

Biochemical evidence for ubiquitin ligase activity of the *Arabidopsis* COP1 interacting protein 8 (CIP8)

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Summary

Arabidopsis COP1 is a negative regulator of photomorphogenesis, which targets HY5, a positive regulator of photomorphogenesis, for degradation via the proteasome pathway in the absence of light. COP1 and its interactive partner CIP8 both possess RING finger motifs, characteristic of some E3 ubiquitin ligases. Here we show that CIP8 promotes ubiquitin attachment to HY5 in E2-dependent fashion *in vitro*. CIP8 exhibits a strong interaction with the E2 enzyme AtUBC8 through its N-terminal domain. Phosphorylation of HY5 by casein kinase II requires the beta subunit 2, but does not affect HY5's susceptibility to ubiquitination. The RING domain of CIP8 is required but is not sufficient for ubiquitin ligase activity. Although the RING domain of CIP8 interacts with the RING domain of COP1, addition of recombinant COP1 fails to affect CIP8's ubiquitin ligase activity towards HY5 *in vitro*. However, recombinant COP1 can pull-down native CIP8 from the extract of dark-grown seedlings, but not from the extract of light-grown seedlings in a column-binding assay, implying a requirement for light-regulated modification *in vivo*. Our data suggest that CIP8 can form a minimal ubiquitin ligase in co-operation with the E2 enzyme AtUBC8. It is possible that the AtUBC8-CIP8 module might interact with COP1 *in vivo*, thereby participating in proteasome-mediated degradation of HY5.

Keywords: CIP8, COP1, ubiquitin, E3 ligase, light regulation, RING finger.

Introduction

The switch from dark-adapted (skotomorphogenic) to light-adapted (photomorphogenic) development in *Arabidopsis* is controlled by a molecular network of negative and positive regulators of seedling photomorphogenesis (Hardtke and Deng, 2000; Nagy and Schäfer, 2000; Neff *et al.*, 2000). A key negative regulator is COP1, a protein with an N-terminal RING finger motif, followed by a coiled coil and a WD40 domain (Deng *et al.*, 1992). Using a variety of approaches, a number of proteins interacting with distinct domains of COP1 have been isolated. Among them, the COP1 Interacting Proteins CIP1, CIP4 and CIP7 interact with the coiled coil domain, while CIP8 specifically interacts with the RING finger domain (Matsui *et al.*, 1995; Torii *et al.*, 1999; Yamamoto *et al.*, 1998; Yamamoto *et al.*, 2001). In addition, a number of transcription factors, which share a common COP1 binding motif, interact with the WD40 repeat domain (Ang *et al.*, 1998; Holm *et al.*, 2001).

Among these interactions, the best characterized occurs between the WD40 domain of COP1 and the bZIP transcription factor HY5, a positive regulator of photomorphogenesis.

We have recently shown that HY5 is targeted for degradation via the proteasome pathway in darkness (Osterlund *et al.*, 2000). This HY5 degradation depends on the presence of COP1 and is most likely regulated by differential nucleo-cytoplasmic partitioning of COP1 in response to light stimulus. While HY5 is constitutively nuclear, COP1 is nuclear localized in darkness but not in light (von Arnim and Deng, 1994). Thus in the dark, COP1 acts in the nucleus to keep HY5 levels low. By contrast, HY5 protein strongly accumulates in response to light due to nuclear exclusion of COP1. Based on these observations it has been suggested that COP1 may act as an E3 ubiquitin ligase or part of an E3 ubiquitin ligase complex, promoting

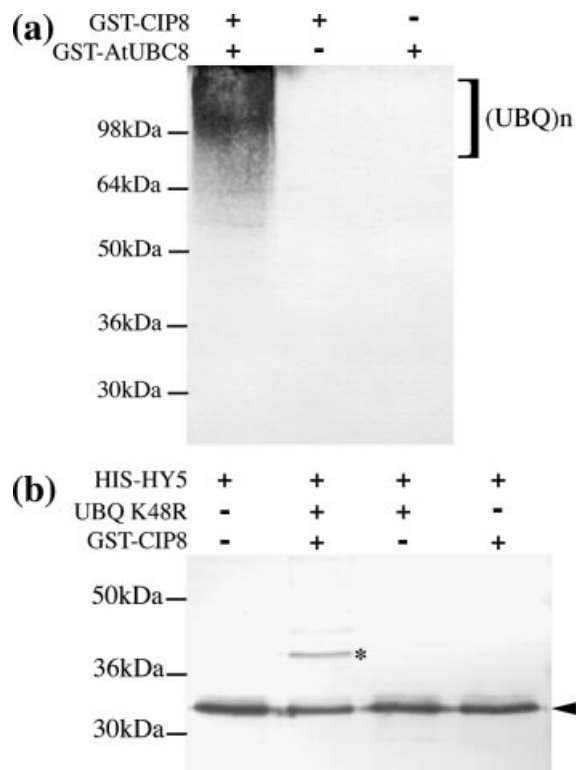


Figure 1. CIP8 has ubiquitin ligase activity *in vitro*.

(a) GST-CIP8 promotes ubiquitin chain formation *in vitro*. Western analysis of ubiquitin conjugation reactions probed with antiubiquitin antibody. Addition of GST-CIP8 to the reaction mix results in the detection of a high molecular weight smear (UBQ_n, indicated by bracket), which represents multi-ubiquitin chains. No such signal is detected if GST-CIP8 or the E2 ubiquitin-conjugating enzyme GST-AtUBC8 are omitted from the assay.

(b) GST-CIP8 promotes ubiquitin attachment to HIS/T7-HY5. Western analysis of HIS/T7-HY5 added to ubiquitin conjugating reactions and visualized by anti-T7-tag antibody. The appearance of a slower migrating band (marked by asterisk) depends on the presence of GST-CIP8 as well as the mutant K48R ubiquitin (this mutant form of ubiquitin does not allow formation of multi-ubiquitin chains). Thus, this band must correspond to a HIS/T7-HY5 conjugated to one ubiquitin molecule.

HY5 ubiquitination and proteasome-mediated degradation in the dark.

E3 ubiquitin ligases are a growing family of mostly heteromeric complexes that provide specificity to the ubiquitination machinery by selecting target proteins bound for degradation. Recently, biochemical and genetic evidence pointing towards a role of RING finger domain proteins in ubiquitination has accumulated (Chen *et al.*, 2000; Fang *et al.*, 2001; Gmachl *et al.*, 2000; Hashizume *et al.*, 2001; Honda and Yasuda, 2000; Joazeiro *et al.*, 1999; Lorick *et al.*, 1999; Matsuda *et al.*, 2001; Xie and Varshavsky, 2000). In particular, proteins of the RING-H2 subtype have been found to be associated with prototypical E3 ubiquitin ligases of the SCF, VBC and APC type *in vivo* (Kamura *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999; for review see Jackson *et al.*, 2000; Joazeiro and Weissman, 2000). *In vitro*, these

RING-H2 components are able to promote E2 ubiquitin conjugating enzyme-dependent ubiquitination of respective target proteins. For some, the essential function of the RING-H2 domain has also been demonstrated *in vivo*.

The RING-H2 protein CIP8 has previously been isolated as an interaction partner for the RING finger domain of COP1 (Torii *et al.*, 1999). In this report we demonstrate that CIP8 forms an ubiquitin ligase in co-operation with an E2 enzyme and can promote ubiquitination of the HY5 transcription factor *in vitro*.

Results

CIP8 promotes ubiquitin chain formation in vitro in E2 conjugating enzyme-dependent fashion

Recently published results demonstrated the involvement of RING-H2 proteins in E3 ubiquitin ligase activity (for review see Jackson *et al.*, 2000; Joazeiro and Weissmann, 2000). Thus we set out to test whether CIP8 could perform a similar enzymatic activity. To this end, we set up ubiquitination assays consisting of *Saccharomyces cerevisiae* E1 ubiquitin activating enzyme, recombinant GST fusion protein of the *Arabidopsis* E2 ubiquitin-conjugating enzyme AtUBC8, ubiquitin and an ATP regenerating system. To monitor the formation of multi-ubiquitin chains, we probed the assays by Western analysis using an ubiquitin antibody. In these reactions no ubiquitin chain formation was observed in the presence of all the above components without CIP8. However, upon addition of recombinant GST-CIP8 fusion protein we observed strong ubiquitin chain formation activity (Figure 1a). By contrast, no ubiquitin chain formation was observed if GST-CIP8 was added but at the same time the E2 enzyme was omitted. Thus, GST-AtUBC8 and GST-CIP8 are able to cooperate in promoting ubiquitin chain assembly.

CIP8 promotes ubiquitin attachment to HY5 in vitro

CIP8 strongly interacts with COP1 (Torii *et al.*, 1999), and COP1 is required for targeted degradation of HY5 by the proteasome pathway. Thus we went on to test whether GST-CIP8 might also be able to aid in ubiquitination of recombinant HIS/T7-tagged HY5 protein (HIS/T7-HY5). In these assays, a mutant K48R ubiquitin was used in order to generate a stronger and sharper signal rather than a smear of substrate conjugated to multi-ubiquitin chains of variable length (K48R ubiquitin can be attached to a target protein but does not support chain formation). The assays were analysed by Western analysis and the recombinant HY5 was visualized with anti-T7 tag antibody. No modification of HIS/T7-HY5 was observed when GST-CIP8 was omitted from the assay. However, in the presence of GST-CIP8 a second band was detected, running approximately

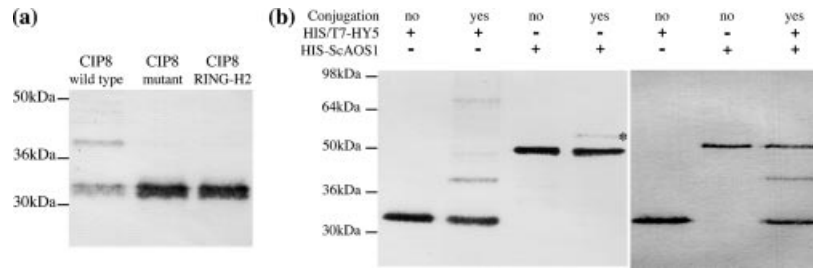


Figure 2. Specificity of CIP8 ubiquitin ligase activity and dependence on the RING-H2 domain. (a) Ubiquitin ligase activity of GST-CIP8 requires the RING-H2 domain, but the RING-H2 domain is not sufficient for the activity. Western analysis of HIS/T7-HY5 added to ubiquitin conjugating reactions and visualized by anti-T7 tag antibody. Ubiquitin conjugate of HIS/T7-HY5 is only observed in the presence of full-length GST-CIP8 (CIP8 wild type), but not in the presence of a GST-CIP8 carrying point mutations that disrupt the RING-H2 finger domain (CIP8 mutant) or a truncated version of the RING-H2 domain of CIP8 only (CIP8 RING-H2). (b) CIP8 ubiquitin ligase activity is promiscuous *in vitro* but prefers HY5 as a substrate. Western analysis of HIS/T7-HY5 (c. 200 ng) or HIS-ScAOS1 (approximately 500 ng) added to ubiquitin conjugating reactions containing GST-CIP8 and visualized by anti-HIS tag antibody. Input (no conjugation reaction) of HIS/T7-HY5 or HIS-ScAOS1 compared to their respective conjugation reactions. An ubiquitin conjugate is observed for both HIS/T7-HY5 and ScAOS1 (asterisk). In a reaction that contains both proteins an ubiquitin conjugate is observed for HIS/T7-HY5 only, not for HIS-ScAOS1.

8 kDa above the HIS/T7-HY5 input (Figure 1b). Since the size of a single ubiquitin moiety is approximately 8 kDa, this would be the expected size of a HIS/T7-HY5 conjugated to one ubiquitin molecule. Indeed, if GST-CIP8 was added to the assay, but the K48R ubiquitin was omitted, the slower migrating band of the HIS/T7-HY5 disappeared. Thus the newly observed band represents a HIS/T7-HY5 ubiquitin conjugate and its formation requires the presence of GST-CIP8.

The RING-H2 domain of CIP8 is required but not sufficient for ubiquitin ligase activity

A RING-H2 domain has previously been shown to be essential for the ubiquitin ligase activity of Rbx1. To determine whether the addition of any RING-H2 domain to our basic assay is sufficient for ubiquitin ligase activity, we replaced GST-CIP8 by a recombinant GST fusion protein of the RING-H2 domain of an unrelated *Arabidopsis* gene, derived from the expressed sequence tag AT2447, or the RING finger protein PRT1, a N-end rule ubiquitin ligase of *Arabidopsis* (Potuschak *et al.*, 1998). No ubiquitination of HIS/T7-HY5 was observed with these proteins (data not shown).

To test a possible requirement of the RING-H2 domain of GST-CIP8 for its ubiquitin ligase activity, we tested a mutant version of CIP8 in our assay. Point mutations destroying the co-ordination of the RING-H2 domain were introduced by site-directed mutagenesis (Torii *et al.*, 1999) and a respective GST fusion protein was purified. Unlike the wild type CIP8, this protein was not able to mediate ubiquitination of HIS/T7-HY5 (Figure 2a). Thus, an intact RING-H2 domain is required for the ubiquitin ligase activity of GST-CIP8. To determine whether the RING-H2 domain might be sufficient for the activity, we purified a GST fusion of the 93 C-terminal amino acids of CIP8 only. Replacing GST-CIP8 with this RING-H2 domain fusion did

not result in any detectable ubiquitination of HIS/T7-HY5. Thus, the RING-H2 domain is essential for ubiquitin ligase activity of CIP8, but not sufficient.

CIP8 ubiquitin ligase activity is promiscuous in vitro but prefers HY5 as substrate

To evaluate the specificity of our assay with regard to the substrate, we tested an unrelated protein, the *S. cerevisiae* SUMO activating enzyme subunit AOS1 (ScAOS1). As for HIS/T7-HY5, we observed a slower migrating band as compared to the input for ScAOS1 as well (Figure 2b). Again, the shift in size indicates that the newly observed band represents an ubiquitin conjugate of ScAOS1. Thus, CIP8 seems to be able to promiscuously ubiquitinate this substrate *in vitro*. On the other hand, the activity towards HIS/T7-HY5 seemed to be stronger in our assay, since the major ubiquitinated HIS/T7-HY5 band had stronger intensity. Also additional bands corresponding to HIS/T7-HY5 protein conjugated to more than one ubiquitin could be observed, which was not the case for ScAOS1. Indeed, when we offered the same molar amounts (approximately 200 ng HIS/T7-HY5 and approximately 500 ng HIS-ScAOS1) of both proteins in the same assay, the ubiquitin ligase activity was preferentially directed towards HIS/T7-HY5 (Figure 3c). Thus, while GST-CIP8 clearly has a strong ubiquitin ligase activity that goes beyond its natural substrates *in vitro*, HY5 is a preferred substrate.

Phosphorylation of HY5 by casein kinase II requires a specific beta subunit

We have previously shown that HY5 is also regulated by phosphorylation, which is very likely mediated by a casein kinase II activity (Hardtke *et al.*, 2000; Oyama *et al.*, 1997). We sought to test whether HY5's phosphorylation status has any effect on its susceptibility to ubiquitination *in vitro*.

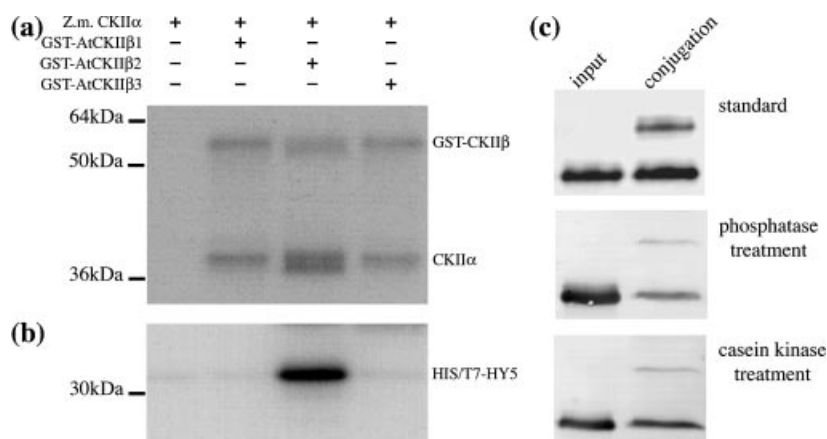


Figure 3. Phosphorylation of HY5 by casein kinase II does not affect its susceptibility to ubiquitin conjugation *in vitro*.

(a) Autoradiographs of kinase assays with bacterially expressed casein kinase II alpha subunit from *Z. mays* and GST fusion proteins of three *Arabidopsis* beta subunits of casein kinase II. No autophosphorylation is observed in the presence of the alpha subunit alone. By contrast, autophosphorylation of both the alpha and beta subunits is observed upon addition of any of the three beta subunits.

(b) Phosphorylation of HY5 by casein kinase II. HIS/T7-HY5 added to the kinase assays in (a) is only effectively phosphorylated in the presence of both the *Z. mays* casein kinase II alpha subunit and the GST fusion protein of the *Arabidopsis* casein kinase II beta subunit 2.

(c) Ubiquitin conjugation of differentially phosphorylated HIS/T7-HY5. HIS/T7-HY5 was phosphorylated in a kinase assay with casein kinase II subunits (*Z. mays* alpha + *Arabidopsis* beta 2) and purified from the reaction with Ni²⁺-sepharose beads (casein kinase treatment). The sample was split in half and one half was treated with the unspecific lambda phosphatase (phosphatase treatment). The samples were then added to ubiquitination reactions as a substrate. No difference in ubiquitin conjugation is observed between the samples when analysed by Western analysis with anti-T7 tag antibody.

However, a bacterially expressed casein kinase II alpha subunit from *Zea mays*, a protein nearly identical to its *Arabidopsis* counterpart, was not able to phosphorylate HIS/T7-HY5 *in vitro*. Casein kinase II is a tetrameric enzyme assembled from two catalytic alpha and two regulatory beta subunits. Thus we isolated cDNAs for three beta subunits described in *Arabidopsis* and constructed respective two hybrid assay as well as GST fusion constructs. Interaction between beta subunits and transcription factors has been observed before (Sugano *et al.*, 1998; Sugano *et al.*, 1999), however, we did not see any significant interaction between a HY5 bait construct and prey constructs for each of the three beta subunits (data not shown). The same was true for *in vitro* interaction assays. Nevertheless, the GST fusions of all three beta subunits were able to strongly enhance the autophosphorylation of the *Z. mays* alpha subunit (Figure 3a). Moreover, in kinase assays based on the *Z. mays* alpha subunit supplemented with GST fusions of the different beta subunits, HIS/T7-HY5 was effectively phosphorylated in the presence of the *Arabidopsis* beta subunit 2 (Figure 3b). Thus, phosphorylation of HY5 by casein kinase II seems to require this specific beta subunit.

Phosphorylation of HY5 does not result in altered susceptibility to ubiquitination

Using the *Z. mays* alpha subunit and the *Arabidopsis* beta subunit 2, we went on to test phosphorylated HIS/T7-HY5

in our ubiquitination assay. First, HIS/T7-HY5 was phosphorylated in a kinase assay by the casein kinase II subunits and purified from the reaction using Ni²⁺-charged sepharose beads. After washing, one half was dephosphorylated by treatment with the unspecific lambda protein phosphatase (Hardtke *et al.*, 2000). Both samples were then washed again and incubated in the ubiquitination assay. No difference in ubiquitination was observed (Figure 3c), indicating that the phosphorylation status of the casein kinase II site is not critical in our assay.

Further, we tested the possibility that lysine residues within the COP1 binding domain might be the targets for ubiquitin attachment. We constructed mutant HY5 versions by site-directed mutagenesis, either changing the lysine residue at position 31 or in addition at position 53 to arginine, preventing any ubiquitin attachment at those residues. All of these mutant proteins could still be modified in our assay (data not shown), indicating that the mutated lysines are unlikely to be exclusive target sites for ubiquitination. Consistently, in transgenic lines overexpressing respective mutant transgenes the proteins did not show altered stability as compared to wild type HY5 (data not shown).

In vitro interaction between CIP8 and HY5 is very weak

We were wondering whether CIP8-mediated HY5 ubiquitination is based on interaction between the two proteins, possibly mediated by a highly basic stretch of amino acids

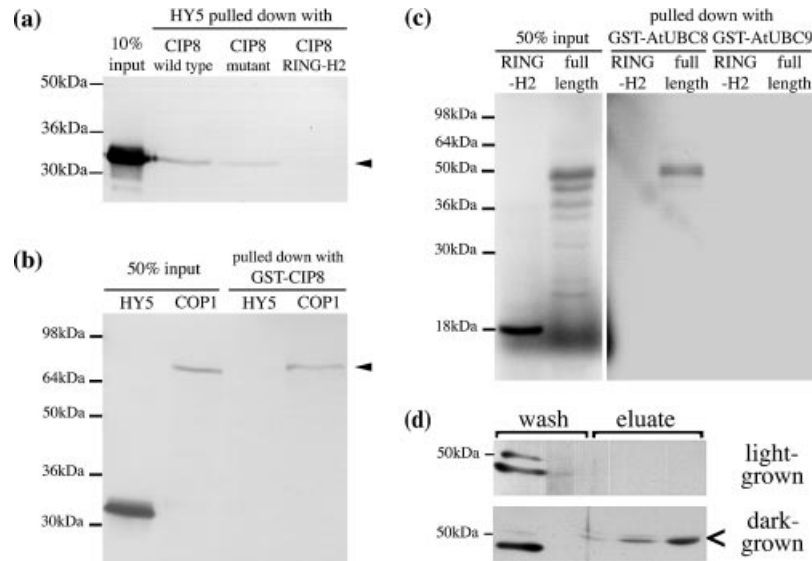


Figure 4. *In vitro* interaction assays between CIP8 and other factors.

(a) Western Analysis of *in vitro* interaction samples of GST fusion proteins of CIP8 (see Figure 3b) and HIS/T7-HY5 (Arrowhead). HIS/T7-HY5 is visualized by anti-T7 tag antibody. Only weak interaction is observed between HIS/T7-HY5 and full-length cip8 constructs, while no interaction is observed with the RING-H2 domain only.

(b) Comparison of the strength of the interaction between GST-CIP8 and HIS/T7-HY5 and T7-COP1-HIS, respectively. Western analysis with anti-t7 tag antibody detects either bacterially purified HIS/T7-HY5 or *in vitro* translated T7-COP1-HIS (Arrowhead). Interaction with the COP1 fusion protein is multiple times stronger than with the hy5 fusion protein.

(c) *In vitro* interaction between GST fusion proteins of E2 enzymes and full-length or truncated CIP8. Autoradiograph of radioactively translated untagged full-length CIP8 or the RING-H2 domain only. The input into the *in vitro* interaction assays is shown after a 6 h exposure, While the assays themselves are shown after a 12 h exposure. GST-Atubc8 pulls down full-length CIP8, but not the RING-H2 domain only. The putative SUMO conjugating enzyme Atubc9 does not interact with either CIP8 version.

(d) Specific interaction of COP1 with CIP8 from dark-grown extract. western analysis of an interaction assay with anti-CIP8 antibody. Protein extract from either light- or dark-grown seedlings was passed over amylose columns that were coupled to a maltose binding protein fusion of the COP1 RING finger domain. Fractions were collected from subsequent wash and elution steps, concentrated and analysed by western blot with anti-CIP8 antibody. The band running at the size of CIP8 (Arrowhead) and a cross-hybridizing band, which might or might not represent a CIP8 isoform, are detected in the wash of light-grown extract but not in the eluate. By contrast, CIP8 that had been bound to the COP1 RING fusion protein on the column is detected in the eluate when extract from dark-grown seedlings is used.

in HY5 and a highly acidic stretch in CIP8. We tested for a possible interaction in both yeast two hybrid and *in vitro* interaction assays. Both full-length CIP8 as well as the RING-H2 domain only were tested against full-length HY5. In yeast two hybrid assays, only very weak interaction was observed for the full length CIP8 but not for the RING-H2 domain only (data not shown). Accordingly, *in vitro* interaction assays (Figure 4a) also reveal a very weak interaction between HIS/T7-HY5 and GST-CIP8. Thus, CIP8 seems to interact very weakly with HY5, pointing to a transient interaction that might be sufficient for ubiquitination preference.

CIP8 interacts with COP1 and the E2 ubiquitin conjugating enzyme AtUBC8 through distinct domains

CIP8 has originally been isolated as a two hybrid interaction clone, whose RING-H2 finger domain interacted with the RING finger domain of COP1 (Torii *et al.*, 1999). This interaction is also very strong *in vitro*, and by comparison considerably stronger than the interaction

with HY5 (Figure 4b). The co-operativity of AtUBC8 and CIP8 suggested that they interact physically, in analogy to other ubiquitin ligases, like Rbx1, A07 or Cbl-b (Fang *et al.*, 2001; Lorick *et al.*, 1999; Seol *et al.*, 1999). We tested this possibility in an *in vitro* interaction assay, using sepharose beads coated with GST fusion protein of AtUBC8 as bait. As interaction partners radioactively labelled untagged full-length CIP8 or the RING-H2 domain only was produced by coupled transcription and translation *in vitro*. Interestingly, despite a predicted size of 37 kDa, *in vitro* translated CIP8 migrated at approximately 50 kDa, similar to its size *in vivo*. In the assay, we observed a strong interaction of AtUBC8 with the full-length CIP8 protein, however, no interaction was seen with the RING-H2 domain only (Figure 4c). Notably, a control GST fusion protein of the likely SUMO conjugating E2 enzyme AtUBC9 did not interact at all with either CIP8 or the RING-H2 domain.

In summary, CIP8 displays strong interactions with both COP1 and AtUBC8. However, while interaction with COP1 is mediated by the RING-H2 domain, interaction with

AtUBC8 is mediated by the more N-terminal portions of CIP8.

In vitro translated COP1 cannot promote ubiquitin ligase activity towards HY5

Some ubiquitin ligases consist of several subunits and have no (e.g. SCF^{Cdc4}) or very little (e.g. SCF^{Grr1}) ubiquitin ligase activity in the absence of the RING-H2 subunit (Seol *et al.*, 1999). However, there are also single molecule E3 ubiquitin ligases, like Cbl-b or Mdm2, which contain a RING finger linked to a protein interaction domain. Analogous to the latter, COP1 contains a RING finger linked to a WD40 domain, which is implicated in substrate recognition (Holm *et al.*, 2001). Thus we investigated whether COP1 could exhibit ubiquitin ligase activity. Since we were not able to obtain soluble and fully integral full length COP1 fusion protein by bacterial expression, we expressed the protein in a coupled transcription and translation system. Our attempts to detect conjugation of ubiquitin to HY5 mediated by this COP1 fusion protein were not successful and there was no evidence of cooperativity between CIP8 and COP1 in these experiments. In summary the results suggest that addition of *in vitro* translated COP1 protein does not result in detectable ubiquitin ligase activity in our assay and fails to affect CIP8's ligase activity.

COP1 preferentially interacts with native CIP8 from dark-grown seedling extract

To determine a possible co-localization of COP1 and CIP8 *in vivo*, we examined protein extracts from light- and dark-grown seedlings by Western analysis of gel filtration chromatography fractions. While the localization of COP1 and CIP8 overlaps in these gel filtrations (data not shown), no significant shift in the CIP8 pattern was observed in a *cop1-4* mutant background. Thus, COP1 and CIP8 do not seem to form a permanent complex, or a complex stable enough to survive the gel filtration procedure.

In order to determine whether native CIP8 can form a complex with COP1 at all, we conducted a column binding experiment using the bacterially expressed RING domain of COP1 to purify CIP8 from light- and dark-grown seedling extracts. Amylose resin columns were coupled to a fusion protein of maltose binding protein to the COP1 RING domain (amino acids 22–107, MBP-COP1R). These columns were then washed and subsequently incubated with protein extract from light- or dark-grown *Arabidopsis* seedlings. The columns were washed again and finally bound protein was eluted. Fractions collected from the wash and the elution were concentrated and analysed by Western analysis using anti-CIP8 antibody. Strikingly, in this experiment the immobilized MBP-COP1R selectively

bound to native CIP8 protein from dark-grown seedling extract (Figure 4d). The binding of COP1 to CIP8 from light-grown seedlings was absent even if an excess of 10 times the total light-grown protein was used. This contrast suggests that the interaction between the RING fingers of COP1 and CIP8 is not due to general aggregation, but that it is specific to the dark-grown extract. We have yet to identify the factors that regulate this important interaction.

Discussion

No ubiquitin ligase activity can be detected from COP1 alone in vitro

The activity of E3 ubiquitin ligases can in principle be divided into two tasks: (i) the selection of target proteins bound for degradation; and (ii) the marking of these proteins by the attachment of multi-ubiquitin chains with the aid of an E2 ubiquitin conjugating enzyme. While some ubiquitin ligases consist of multiple subunits, like those of the SCF, VBC and APC families, others are single polypeptides, like those of the HECT type (e.g. E6-AP) or RING finger type (e.g. Cbl, Mdm2) families (Jackson *et al.*, 2000). In multimeric E3 ligases, the two functions are frequently performed by individual subunits. By contrast, in HECT and RING finger type E3s both functions are retained in a single molecule. Based on the observation that COP1 contains a RING finger domain and is required for proteasome-mediated degradation of HY5, we originally proposed that COP1 might be a single polypeptide E3 (Osterlund *et al.*, 2000). Our results, however, indicate that *in vitro* translated COP1 fusion protein fails to promote ubiquitin chain formation *in vitro* and ubiquitin conjugation to HY5. Nevertheless, it is important to note that COP1 might not be fully functional in our *in vitro* system. For instance, COP1 might require post-translational modifications for ubiquitin ligase activity that are not provided in a wheat germ extract translation system. Thus, the technical limitations of working with recombinant COP1 protein prevent a final verdict on this issue, which will be the subject of future investigations.

CIP8 possesses strong ubiquitin ligase activity

In an alternative scenario, COP1-mediated degradation might require accessory factors. Analogous to F-box proteins in SCF complexes, COP1's primary role could be the recruitment of substrate proteins to an E3 complex by contacting them through its WD40 domain. A prime candidate for an accessory factor of COP1 is the RING-H2 finger protein CIP8. Some RING-H2 proteins have been shown to be either absolutely required for ubiquitin ligase activity of their respective complexes (e.g. SCF^{Grr1}), or strongly enhanced the activity of otherwise only weakly

active E3s (e.g. SCF^{Cdc4}) (Seol *et al.*, 1999). Indeed, co-operation of the SCF complex RING-H2 subunit Rbx1 with a cullin subunit and an E2 ubiquitin conjugating enzyme is sufficient for ubiquitin ligase activity *in vitro*. A number of other RING finger proteins require a cooperating E2 enzyme only (Lorick *et al.*, 1999). Even more striking, the RING-H2 subunit of the APC complex is capable of ubiquitinating APC substrates *in vitro* with the sole help of an E2 enzyme (Gmachl *et al.*, 2000). In analogy, CIP8 has E2 enzyme-dependent ubiquitin ligase activity and can mediate ubiquitin attachment to HY5.

The RING-H2 domain of CIP8 is required but not sufficient for ubiquitin ligase activity

The analogy also applies to the role of the RING-H2 domain of CIP8, which is required for ubiquitin ligase activity. However, it is not sufficient, most likely because the N-terminus is required to mediate interaction with AtUBC8. By contrast, BRCA1 and Cbl-b interact with the E2 enzyme directly through their RING domains (Fang *et al.*, 2001; Hashizume *et al.*, 2001), while AO7, like CIP8, uses a domain next to its RING-H2 finger (Lorick *et al.*, 1999). Even more complex, Rbx1 seems to require cullin as an interaction partner for recruitment of the E2 enzyme Cdc34 (Kamura *et al.*, 1999; Seol *et al.*, 1999; Skowrya *et al.*, 1999; Tan *et al.*, 1999). Thus, the direct interaction of N-terminal regions of CIP8 with AtUBC8 represents a variation of the theme.

The RING-H2 domain of CIP8 serves multiple tasks

The RING-H2 domain of CIP8 is in addition required to mediate interaction with COP1 and thus seems to serve multiple tasks. Indeed, dual use of the RING domain has also been observed in other cases. For instance, while all point mutations in the conserved RING-H2 residues of Rbx1 abolish its ubiquitin ligase activity, some also affect interaction with the E2 enzyme Cdc34 as well as with cullin (Chen *et al.*, 2000). Also, BRCA1 uses its RING domain for interaction with the E2 enzyme as well as for ubiquitin ligase activity (Hashizume *et al.*, 2001). Thus, the two functions in COP1 interaction and ubiquitin ligation of the RING-H2 finger of CIP8 must not be mutually exclusive.

CIP8 mediates ubiquitination of HY5

The genetic and physical interaction between the COP1 and HY5 proteins is well documented, and their physical interaction leads to the proteasome-mediated degradation of HY5 (Ang *et al.*, 1998; Osterlund *et al.*, 2000). Should CIP8 indeed co-operate with COP1 *in vivo*, it is not surprising that it is able to ubiquitinate HY5 *in vitro* by itself. In fact, this would again be in line with the findings

for other RING subunits of multimeric ubiquitin ligases. However, CIP8 also aids ubiquitination of an unrelated control protein, *S. cerevisiae* AOS1. Though this result casts some doubt on the specificity of HY5 ubiquitination through CIP8, it is conceivable that in a cellular environment the rate-limiting factor in ubiquitination of a particular substrate is the physical proximity of all components required. Consequently, in a test tube reaction any substrate might be ubiquitinated due to an excess of substrate and minimum complexity of the mixture. Thus, preference of CIP8 for ubiquitinating HY5 if both proteins are presented in the same reaction is the more remarkable and hints to the possibility that HY5 could also be a preferred substrate *in vivo*. Moreover, other factors (such as COP1) might exist that give additional specificity to the target selection *in vivo*. For instance, SGT1 has been described as an essential gene that is part of an SCF complex for a specific function but is not needed for reconstitution of generic ubiquitin ligase activity *in vitro* (Kitagawa *et al.*, 1999).

Relevance of the in vitro interactions between CIP8, COP1, HY5 and AtUBC8 for an in vivo model

The strong interactions between CIP8 and both COP1 and AtUBC8 provide circumstantial evidence that these proteins might co-operate *in vivo*. The fact that CIP8 and COP1 do not co-localize exclusively in gel filtration experiments argues that if such a scenario is true, it might not represent a constant state in the cell. Thus, interactions between CIP8 and COP1 might be transient. Further, CIP8 might be a very basic factor that transiently associates with different specificity factors, in analogy to Rbx1, which is found in different SCF as well as VHL complexes. Indeed, our unsuccessful attempts to regenerate transgenic plants overexpressing a CIP8 antisense RNA and the lack of overexpression in regenerated transgenics carrying a CIP8 overexpression construct (H. Okamoto, unpublished) hint to the possibility that CIP8 performs an essential cellular function.

Additional evidence further strengthens a case for *in vivo* interaction between CIP8 and COP1. Our finding that recombinant MBP-COP1R specifically binds native CIP8 from dark-grown seedlings, but not from light-grown seedlings, implies that their interaction might be dependent upon a limited set of physiological conditions or other proteins. For instance, although CIP8 is just small enough to possibly nuclear localize by diffusion through nuclear pores, it does not contain an effective nuclear localization signal itself (Torii *et al.*, 1999). Conceivably, CIP8-COP1 interaction depends on their simultaneous presence in a subcellular compartment. This could itself be subject to developmental control, which is at least the case for COP1 (von Arnim and Deng, 1994).

Conclusion

Our results provide biochemical evidence that CIP8 possesses a strong ubiquitin ligase activity, which requires an E2 ubiquitin-conjugating enzyme. This ubiquitin ligase activity can be directed towards HY5. Since CIP8 also strongly interacts with COP1, we speculate that *in vivo* a module of AtUBC8 and CIP8 can team up with COP1 to ubiquitinate substrates recruited by COP1's WD40 domain.

Clearly, future experimental designs have to verify this working hypothesis *in vivo*. To conduct these experiments, a number of technical constraints have to be overcome. For instance, immunoprecipitation experiments from plant extract are not yet feasible due to the failure of our antibodies to immunoprecipitate their primary antigen (C. Hardtke, unpublished). An alternative approach might be the use of transgenic or transient expression systems with antigen-tagged expression constructs. Also, in the absence of a mutant the physiological role of CIP8 is hard to determine. An insertion in the 3' region of CIP8 reported previously (Vielle-Calzada *et al.*, 2000) did not affect CIP8 mRNA or protein levels (C. Hardtke and H. Okamoto, unpublished).

Nevertheless, in several attempts to isolate COP1 interacting proteins by different methods, CIP8 is the only protein isolated so far that specifically interacts with the RING finger domain of COP1. The requirement of this domain for COP1 function has been clearly demonstrated (Stoop-Myer *et al.*, 1999; Torii *et al.*, 1998). Thus, while caution must be exercised in the interpretation of our results, they provide a basis of testable predictions for future investigations in an *in vivo* system.

Experimental procedures

Protein purification from bacterial extracts

The cDNAs of AT2447, AtUBC8, CIP8, CKII β 1, CKII β 2, CKII β 3, HY5, PRT1 and ScAOS1 as well as truncated or mutated CIP8 and HY5 sequences were cloned into vectors of the pGEX (Amersham-Pharmacia, Little Chalfont, Buckinghamshire, UK; AtUBC8, AtUBC9, CIP8, CKII β 1, CKII β 2, CKII β 3, PRT1 constructs) or pET28 (Novagen, Madison, WI, USA; HY5, ScAOS1) series to give in frame fusions with the GST (pGEX) or HIS/T7 (pET) tags. The fusion proteins were expressed in bacterial cultures of the *E. coli* strains XL1B (pGEX) or BL21(DE3) (pET) in a volume of 100 ml. 5 ml extracts were prepared from the cell pellets in 1 \times PBS (pGEX) or 25 mM Tris-HCl pH 7.5, 500 mM NaCl, 5 mM imidazole (pET) and 0.01% Triton X-100. Fusion proteins were bound to either glutathione sepharose beads (pGEX; Amersham-Pharmacia) or charged Ni²⁺ sepharose (pET; ProBond resinTM, Invitrogen) by adding a bed volume of 100 μ l and incubating for 15 min at RT. The beads were then washed four times with 1.5 ml of 25 mM Tris-HCl pH 7.5, 300 mM NaCl and 0.01% Triton X-100. Proteins were eluted in 300 μ l of 25 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.01% Triton X-100 supplemented with 10 mM reduced glutathione (pGEX) or 1 M imidazole (pET). Finally, 200 μ l of glycerol was added and the proteins were stored at -80°C.

Integrity and concentration of the fusion proteins were determined by running aliquots on SDS-PAGE and staining them with Coomassie blue.

Ubiquitination assays

Ubiquitination reactions were done in a total volume of 25 μ l in conjugation buffer (50 mM Tris-HCl pH 7.5, 10 mM phospho creatine, 10 mM MgCl₂, 1 mM ATP, 0.2 mM DTT, 0.05 mM ZnCl₂) supplemented with 500 ng of E1 ubiquitin activating enzyme from yeast (Affiniti, Exeter, UK), 0.1 units of phospho creatine kinase (Sigma, Oakville, Ontario, Canada) and 2 μ g of ubiquitin (Sigma; chain formation assays) or mutant K48R ubiquitin (Affiniti). As E2 ubiquitin conjugating enzyme, approximately 500 ng of GST-AtUBC8 was added. Both *Arabidopsis* UBC8 and UBC9 were produced by bacterial expression. GST-CIP8 or other RING finger components were added at approximately 100 ng, while HIS/T7-HY5 or ScAOS1 substrate was added at approximately 200 ng or 500 ng, respectively. Reactions were incubated at 30°C for 1–2 h and stopped by adding an equal amount of 2 \times sample buffer (100 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 2% beta-mercaptoethanol, 0.001% bromophenol blue).

In vitro translations

For coupled *in vitro* transcription/translation, a fragment encoding a fusion of the T7 tag to amino acid 7 of the full-length COP1 cDNA or the RING finger domain only (amino acids 7–103) was cloned into pCRT7/CT-TOPO (Invitrogen, Burlington, Ontario, Canada) in frame with the c-terminal HIS tag. To produce untagged *in vitro* translated CIP8 or its RING-H2 domain only, respective fragments were cloned into the same vector, however, not in frame with the HIS tag. *In vitro* translations were performed in the TNT T7 Coupled Wheat Germ Extract System (Promega, Madison, WI, USA) according to the manufacturer's instructions. To purify COP1 fusion proteins from these reactions, they were filled up to 1 ml with 25 mM Tris-HCl pH 7.5, 300 mM NaCl and 0.01% Triton X-100, and the protein was captured with a bed volume of 20 μ l ProBond Ni²⁺-sepharose (Invitrogen). The beads were washed twice in ubiquitination assay buffer before they were added to conjugation reactions.

Gel filtration assays, Western analysis and antibodies

Gel filtration assays were performed as described (Torii *et al.*, 1998). SDS-PAGE and Western analysis was done according to standard procedures and proteins were visualized using either a chemiluminescent detection system (Amersham-Pharmacia; to detect native COP1 and CIP8) or alkaline phosphatase based chromogenic detection. The primary antibodies for the detection of native COP1 and CIP8 have been described previously (Torii *et al.*, 1998; 1999). To detect T7- or HIS-tagged proteins in our assays either alkaline phosphatase coupled anti-T7 tag antibody (Novagen) or anti-HIS tag antibody (Clontech, Palo Alto, CA, USA) was used. To detect ubiquitin chains, an antiubiquitin antibody (Affiniti) was employed.

Kinase assays

Kinase assays were done as described (Hardtke *et al.*, 2000) in 50 mM HEPES pH 7.5, 1 mM MgCl₂, 0.2 mM CaCl₂ and 0.1 mM EGTA in a total volume of 25 μ l. Bacterially expressed casein

kinase II alpha subunit from *Zea mays* (Calbiochem, San Diego, CA, USA) was added at 10 ng, while GST fusion protein of individual *Arabidopsis* beta subunits was added at approximately 50 ng. To test autophosphorylation of the kinase, or to test phosphorylation of HIS/T7-HY5 (200 ng were added) the reactions were performed in the presence of ^{32}P - γ -ATP. Phosphorylated proteins were detected by autoradiography.

To test a possible influence of the phosphorylation on HIS/T7-HY5 ubiquitination, 1 μg of HIS/T7-HY5 was phosphorylated using 250 ng of *Zea mays* CKII α and 500 ng of *Arabidopsis* CKII β 2 in the presence of 5 mM ATP for 30 min. at RT in a total volume of 50 μl . This reaction was then filled up to 1 ml with 25 mM Tris-HCl pH 7.5 and 150 mM NaCl, and HIS/T7-HY5 was captured with a bed volume of 10 μl and washed twice in the same buffer. The beads were resuspended to give a total volume of 20 μl and half of it was transferred into a new tube. MnCl_2 was added to both tubes to 2 mM, and one tube was supplemented with 800 units of lambda protein phosphatase (New England Biolabs, Beverly, MA, USA). After 15 min incubation at RT, the bead-bound HIS/T7-HY5 was washed twice more and finally added as a substrate to ubiquitination assays containing GST-CIP8.

In vitro interaction assays

To test *in vitro* interactions, glutathione sepharose beads (Amersham-Pharmacia) were washed in interaction buffer (25 mM Tris-HCl pH 7.5, 5 mM MgCl_2 , 1 mM DTT, 150 mM NaCl, 0.05 mM ZnCl_2 , 1% BSA, 0.01% Triton X-100) and resuspended in 1 ml of the buffer. An excess of the GST fusion protein in the assay was added to the beads and the samples were nutated for 30 min at RT. The beads were washed in the buffer once more and resuspended again in 1 ml. The protein to be tested for interaction was then added and reactions were then nutated o/n at 4°C. Finally, the beads were washed three times in 1.5 ml of ice-cold NTET buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.01% Triton X-100) and resuspended in 20 μl . An equal amount of 2 \times sample buffer was added, and the amount of bound test protein was determined by Western analysis or autoradiography.

Column binding experiment

The RING finger of COP 1 (amino acids 22–107) was expressed as a maltose binding protein fusion in *E. coli* (MBP-COP1R). The cell extract was split into two and bound to amylose resin columns (New England Biolabs). The columns were then washed with 1 M NaCl to insure that only MBP-COP1R was bound. For plant extracts, 6-day-old seedlings grown in continuous light or dark were ground in liquid nitrogen with grinding buffer (400 mM sucrose, 50 mM Tris-HCl pH 7.5, 10% glycerol, 2.5 mM EDTA, 1 mM PMSF). The extracts were spun for 10 min in a microcentrifuge to remove cellular debris, filtered through a 0.2- μm filter and loaded onto the previously prepared MBP-COP1R columns. The columns were then washed with 200 mM NaCl (Column buffer: 20 mM Tris-HCl pH 7.5, 3 mM β -mercaptoethanol and 0.1 μM ZnCl_2) and the bound proteins eluted with column buffer supplemented with 10 mM maltose and 50 mM NaCl. 1 ml fractions were collected and concentrated using Strataclean beads (Stratagene, La Jolla, CA, USA), separated by SDS-PAGE (10%), and analysed by Western blot.

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