–4 in yeast (Figure 2b) indicating that W44 is required for interaction with CIP proteins. However, the mutant protein LexA::COI1$_{L11A}$ interacted with CIP1–4 (Figure 2b), indicating that L11 is not required for interaction with the CIP proteins.

The mutant *coi1*-1 encodes the protein COI1-1 (p3′Δ), in which the two C-terminal leucine-rich repeats (amino acids 479–592) are deleted (Xie *et al.*, 1998). The mutant *coi1*-16 encodes a protein (COI1$_{L245F}$), in which Leu245 in the sixth leucine-rich repeat is replaced by a phenylalanine (Ellis and Turner, 2002). These mutant *coi1* alleles were obtained from the plasmid pLexA-COI1 and tested for interaction with CIP1–4 in the yeast two-hybrid assay. Figure 2(b) shows that COI1-1 (p3′Δ) did not interact with CIP1–4, and that COI1$_{L245F}$ interacted with CIP1 and 2, but not with CIP3 and 4. Immunoblots of yeast extracts probed with αLexA monoclonal antibody indicated that the mutant proteins were expressed in yeast and were stable (data not shown).

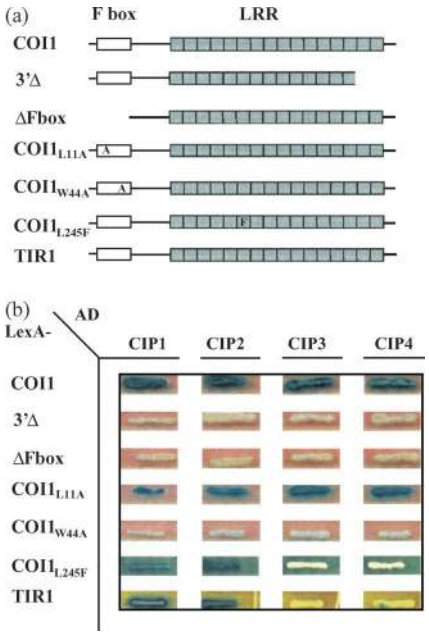


**Figure 2.** Analysis of the interaction between COI1 and CIPs in yeast.
(a) Schematic representation of the domain structure of the *Arabidopsis* COI1, derived deletion and point mutants, and TIR1. The F-box (white box) and the LRR domains (grey boxes) are indicated. Numbers refer to amino acid position in the full-length protein. Letters in the corresponding boxes show the position of the mutations L11A, W44A, and L245F. 3′Δ: 3′ deletion mutant corresponding to the *coi1*-1 mutant. (b) Two-hybrid interactions between COI1 and CIPs in comparison to TIR1. Full-length wild-type or mutant proteins of COI1 were tested for specific interaction. Positive interactions are defined by activation of the LacZ reporter gene, in blue. LexA, LexA fusion proteins; AD, fusion proteins with the activator domain).

**Table 1** β-galactosidase activity measured for *S. cerevisiae* EGY48 independently transformed with pLexA-COI1 or pLexA-TIR1 and the pB42AD-CIPs

| | pLexA-COI1 | | pLexA-TIR1 | |
|---|---|---|---|---|
| | Galactose | Glucose | Galactose | Glucose |
| pB42AD-CIP1 | 8.24 ± 3.90 | 0.55 ± 0.48 | 2.38 ± 0.72 | 0.60 ± 0.22 |
| pB42AD-CIP2 | 3.73 ± 1.55 | 0.14 ± 0.17 | 6.02 ± 3.12 | 0.74 ± 0.21 |
| pB42AD-CIP3 | 2.77 ± 1.19 | 0.43 ± 0.29 | 0.69 ± 1.17 | 0.24 ± 0.29 |
| pB42AD-CIP4 | 16.94 ± 5.87 | 0.27 ± 0.20 | 0.17 ± 0.14 | 0.13 ± 0.12 |

The *Arabidopsis* F-box protein TIR1 (Ruegger *et al.*, 1998) has 33.3% identity to the amino acid sequence of COI1 (Xie *et al.*, 1998), and interacts with ASK1 and ASK2 (Gray *et al.*, 1999). We confirmed the interaction between LexA::TIR1 and CIP1 (ASK1) and CIP2 (ASK2) in yeast (Figure 2b). *CIP3* and *4*, encoding a histone deacetylase and a small subunit of Rubisco, respectively, did not interact with LexA::TIR1 (Figure 2b), suggesting specificity of the interaction between COI1 and CIP3 and 4. To compare the strength of interaction between COI1 and CIP1–4 and between TIR1 and CIP1–4, we measured β-galactosidase activity (Table 1). COI1 interacted with CIP1 more strongly than did TIR1, but TIR1 interacted more strongly with CIP2 than did COI1. Significantly, COI1 but not TIR1, interacted with CIP3 and 4.

*Interaction between COI1 and SKP1-like proteins, cullin and a histone deacetylase,* in planta

We tested for interaction between COI1 and other proteins *in planta*. For this, wild-type *COI1* was fused to an N- and a C-terminal haemagglutinin (HA) (HiA) intron–epitope tag (Ferrando *et al.*, 2000), and the fusions were expressed from the CaMV 35S promoter. The constructs 35S::HiA::COI1 and 35S::COI1::HiA were introduced into *Arabidopsis* cell cultures and plants by transient and stable transformation. To test whether the fusions were functional, three independent stable transgenic lines containing the 35S::HiA::COI1 and three containing the 35S::COI1::HiA constructs were crossed to a *coi1*-1 mutant, and the F2 seeds were collected. All F2 progeny resistant to kanamycin were fertile

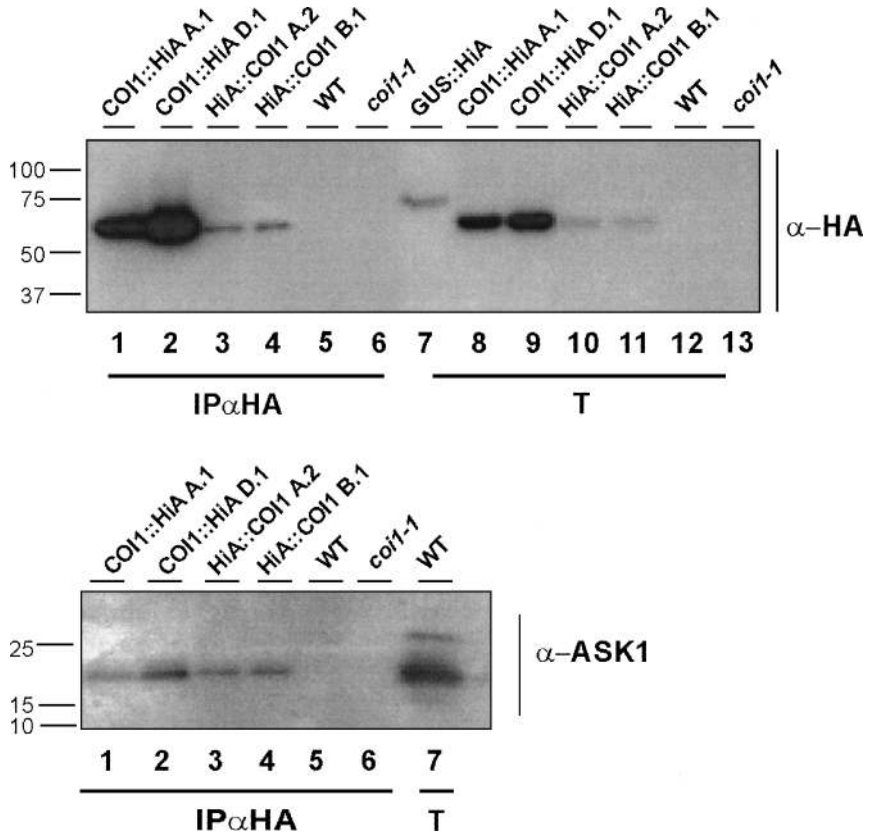


**Figure 3.** *In planta* protein interaction between COI1 and the SKP1-like protein ASK1.
Western blot analysis of α-HA immunoprecipitates (IPα-HA) (lanes 1–6. top and bottom panels) or total protein extracts (T) (lanes 7–12, top panel; lane 7, bottom panel) obtained from methyl jasmonate-treated seedlings of four independent transgenic *A. thaliana* T2 lines expressing COI1 as haemagglutinin (HA) C- or N-terminal fusion proteins (COI1::HiA or HiA::COI1) (lanes 1–4, 8–11, top panel; lanes 1–4, bottom panel), wild-type (lanes 5 and 12, top panel; lane 5, bottom panel) or *coi1*-1 (lanes 6 and 13, top panel; lane 6, bottom panel). Top: detection with monoclonal α-HA antibody (Roche). Bottom: detection with polyclonal antisera raised against ASK1. GUS::HiA, *Arabidopsis* transgenic lines expressing GUS and HA C-terminal fusion (lane 7).

and approximately one quarter of these were homozygous for the *coi1*-1 mutation as verified by PCR (Xie *et al.*, 1998). This indicates that both constructs complement the *coi1*-1 mutation.

Clarified extracts of transformed cell cultures or transgenic plants were incubated with the anti-haemagglutinin (HA) affinity matrix and the immunoprecipitated proteins were collected and analysed by Western blot. To detect the COI1::HiA fusion, we used a peroxidase-coupled monoclonal anti-HA antibody (Roche); to detect ASK1 we used an antibody demonstrated to be specific by Ferrando *et al.* (2000) and one we raised against the full-length ASK1 protein; to detect the cullin we used the antibody raised by Gray *et al.* (1999). Our results show that COI1, ASK1 and cullin co-immunoprecipitate with COI1::HiA (Figures 3 and 4). The cullin protein band migrated as a doublet as previously observed by Gray *et al.* (1999) in wild-type crude extracts and less obviously in the extracts from 35S::COI1::HiA or 35S::HiA::COI1 transgenic lines (Figure 4). Co-immunoprecipitation experiments using independent 35S::COI1::HiA or 35S::HiA::COI1 transgenic lines gave identical results (Figures 3 and 4). Results similar to those shown in Figures 3 and 4 were obtained with plants not treated with JA and with transiently transformed *Arabidopsis* cell cultures.

Western blots were also probed with histone deacetylase antiserum. The histone deacetylase co-immunoprecipitated with COI1::HiA from cell cultures but not from plants (Figure 5). Total protein extracts obtained either from wild-type, COI1-over-expressing or *coi1*-1, showed that the band corresponding to the histone deacetylase in green tissues was not detectable on immunoblot (Figure 5).

As controls, extracts from wild-type plants or cell cultures were incubated with anti-HA affinity matrix, but Western analysis of immunoprecipitates indicated that ASK1, cullin and histone deacetylase were not detectable, indicating that immunoprecipitation was specific for COI1. In addition, no signal was detected when co-immunoprecipitation was performed with protein extracts from *coi1*-1 plants. We tested the identity of the 52.6 kDa signal and addressed the specificity of the antibody by expressing a 3′ histidine-tagged histone deacetylase truncation of 333 amino acids which generated a signal of the expected molecular mass of 37 kDa (Figure S1).

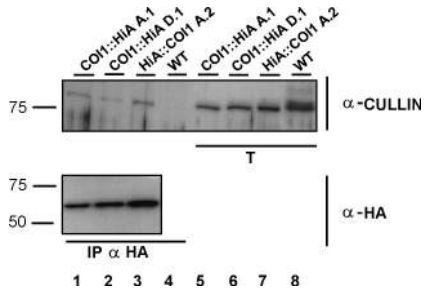


**Figure 4.** *In planta* protein interaction between COI1 and cullin.
Western blot analysis of α-HA immunoprecipitates (IPα-HA) (lanes 1–4) or total protein extracts (T) (lanes 5–8) obtained from untreated healthy seedlings of three independent transgenic *A. thaliana* T2 lines expressing COI1 as haemagglutinin (HA) C- or N-terminal fusion proteins (COI1::HiA or HiA::-COI1) (lanes 1–3, 5–7) or wild-type (lanes 4 and 8). Top: detection with polyclonal antisera raised against cullin. Bottom: detection with monoclonal α-HA antibody (Roche).

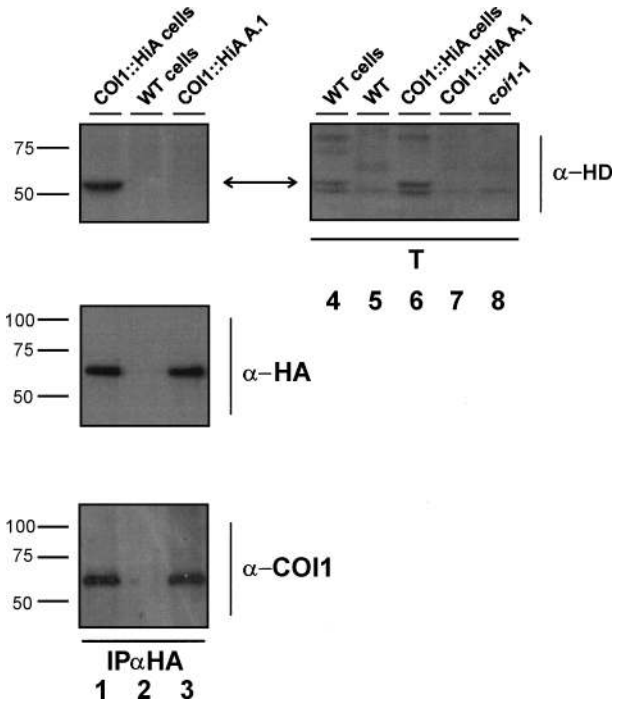


**Figure 5.** *In planta* protein interaction between COI1 and histone deacetylase.
Western blot analysis of α-HA immunoprecipitates (IPα-HA) (lanes 1–3) or total protein extracts (T) (lanes 4–8) obtained from *Arabidopsis* cell cultures (lanes 1, 2, 4, 6) or untreated healthy seedlings of a transgenic *A. thaliana* T2 line expressing COI1 as haemagglutinin (HA) C-terminal fusion proteins (COI1::-HiA) (lane 3 and 7), or wild-type (line 5) or *coi1*-1 (lane 8). Top: detection with polyclonal antisera raised against histone deacetylase; the double arrow indicates the corresponding band. Middle: detection with monoclonal α-HA antibody (Roche). Bottom: detection with polyclonal antisera raised against COI1.

### *Meiosis occurs regularly in* coi1-1

The *Arabidopsis ASK1* gene controls homologue association before anaphase II, is essential for male meiosis, and *ask1* mutants are male-sterile (Yang *et al.*, 1999). Because *coi1*-1 mutants are also male-sterile, we examined whether homologue association before anaphase II was affected in these plants. Meiosis in microspore mother cells of wild-type and *coi1*-1 plants was by examined by fluorescence microscopy of DAPI-stained nuclei. Figure 6(a) shows that formation of the tetrads in telophase II occurs regularly in *coi1*-1, leading to the formation of four microspores of uniform size (Figure 6c). All other stages of male meiosis I and II in *coi1*-1 appeared to be normal (data not shown). These results indicate that pollen development is aborted at a later stage in *coi1*-1 than in *ask1*.

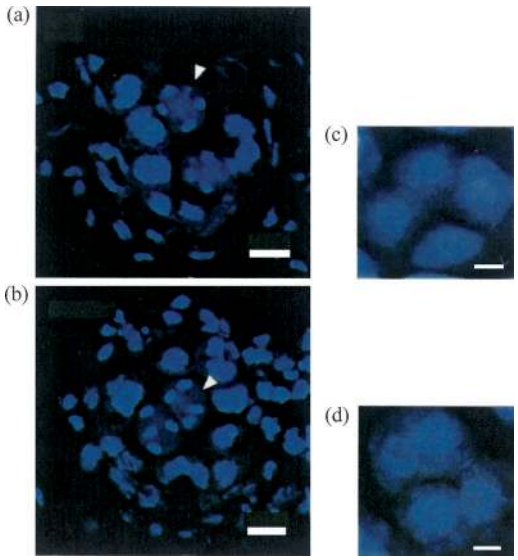


**Figure 6.** *coi1*-1 produces normal tetrads and microspores.
DAPI-stained cross-section of immature anthers (locule) of *coi1*-1 and wild-type *Arabidopsis* showing meiocytes in late telophase II in one of the four locules. (a,b) *coi1*-1 and wild-type anther locules, respectively. (c,d) Details of the separate microspores of *coi1*-1 and wild-type, respectively. Arrows indicate separated tetrads. Scale bars: 10 μm (a,b); 5 μm (c,d).

*The axe1-5 histone deacetylase mutant has a partial, constitutive JA phenotype*

The COI1-interacting protein CIP3 is the *Arabidopsis* histone deacetylase HDA6 (also named RPD3b by Wu *et al.*, 2000), and the corresponding gene is mutated in *axe1*-5 (Murfett *et al.*, 2001). *axe1*-5 seedlings were germinated on MS media (MS) and transferred to vertical plates containing MS or MS containing 50 μM MeJA. MeJa reduced root growth and increased numbers of root hairs. Untreated *axe1*-5 plants had a higher number of root hairs in the immature region than wild-type plants, but after treatment with MeJA the numbers of root hairs was similar in *axe1*-5 and wild-type plants (Figure 7). Treatment with MeJA increased anthocyanin content and inhibited root growth in both *axe1*-5 and wild-type plants, and treatment with 1-naphthalene acetic acid (NAA) reduced root growth and enhanced formation of lateral root primordia in *axe1*-5 and wild-type plants (data not shown).

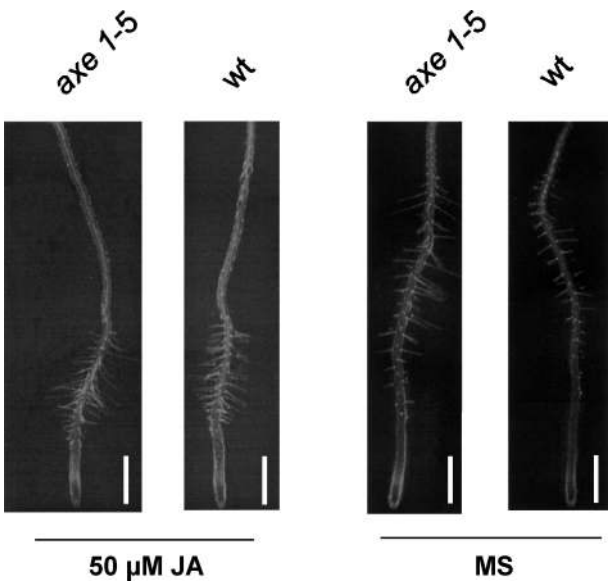


**Figure 7.** Roots of 14-day-old *axe1*-5 mutant and wild-type *Arabidopsis* seedlings in the presence or absence of MeJA.
*axe1*-5 or *Arabidopsis* Col-0 seedlings were grown for 12 days on MS (Murashige and Skoog, 1962) agar and then transferred to MS 50 μM MeJA (left) or unsupplemented MS for 48 h (right). Scale bars: 500 μm.

## Discussion

COI1 is an *Arabidopsis* F-box protein required for responses to JA, and was predicted to function in an SCF complex that recruits regulators of JA responses for ubiquitination (Xie *et al.*, 1998). To test this hypothesis, we used COI1 as bait to screen an *Arabidopsis* cDNA expression library in yeast, and identified four CIP proteins as candidates for COI1 function.

CIP1 and 2 were *Arabidopsis* SKP1-like proteins, and bound COI1 *in planta*. These two proteins were previously identified as ASK1 and ASK2, respectively, and bind to the F-box proteins TIR1, which regulates response to auxin (Gray *et al.*, 1999), and UFO, which regulates flower development (Samach *et al.*, 1999). Although a cullin was not recovered in the yeast two-hybrid screen, we demonstrated by immunoprecipitation that COI1 also associated with cullin in *Arabidopsis* cells. This indicates that COI1 may form part of an $SCF^{COI1}$ complex in *Arabidopsis*.

Analysis of the F-box motif in COI1 indicated that it was required for binding to all CIP proteins. Thus, the mutant COI1 protein lacking the entire F-box, ΔFbox, and the mutant protein in which the F-box amino acid W44 was mutated to alanine, $COI1_{W44A}$, did not bind to CIP1, 2, 3 or 4 in yeast. Significantly, W44 corresponds to tryptophan 139 in the F-box of the F-box protein, human SKP2, and it interacts directly with SKP1 (Schulman *et al.*, 2000 and Figure 2). CIP3 and 4 are not SKP1-like proteins however, indicating that the F-box motif is required for binding proteins besides SKP1-like proteins. Similarly, the mutant COI1 protein 3′Δ in which the C-terminus was deleted also failed to bind any of the four CIP proteins in yeast, confirming other evidence that sequences outside the F-box can moderate binding to SKP1 proteins (Gray *et al.*, 1999).

COI1 and CIP1 (which is the same as ASK1) interacted *in planta*. Because *coi1*-1 and *ask1* mutants are both male-sterile (Feys *et al.*, 1994; Yang *et al.*, 1999; Zhao *et al.*, 1999), this raised the possibility that male sterility has a common physiological basis in these mutants. Male sterility in *ask1* has been attributed to the failure of chromosomes

to separate during male anaphase I, and we therefore examined meiosis in microspore mother cells of *coi1*-1 anthers. However, meiotic divisions occurred regularly in *coi1*-1, and four microspores of uniform size were formed (Figure 6C). This demonstrates that the defect in pollen formation in *coi1*-1 occurred at a later developmental stage than in *ask1.*

CIP3 and 4 are the *Arabidopsis* proteins histone deacetylase RPD3b and the small subunit of Rubisco. Neither has previously been detected as components of SCF complexes. These proteins are therefore candidates for regulators of the JA response as substrates for COI1-mediated ubiquitination. Significantly, CIP3 and 4 differ from CIP1 and 2 in their binding to $COI1_{L245F}$ and TIR1. $COI1_{L245F}$ is the gene product of *coi1*-16, a novel mutant *COI1* allele that displays a jasmonate-insensitive phenotype intermediate between that of wild-type and *coi1*-1 plants (Ellis and Turner, 2002). The specificity of binding of F-box proteins to targets for ubiquitination is likely to be determined by sequences in the leucine-rich repeats, and it may therefore be significant that the mutation in $COI1_{L245F}$ occurs in a predicted $\alpha$-helix of the sixth leucine-rich repeat of the COI1 protein. $COI1_{L245F}$ is required for the regulation of *Arabidopsis* responses to JA, and binds to CIP1 and 2, but not to the putative targets for COI1-mediated degradation CIP3 and 4. This therefore provides indirect evidence that CIP3 and 4 bind to the leucine-rich repeats of COI1. Similarly, TIR1, which regulates response to auxin but not to JA, binds to CIP1 and 2, but not to CIP3 and 4. These results are consistent with CIP3 and 4 as targets for COI1-mediated modifications that regulate the response to JA.

The small subunit of Rubisco is encoded by the nuclear genome, is translated in the cytoplasm, and translocated into the chloroplast. Application of JA to plant leaves reduces expression of the small subunit of Rubisco, reduces translation and induces degradation of Rubisco, and enhances chlorophyll loss (Parthier, 1990). In *Arabidopsis*, senescence is associated with increased JA levels in leaves, and treatment of *Arabidopsis* with JA induces *COI1*-dependent senescence (He *et al.*, 2002). When soybean leaves are treated with JA, Rubisco accumulates in a cytosolic complex (Staswick, 1997), possibly prior to its degradation. Therefore, the small subunit of Rubisco, CIP4, may be a substrate for COI1- and JA-dependent degradation or modification.

COI1 bound to CIP3, the histone deacetylase RPD3b, both in yeast and in *Arabidopsis* cells. Acetylation and deacetylation of histones is an important mechanism in the regulation of gene transcription in eukaryotes (Hassig *et al.*, 1997; Lusser *et al.*, 2001; Pazin *et al.*, 1998). Histone acetylation is believed to increase the accessibility of chromatin to the transcription machinery, and the removal of acetyl groups from acetylated histones has the opposite effect and represses transcription. The acetylation level of histones is determined by the relative activities of histone acetyltransferases and histone deacetylases (HDACs). Yeast and mammals have two types of HDACs, which comprise the RPD3 and the HDA1 families of proteins. Homologues of RPD3 have also been found in maize and *Arabidopsis* (Murfett *et al.*, 2001; Rossi *et al.*, 1998; Wu *et al.*, 2000). In *Arabidopsis*, RPD3b transcripts are generally present at a low level (Wu *et al.*, 2000). We observed that the expression of histone deacetylase was higher in actively dividing *Arabidopsis* cells than in leaf tissues (Figure 5). Moreover, we could recover histone deacetylase by immunoprecipitation with COI1 from the cell cultures, but not from leaf tissues.

The *Arabidopsis* mutant *axe1* is defined by a mutation in the gene for histone deacetylase RPD3b (Murfett *et al.*, 2001). The *axe1* mutant was isolated in a screen for altered expression of a transgene (Murfett *et al.*, 2001). However, *axe1* also had a distinctive phenotype and produced a higher number of root hairs. We showed that JA also increased root hair development in *Arabidopsis* (Figure 7). This raises the possibility that RPD3b is a repressor of JA-dependent root hair development, and therefore a potential target for the $SCF^{COI1}$ complex. Intriguingly, mammalian homologues of yeast proteins known to interact with each other and involved in the ubiquitin signalling pathway, have been recently shown to associate with a cytoplasmic murine histone deacetylase 6 (Seigneurin-Berny *et al.*, 2001), establishing a link between protein acetylation and protein ubiquitination. We are currently examining whether COI1 regulates the ubiquitination and modification, or proteolytic destruction, of CIP3 and 4.

## Experimental procedures

### *Nucleic acid analysis*

Recombinant plasmids were prepared according to standard procedures (Ausubel *et al.*, 1996). DNA inserts were sequenced using the automated Thermo Sequenase Dye Terminator Cycle Sequencing kit (Amersham) and run on a 377 DNA sequencer (Applied Biosystems). DNA sequences and predicted gene products were analysed by using the University of Winsconsin Genetic Computer Group (GCG) computer packages (Devereux *et al.*, 1984).

### *Constructs used in the yeast-two hybrid assay*

Point mutations to obtain the plasmids $pCOI1_{L11A}$ and $pCOI1_{W44A}$ were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The plasmid pΔFbox was obtained by replacing a *Bam*HI–*Not*I fragment of the *Coi1* coding sequence in the plasmid pLexA-COI1 with a PCR fragment amplified with the primers FboxdelR (5′-GCAGGATCCTATGGCGCTTTGCTACAC) and pET86.3′ (5′-ACTCGAGCGGCCGCATATTGGCTCCTTCAGGACTCT) purified and digested with the same enzymes.

The mutant protein corresponding to COI1-1 in the plasmid p3′Δ was obtained by deletion of the last two leucine-rich repeats and

the C-terminus. The construct p3′Δ was obtained by replacing a *Bam*HI–*Not*I fragment of pLexA-COI1 with a PCR fragment amplified with the primers ATG.AR (5′-TTTTCGTGATCATGGAG-GATCCTGAT) and 86A3DelF (5′-AACGCGGCCGCATTCATCTCAC-GTTTGGACT) purified and digested with same enzymes.

### *Yeast two-hybrid analysis*

We used the LexA two-hybrid system (Gyuris *et al.*, 1993). Details of the construction of the two-hybrid cDNA library are given by van der Biezen *et al.* (2000). Two-hybrid analyses were performed according to the method described by Golemis *et al.* (1998).

The entire coding sequence of *COI1* cDNA or the various PCR-generated *COI1* domains (Xie *et al.*, 1998) were subcloned into pLexA and/or pB42AD. All pLexA constructs were verified by sequencing and tested for auto-activation of the LacZ and LEU2 reporters. Repression assays with JK101 confirmed protein fusion synthesis and nuclear localization of the LexA fusion protein. All two-hybrid combinations were peformed in the standard yeast strain EGY48. cDNA inserts were amplified with PCR primers complementary to regions flanking the insert: pB42AD5′ (5′-GATACCAGCCTCTTGCTGAG) and pB42AD3′ (5′-TTCTGGCAAGG-TAGACAAGC). Amplified inserts were analysed by *Rsa*I restriction analysis to identify related clones and sequenced. Western blot analysis was performed on total yeast protein extracts, derived from galactose-induced cultures, using anti-LexA antibody (Santa Cruz Biotechnology). Quantitative β-galactosidase assays were performed on a minimum of six independent yeast transformants for each combination of interacting partners, and the average for each interaction was calculated as described by Ausubel *et al.* (1996). The experiment was done twice with similar results.

### *Antibody production*

COI1 and CIP3 were expressed in *E. coli* BL21 (pET21) as a fusion to a histidine tag and purified by electro-elution from a preparative SDS–PAGE gel. CIP1 and 2 were expressed and purified as GST fusion proteins in *Saccharomyces pombe*, using the ESP Yeast Protein expression and Purification System with pESP-1 (Stratagene). The coding sequences were introduced into the expression vector pESP-1 and the constructs were established in *E. coli* strain XL-10 GOLD and transformed into *S. pombe*, SP-Q01, for expression. GST fusion proteins were purified in non-denaturing conditions by affinity chromatography according to the manufacturer's instructions. New Zealand white male rabbits were immunized and sera were collected. Antibodies against ASK1 and cullin were kindly donated by Dr C. Koncz and Dr M. Estelle, respectively.

### *Construction of intron-tagged COI1*

The *COI1* coding sequence was PCR-amplified as *Nco*I/*Sma*I and *Sal*I/*Sal*I fragments, and cloned into the vector pPily (Ferrando *et al.*, 2000) to obtain C-terminal and N-terminal translational fusions, respectively. The *Kpn*I cassettes containing the fusions, a double CaMV35S promoter and a NOS terminator, were transferred into the binary kanamycin-resistant plasmid pBin19PLUS (van Engelen *et al.*, 1995). The plasmid pHiA-GUS (Ferrando *et al.*, 2000) was used as a positive control for *Arabidopsis* cell suspension culture transformation and in Western blot analysis. Binary vectors were transferred into *Agrobacterium tumefaciens* GV3101 by electroporation.

### *Transformation of Arabidopsis cell suspensions and plants with Agrobacterium tumefaciens*

Culture media and conditions for *Agrobacterium*-mediated transformation of *Arabidopsis* cell suspensions were described previously by Ferrando *et al.* (2000). *Arabidopsis* cells were infected with *Agrobacterium* cells 4 days after subculture, and growth of infected cells was monitored against a mock-inoculated control by checking packed-cell volume. Cells were collected 3 days after transformation (when in the exponential growth phase) to prepare protein extracts for immunoassay. *A. thaliana* Col-*gl1* plants were transformed using the flower-dip method (Clough and Bent, 1998). T2 independent homozygous lines were used for analysis. Growth conditions and treatment of plant material with MeJA have been described previously (Ellis and Turner, 2001).

### *Protein extraction, immunoaffinity binding and Western blot analysis*

Total protein extracts were prepared from either plant cell suspension or 2-week-old seedlings. Plant cells were collected by filtration using a Millipore holder system, frozen in liquid nitrogen and stored at −70°C. Plant material was ground to powder in liquid nitrogen and resuspended in IP/extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.15% IGEPAL containing 5 mM DTT, 1 × COMPLETE mini (Roche), 1 mM pefabloc (Roche), 2 μg ml$^{-1}$ aprotinin (Roche). Samples were spun at 10 000 ***g*** for 15 min and the protein concentration of the soluble fraction was determined using Bradford reagent (BioRad) and BSA as a standard. For immunoprecipitation reactions, 1–2 mg total protein was pre-cleared by incubating with 50 μl of protein A agarose (Roche) for 2 h at 4°C on a rotating shaker. Then, 20 μl of anti-HA affinity matrix (Roche) were added to the pre-cleared extract and incubated for 2–16 h in a total volume of 1.4 ml IP/extraction buffer. IP reactions were washed four times with 1 ml ice cold IP/extraction buffer, resuspended in 100 μl SDS–PAGE sample buffer, boiled for 3 min, and 15 μl aliquots run on 10 or 12% SDS–PAGE gels (BioRad). For standard Western blot analysis, 10–15 μg of total protein was loaded. Proteins were electroblotted to PVDF membranes (Roche), blocked for 1 h at room temperature in TBS-Tween containing 5% w/v non-fat dried milk. Incubation with primary antibodies was in TBS-Tween containing 5% w/v non-fat dried milk using the following dilutions: peroxidase-coupled monoclonal anti-HA antibody 3F10 (1:1000, Roche); cullin antiserum (1:2000); COI1 antiserum (1:100); histone deacetylase antiserum (1:100), AtSKP1 antiserum (1:1000, Ferrando *et al.*, 2000); SKP antiserum (1:1000); HRP antiserum (1:5000, Amersham). Blots were developed using the ECL kit (Amersham). Both the anti-HA antibody (Roche) and the anti-COI1 polyclonal antiserum we generated (see Antibody production) detected single protein bands of the expected size in total soluble extracts of the transgenic lines, but not in protein extracts from the *coi1*-1 mutant (data not shown). The molecular weight of the fusion proteins between COI1 and the HA tag was approximately 68 kDa (Figures 3, 4 and 5), and that of COI1 was approximately 67 kDa). No protein cross-reacting with the anti-HA antibody was found in bacterial cells carrying the intron-tagged COI1::HiA or HiA::COI1 epitope constructs (data not shown).

### *Immunofluorescence microscopy*

Immature inflorescences of *Arabidopsis* wild-type and *coi1*-1 were fixed, embedded and sectioned in paraffin wax according to the

method described by Leitch *et al*. (1994), with the exception that the slides used were aminopropyltuetoxysilane (APES)-coated. After ethanol dehydration, the sections were stained with 1 μg ml$^{-1}$ DAPI (4′,6-diamidino-2-phenylindole) for 10–15 min, washed in distilled water and mounted in Vectashield anti-fade solution (Vector Laboratories Inc.). The sections were viewed by confocal microscopy (MRC-1024, BioRad) and data were collected for individual anther locules containing meiocytes. Three-dimensional image projections were obtained using BioRad Confocal Assistant software (ftp://ftp.genetics.bio-rad.com/Public/confocal/cas/). Further image processing was carried out in Adobe Photoshop 5.0 (Adobe Systems Inc.).

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## Supplementary Material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/TPJ/TPJ1432/TPJ1432sm.htm

**Figure S1** The antibody raised against the hystone deacetylase recognises a 3′histidine-tagged protein truncation. As described in the Experimental procedures, polyclonal antibodies were raised in rabbit against denatured CIP3-His protein. A partial cDNA (999 nt) was cloned into pET21 expression vector and over-expressed in E. coli as a fusion to histidine tag. M: rainbow marker.

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