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**Developmental Regulation and Light Signal
Transduction in Plants: The FUS5 Subunit of the
COP9 Signalosome**

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- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

The collaboration was primarily complementary and supportive. There was free exchange of materials and information between the two laboratories. This resulted in six publications. However, by most standards, these publications should be considered joint publications. For example, Figure 6 in Serino et al., (2003) employed materials provided by the Chamovitz lab, while figures in the two manuscripts coming out of the Chamovitz lab used materials provided by the Deng lab.

Dr. Chamovitz visited the Deng lab once during the funding period. Students from Prof. Deng's lab were supposed to visit the Chamovitz lab, but unfortunately the security situation during the funding period were not conducive for the visit. Because of this, Dr. Chamovitz met with two scientists from the Deng lab, Drs. Wei and Schwechheimer, at a meeting in Turkey (which was originally supposed to be held in Israel).

Introduction

Plants adjust their growth and development in a manner optimal for the prevailing light conditions. The molecular mechanisms by which light signals are transduced and integrated with other environmental and developmental signals are an area of intense research. (Batschauer, 1999; Quail, 2002) One paradigm emerging from this work is the interconnectedness of discrete physiological responses at the biochemical level, for instance, between auxin and light signaling (Colon-Carmona et al., 2000; Schwechheimer and Deng, 2001; Tian and Reed, 1999) and between light signaling and plant pathogen interactions (Azevedo et al., 2002; Liu et al., 2002).

The COP9 signalosome (CSN) protein complex has a central role in the light control of plant development. Arabidopsis mutants that lack this complex develop photomorphogenically even in the absence of light signals (reviewed in (Karniol and Chamovitz, 2000; Schwechheimer and Deng, 2001). Thus the CSN was hypothesized to be a master repressor of photomorphogenesis in darkness, and light acts to bypass or eliminate this repression. However, the CSN regulates more than just photomorphogenesis as all mutants lacking this complex die near the end of seedling development. Moreover, an essentially identical complex was subsequently discovered in animals and yeast, organisms whose development is not light responsive, exemplifying how plant science can lead the way to exciting discoveries in biomedical model species (Chamovitz and Deng, 1995; Freilich et al., 1999; Maytal-Kivity et al., 2002; Mundt et al., 1999; Seeger et al., 1998; Wei et al., 1998).

Our long-term objective is to determine mechanistically how the CSN controls plant development. We previously found that this complex contains eight subunits (Karniol et al., 1998; Serino et al., 1999) and that the 27 kDa subunit is encoded by the *FUS5/CSN7* locus (Karniol et al., 1999). The CSN7 subunit also has a role extraneous to the COP9 signalosome, and differential kinase activity has been implicated in regulating CSN7 and the COP9 signalosome (Karniol et al., 1999). In the present research, we further analyzed CSN7, both in terms of interacting proteins and in terms of kinases that act on CSN7. Furthermore we completed our analysis of the CSN in Arabidopsis by analyzing the remaining subunits.

Outline of Original Objectives and Subsequent Modifications

The general goal of the proposed research was to study the CSN7 (*FUS5*) subunit of the COP9 signalosome. To this end we specifically intended to:

1. Identify the residues of CSN7 that are phosphorylated.
2. Monitor the phosphorylation of CSN7 under different environmental conditions and under different genetic backgrounds.
3. Generate transgenic plants with altered CSN7 phosphorylation sites.
4. Purify CSN7 kinase from cauliflower.
5. Clone the Arabidopsis cDNA encoding CSN7 kinase
6. Isolate and characterize additional CSN7 interacting proteins.
7. Characterize the interaction of CSN7 and the COP9 signalosome with the HY5-COP1 transcriptional complex.

Throughout the course of the research, emphasis shifted from studying CSN7 phosphorylation (Goals 1-3), to studying the CSN7 kinase (Goal 4 and 5), an in depth analysis of CSN7 interactions (Goal 6), and the study of additional CSN subunits. Goal 7 was also abandoned as no data was found to support this interaction.

Results

Goals 4 and 5. Analysis of the CSN7 kinase

In our previous BARD-funded research, we showed that the CSN7 subunit is a phospho-protein. (Karniol et al., 1999) To identify the kinase(s) which act on CSN7, we adopted a biochemical approach to purify the kinase activity. Using cauliflower as a model system for

Arabidopsis, total soluble proteins were fractionated over an ion exchange column, and the eluted fractions were assayed for CSN7 kinase activity. Two peaks of CSN7 kinase activity were detected, one which elutes at low salt, and one which elutes at high salt, suggesting that two distinct kinases, which we have designated CSN7 kinase 1 and CSN7 kinase 2, phosphorylate CSN7.

CSN7 kinase 1 was further purified over several columns. Gel filtration analysis indicates that CSN7 kinase 1 is present in a 250 kD fraction. This suggests that FFK1 is a part of a protein complex. This protein complex was further purified over a MonoQ column, and silver staining of the resulting CSN7 kinase 1 -active fraction revealed a small number of proteins. These bands were excised and analyzed by MALDI-TOF to identify the proteins.

The identification of a putative ATM-like protein as a CSN7 kinase in Arabidopsis is especially intriguing. ATM is known to be a master regulator of cellular responses to DNA damage, and one of its modes of action is through selective phosphorylation of key signaling molecules (REF). Furthermore, ATM from humans was recently reported to bind the COP9 signalosome (REF). Because of this, we have chosen to further analyze the Arabidopsis ATM. Two Arabidopsis mutant strains that contain T-DNA insertions in ATM were obtained from the Arabidopsis Stock center. These strains have been confirmed as being null for ATM. We are now monitoring these plants for CSN7 kinase activity.

Goal 6 - Analysis of CSN7-interacting proteins

To identify the immediate interacting partners of CSN7, an interaction-trap screen using the yeast two-hybrid assay to isolate CSN7 interacting proteins. *Csn7* was cloned as a *lexA* fusion, and transformed in yeast cells together with an Arabidopsis library that was constructed in the plasmid pJG4-5. This plasmid contains a transcriptional activation domain followed by the library cloning site. 1200 leucine auxotrophs were isolated following transformation of 5×10^6 yeast cells. Out of these, 44 showed β -galactosidase activity strictly dependent of galactose. These strains were chosen for further analysis.

These results together with our comprehensive study of all COP9 signalosome subunits, indicates that CSN7 interacts with at least ten proteins – four CSN subunits and five other proteins. To identify the regions on CSN7 responsible for these interactions, a series of CSN7 deletion clones were constructed and their interactions with these proteins were tested in a direct yeast two-hybrid assay. As seen in Figure 1 and 2, the three PCI-containing CSN subunits interact with CSN7's PCI domain, while CSN6, which does not have a PCI domain, interacts with the CSN7 COOH-terminus. The CSN PCI-mediated interactions seem specific, as two non-CSN PCI proteins, RPN6 and eIF3e, interact with the CSN7 N-terminus. RNR2a and an unknown protein that we have called PCIP2 (see below), interact with the CSN7 COOH-terminus. The non-PCI regions thus appear to mediate interactions with extra-complex proteins.

Figure 1. CSN7 interacts with different proteins through different regions:

A Direct yeast two hybrid β -galactosidase assay for interaction between CSN7 and: (upper panel) other proteins; (lower panel) other CSN subunits. Suspensions of yeast (equal densities) were grown on a galactose inductive media with X-gal for 3 days at 30 °C.

Figure 2 **Model for CSN7 interactions.** Based on the results in Figure 1. The darker blue rectangle symbolizes PCI domains.

Two proteins were chosen for further confirmation of the interaction, PCIP2 and RNR2a.

PCIP2 interacts with CSN7 and eIF3e in vitro

One of the CSN7 interacting genes encodes a protein that we have named PCI Complex Interacting Protein 2 (PCIP2) as it was also identified in an independent project to identify eIF3e interacting proteins. As PCIP2 was identified in two independent yeast two hybrid screens using baits that are known to interact with each other, this clone was chosen for further study. A manuscript describing the analysis of PCIP2 is currently in review:

PCIP2, a novel plant-specific protein, interacts with both eIF3e/INT6 and COP9 signalosome subunits 7 and 8 from Arabidopsis

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Abstract

Background

eIF3e/Int6 has been described as a component of eIF3, as an interactor of the COP9 signalosome (CSN), and as a regulator of protein turnover through binding components of the proteasome regulatory lid. Integration of mouse mammary tumor virus into the mouse *eIF3e/Int6* gene is tumorigenic. eIF3e/INT6 has a carboxy terminal PCI domain.

Results

Ten putative interactors of eIF3e/INT6 have been identified. These include five subunits of the CSN, a subunit of the proteasome regulatory lid, and a component of the ribosome, all consistent with proposed roles for eIF3e/Int6. The PCI domain of eIF3e/Int6 mediates interaction with other proteins that contain this amino acid motif. eIF3e/INT6 also interacts with a novel protein termed here PCI Complex Interacting Protein 2 (PCIP2). PCIP2 was also identified in a screen for proteins that interact with CSN7. PCIP2 binds eIF3e/INT6 and CSN7 both in yeast and in vitro. Database searches indicate the PCIP2 is a plant-specific protein, with related proteins found also in eubacteria. The bacterial *Pcip2*-related genes are found in the *dnaX-recR* operon.

Conclusion

Our results, together with those garnered from other systems, indicate that eIF3e/INT6 is a multifunctional protein, interacting with the three PCI complexes, the proteasome, CSN and eIF3, as well as PCIP2. PCIP2 is a plant-specific gene with a putative role in DNA repair.

Background

The PCI complexes are family of multimeric protein complexes that regulate signaling at the level of protein degradation and translation. (Kim et al., 2001) They include the regulatory lid of the 26S proteasome ("P"), the COP9 signalosome ("C"), and the eukaryotic translation initiation factor eIF3 ("T"). Subunits of all three complexes contain one of two domains, the PCI domain or the MPN domain. (Aravind and Ponting, 1998; Hofmann and Bucher, 1998) Six lid, six CSN and three eIF3 subunits contain the PCI motif, which is characterized by a conserved predicted α -helical secondary structure, but with a divergent primary sequence. This motif is proposed to have a role in stabilizing the general architecture of the complexes. (Freilich et al., 1999; Kapelari et al., 2000; Tsuge et al., 2001) The shared domains suggest that the three complexes or their subunits have a common evolutionary ancestor. Indeed, subunits of the COP9 signalosome are paralogous in a one-to-one relationship to subunits of the proteasome lid, whereas eIF3 is more distantly related. Data from diverse systems indicate that the relationship extends beyond evolution, as the three complexes may have a common agenda in regulating protein stability, and certain subunits from one complex can interact with specific subunits for a second complex. (Kim et al., 2001; Schwechheimer and Deng, 2001)

One protein that has been shown to interact with all three complexes is eIF3e/INT6. (von Arnim and Chamovitz, 2003) eIF3/INT6 has been identified independently at least four times: 1) As an integration site for mouse mammary tumor virus (MMTV); (Marchetti et al., 1995) 2), as a subunit of eIF3; (Asano et al., 1997) 3) as an interactor of the COP9 signalosome (CSN); (Karniol et al., 1998) 4) and as a mediator of multi-drug resistance in *S. pombe*. (Crane et al., 2000) Recently, eIF3e/INT6 was also identified as an interactor of the proteasome lid. (Yen et al., 2003) It is a conserved 48 kDa protein that has been recognized in most eukaryotes, with the noted exception of *S. cerevisiae*, which has a related protein. (Shalev et al., 2001) Overexpression of a truncated gain-of-function alleles of eIF3/INT6 are tumorigenic. (Mayeur and Hershey, 2002)

eIF3 is responsible for loading of the 40S small ribosomal subunit with the ternary complex, composed of eIF2, GTP, and tRNA^{Met}, and for recruiting the resulting 43S complex to the mRNA marked by the cap-binding complex, eIF4F. The plant eIF3 at present is thought to contain eleven subunits. (Burks et al., 2001) eIF3e/Int6 persistently copurifies as a subunit of eIF3 in numerous systems, and physically interacts with a number of eIF3 subunits. While not essential for translation, a regulatory role for eIF3e/INT6 has been proposed in mammalian cells, fission yeast, and plants (reviewed in) (von Arnim and Chamovitz, 2003).

However eIF3e/INT6 has roles that are independent of eIF3. While eIF3e/INT6 may interact with eIF3 in the cytoplasm, it also has a nuclear role in plants and animals. (Desbois et al., 1996; Guo and Sen, 2000; Yahalom et al., 2001) We previously showed that the subcellular localization of eIF3e/INT6 in Arabidopsis is tissue specific, being nuclear in roots and cytoplasmic in leaves. We further showed that eIF3/INT6 copurifies with CSN and directly interacts with CSN subunit 7 (CSN7). (Karniol et al., 1998; Yahalom et al., 2001) These results were confirmed also for mammalian cells. (Hoareau Alves et al., 2002) Recent work in *S. pombe* has indicated that eIF3e/INT6 has a major role in regulating protein turnover through directly binding to and regulating the nuclear import of a proteasome lid subunit, thus specifically interfering with the regulated degradation of cell cycle regulators such as CDC13 and CUT2. (Yen et al., 2003)

To further elucidate the role of eIF3e/INT6 in Arabidopsis, we have identified eIF3e/INT6 interacting proteins. Our results show that eIF3e/INT6 interacts with CSN, proteasome and ribosomes. We also identified a novel unannotated Arabidopsis protein that may bind both eIF3 and CSN.

Results

Isolation of eIF3e interacting proteins

To identify proteins that interact with eIF3e/INT6, we have undertaken an interaction-trap screen using the yeast two-hybrid assay. Since eIF3e fused to LexA activates the *LacZ* reporter gene by itself (not shown), two partial eIF3e constructs, encoding the N-terminal 292 amino acids, and the C-terminal 153 amino acids that contains the PCI domain, each fused to LexA, were used as baits. These fusion proteins do not activate the reporter genes. *eIF3e-PCI* or *eIF3e-N48*, each cloned as a *LexA* fusion, was transformed in yeast cells together with a cDNA expression library extracted from a six-day-old light-grown *Arabidopsis* seedlings [21]. Following screening of 1.6×10^6 and 6×10^9 yeast colonies for *eIF3e-N48* and *eIF3e-PCI* respectively, seven and 152 independent putative interacting clones were identified as leucine auxotrophs and showed β -galactosidase activity strictly dependent on galactose. Restriction analysis indicated that these clones represent two and three distinct cDNAs for eIF3-Nterm and eIF3-PCI respectively. The putative eIF3e interacting proteins were retransformed back into the original yeast strain to ensure that they do not activate the reporter genes by themselves, or with a non-specific bait.

The five eIF3e interactors are detailed in Table 1. These interactors fall into two classes – expected interactors, i.e. those whose interaction with eIF3e can be readily explained, and those whose function is not obvious. As examples of the first class, the amino terminal half of eIF3e interacts with the S9 protein subunit of the 40S ribosome small subunit (clone N1), while the PCI-carboxyl terminus interacted with RPN12a, (Smalle et al., 2002) the *Arabidopsis* homologue of subunit 12 of the 19S regulatory particle of the 26S proteasome (clone P1). These results are consistent with the role of eIF3 binding the 40S ribosomal subunit in the translation preinitiation complex, (Chaudhuri et al., 1997) and with the reported interaction between eIF3 and the proteasome. (Hoareau Alves et al., 2002; Yen et al., 2003) Both the eIF3e amino terminus and the PCI containing clones interact with proteins whose genes have yet to be studied.

Table 1 - eIF3 interacting proteins isolated in the yeast interaction trap screen

Clone	At # / common name	Function
N1	At5g39850 / 40S ribosomal protein S9	Subunit of the 40S ribosomal small subunit
N2	At1g27930	Unknown
P1	At1g64520 / RPN12	Non ATPase subunit of the 19S regulatory complex from 26S proteasome
P2	At5g14200 / IMDH-3	Isopropylmalate dehydrogenase precursor, used in the leucine biosynthetic pathway
P3	At4g30620 / PCIP2	Unknown

N clones interact with eIF3e-N48; P clones interact with eIF3e-PCI.

eIF3e interacts with CSN subunits

We previously showed the eIF3e interacts with CSN7. (Yahalom et al., 2001) As no CSN subunits were identified in the interaction-trap screen, a direct two-hybrid assay was performed between CSN subunits and eIF3e-PCI and eIF3e-Nterm domains as baits. As can be seen in figure 1, the two eIF3e clones did not interact with the Gal4 activation domain. Both constructs show β -galactosidase activity when co-expressed with CSN1, CSN4 and CSN6. The eIF3-PCI construct

also interacts with CSN8, while the N-terminus construct also interacts with CSN7. eIF3e does not interact with CSN5 in these assays.

Figure 1 - Direct yeast two-hybrid of eIF3e/INT6 with CSN subunits

A representative colony for each strain was suspended in YPD, and a 10 μ l sample was placed on Gal/Raf X -Gal plates. Blue color indicates lacZ reporter gene activity. For eIF3e-Nterm, empty pJG4-5 served as the negative control for activation (-) and pJG-S9 served as the positive control for activation (+). For eIF3e-PCI, empty pJG4-5 served as the negative control for activation (-) and pSH17-4 served as the positive control for activation (+).

eIF3e interacts with PCIP2 in vitro

Clone P-3, which interacts with the PCI-containing half of eIF3e, encodes an unannotated protein, At4g30620. This protein was also identified in an independent project to identify CSN7 interacting proteins, (Karniol, 2001) and thus was named PCI Complex Interacting Protein 2 (PCIP2). As PCIP2 was identified in two independent yeast two hybrid screens using baits that are known to interact with each other, this clone was chosen for further study. To substantiate the interaction, an in-vitro pull-down assay was performed between PCIP2 and CSN7 or eIF3e. Binding assays were carried out using constant amounts of recombinant radio-labeled CSN7 or eIF3e and increasing amounts of the recombinant GST-PCIP2 protein. The proteins pulled down by glutathione Sepharose 4B beads were visualized by auto-radiography. As can be seen in figure 2, an increasing CSN7 and eIF3e signal is visible as the amount of recombinant GST-PCIP2 protein increases, while no CSN7 or eIF3e is pulled down with the GST negative control. These results corroborate the interaction detected in yeast.

Figure 2 - PCIP2 interacts in-vitro with eIF3e/INT6 and CSN7

Increasing amounts of recombinant GST-PCIP2 were bound to glutathione sepharose 4B beads and incubated with ~0.3 μ g recombinant 35 S labeled CSN7 (C-F) or eIF3e/INT6 (H-J). Precipitated proteins were eluted

from the beads by boiling in SDS-sample buffer, separated through 12.5% SDS-PAGE, blotted onto a PVDF membrane, and exposed to film. A-B: in vitro transcription/translation product for CSN7 (A) and eIF3e (B). 2.5 μ g (C), 5 μ g (D), 10 μ g (E), 5 μ g (G), 15 μ g (H). F, I: Empty vector controls, 15 μ g GST.

PCIP2 interacts with other CSN subunits

In order to determine if PCIP2 can interact with other CSN subunits, a direct two hybrid assay was carried out with PCIP2-Gal4 as the prey and CSN1, CSN7 and CSN8 fused to LexA binding domain as baits. The reciprocal experiment was impossible as the LexA-PCIP2 fusion protein was unstable (not shown). As shown in figure 3, PCIP2 did not interact with the LexA binding domain, or with CSN1, but did interact with CSN7 and CSN8. The expression of the reporter genes was dependent upon growth on galactose-containing medium.

Figure 3 - PCIP2 interacts in yeast with CSN7 and CSN8

A. Interaction in yeast. A representative colony for each strain was suspended in YPD, and a 10 μ l sample was placed on Gal/Raf X -Gal plates. Blue color indicates lacZ reporter gene activity. Empty pJG4-5 served as the negative controls for activation (-) and pSH17-4 served as the positive control for activation (+). For diploid mating assay, yeast carrying pJG-PCIP2 were mated with yeast carrying pEG-CSN7, pEGCSN8, or empty pEG202 (-). **B. PCIP2 interacts with CSN7 carboxyl terminus.**

CSN7 was cloned in three sub-fragments in pEG202 (Levanon and Chamovitz, in preparation), and checked for their interaction with pJG-PCIP2. The negative controls (-) are empty pEG202 and empty pJG4-5.

To confirm the specificity of the PCIP2 interaction with CSN7 and CSN8 in yeast, the two hybrid assay was repeated in diploid cells. PCIP2 fused to the Gal4 activation domain was transformed into yeast strain L-40 (MAT α), while CSN7 and CSN8 subunits fused to LexA binding domain and the negative control (Empty PEG202) were transformed into EGY48 (MAT α). As can be seen in figure 3, the interaction of PCIP2 protein with both CSN7 and CSN8 appears specific since it does not interact with the DNA binding domain portion of the bait alone.

As CSN7 interacts with eIF3e (Yahalom et al., 2001) and like eIF3e, CSN7 also contains a PCI domain, we checked if PCIP2 interacts with the CSN7-PCI domain. As seen in figure 4c, PCIP2 does not interact with the CSN7-PCI domain by itself, nor with a partial clone comprising the N-terminal region and PCI domain. PCIP2 does interact with a carboxyl terminal clone of CSN7. As the PCI construct alone is sufficient to mediate interaction between CSN7 and other CSN subunits (N. Levanon and D. Chamovitz, unpublished), we conclude that PCIP2 interacts with the carboxyl terminal region of CSN7.

PCIP2 encodes a novel plant protein

At4g30620, *PCIP2*, encodes a novel unannotated Arabidopsis protein of 180 amino acids and pI of 9.7 on chromosome 4. A second gene on chromosome 2, At2g24020, encodes a protein that is 77% identical to PCIP2 (Figure 4). These proteins have no obvious motifs and appear to be plant specific as there are no similar genes in animals.

While no obvious related proteins were identified in animals, most bacteria have one protein related to PCIP2. The region of similarity corresponds to the carboxyl-terminus half of PCIP2 (Figure 4a). The closest relatives of the Arabidopsis genes are from cyanobacteria, suggesting that PCIP2 is originally a prokaryotic gene that was transferred to the plant nuclear genome following endosymbiosis (Figure 4b). While cyanobacterial in origin, PCIP2 does not contain an obvious chloroplast leader sequence, so its functional location is unclear. In several bacteria, including *E. coli* and *B. subtilis*, the bacterial gene is typically found in an operon immediately downstream of the *dnaX* and immediately upstream of the *RecR* genes (Figure 5).

Discussion

To further understand the role of eIF3e/INT6 in Arabidopsis, we have identified proteins that interact with eIF3e/INT6 using the yeast interaction-trap screen. Our results, together with those garnered from other systems, indicate that eIF3e/INT6 is a multifunctional protein, interacting with the three PCI complexes, the proteasome, CSN and eIF3. Consistent with the interaction between

Figure 4 - Sequence comparison and phylogenetic analysis of PCIP2

A. Lineup of the conserved region of PCIP2 with similar bacterial protein. White on black designates conserved residues; white on grey designates similar residues. **B.** Phylogenetic tree based on the alignment in A.

eIF3 and the ribosome, eIF3e/INT6 also interacts with a ribosomal protein. We also present here the first characterization of a novel unannotated Arabidopsis protein that we term PCIP2. eIF3e/INT6 clearly associates with the proteasome, as seen through its interaction with the proteasome lid subunits RPN12 in Arabidopsis (this work), RPN5 in *S. pombe*, (Yen et al., 2003) and with Rpt4, one of the six ATPases in the base of the proteasome lid, in mammalian cells. (Hoareau Alves et al., 2002) In budding yeast, RPN12 has a role in cell cycle control, (Bailly and

*E. coli**B. subtilis*

Figure 5 - Operon structure of PCIP2-like proteins in *E. coli* and *B. bacillus*

Reed, 1999; Kaiser et al., 1999) and in numerous cytokinin-regulated growth responses in

Arabidopsis. (Smalle et al., 2002) Yen et al., (2003) reported that the role of eIF3e/INT6 in binding RPN5 is to regulate its nuclear import, which affects the degradation of specific cell cycle regulators. It is tempting to speculate that eIF3e/INT6 has a similar relationship with RPN12 in Arabidopsis.

One caveat of this study is the use of partial eIF3e/INT6 constructs in the yeast interaction studies. This was necessary due to the self-activation properties of the full length eIF3e/INT6 –LexA fusion protein. However, earlier studies have clearly shown that eIF3e/INT6 clearly binds to CSN subunits in both Arabidopsis, mammalian cells, and *S. pombe*. We previously reported that full length eIF3e/INT6 copurifies with CSN from cauliflower and directly binds the Arabidopsis CSN7 in yeast, in vitro and in planta. (Karniol et al., 1998; Yahalom et al., 2001) We further show here that eIF3e/INT6 also binds Arabidopsis CSN1, 4, 6 and 8, but not CSN5. Horeau et al. (2002) showed an interaction in mammalian cells between full length human eIF3e/INT6 and CSN6, and 7, but not with CSN5, lending support to the interactions reported here.

RPN12, CSN1, CSN4 and CSN8 all contain PCI domains and their interaction with eIF3e/INT6 is dependent on the eIF3e/INT6 PCI domain, further supporting the role for this domain in stabilizing protein-protein interactions in the PCI complexes. However the interactions between eIF3e/INT6 and the proteasome and CSN are not only solely based on PCI-PCI interactions. For example, neither CSN6 nor RPT4 contain the PCI domain. Indeed, CSN6, like PCIP2 and S9, interacts with the N-terminal half of eIF3e, which does not contain the PCI domain. CSN7, which has a smaller PCI domain, interacts well with a full-length eIF3e/INT6, (Yahalom et al., 2001) but only weakly interacts with the N-terminal part of eIF3e, and not at all with the eIF3e/INT6 PCI domain.

PCIP2 was identified in two separate interaction-trap screens using either eIF3e/INT6 (this study) or CSN7 as baits. (Karniol, 2001) The interactions were confirmed by in vitro studies, so they appear biochemically valid. PCIP2 also interacts with a second CSN subunit, CSN8. While the interaction of PCIP2 with eIF3e/INT6 is dependent on the eIF3e/INT6 PCI domain, PCIP2 does not bind all PCI domains. For example, PCIP2 does not bind CSN1, and does not interact with the CSN7 PCI domain, but rather binds its carboxyl terminus. Unfortunately, attempts to identify in plant interactions have been hampered by a lack of efficient anti-PCIP2 antibodies.

As PCIP2 was identified through independent interaction trap screens, it is tempting to speculate that PCIP2 binds two protein complexes, eIF3 and CSN, perhaps with some coordinated function. While the function of PCIP2 remains unknown, genetic analysis of its prokaryotic related proteins hints that it has a function in DNA repair. As eIF3 and CSN have not been found in prokaryotes, PCIP2 function apparently predates any interaction with these complexes. A gene encoding a PCIP2-like protein is found in the bacterial *dnaX/RecR* operon. *dnaX* encodes two different subunits of the Pol-III complex, γ and τ . The *RecR* gene product works together with *RecF* and *RecO* in the process of gap repair. It is not totally clear, why these three genes form an operon, but they apparently do. (Flower and McHenry, 1991) As both CSN and eIF3e/INT6 have recently been implicated in regulating DNA damage responses, (Liu et al., 2003; Yen et al., 2003) this placement of PCIP2 is in the least intriguing, suggesting that it has some role in DNA damage

responses that may be regulated by its interaction with CSN and/or eIF3e/Int6. Further experimental work is needed to test this hypothesis.

Methods

Interaction trap

eIF3e/INT6 interacting proteins were isolated using the yeast interaction-trap essentially as described. (Kwok et al., 1999) The N-terminal 876 bp coding region of Arabidopsis eIF3e/INT6 was cloned into the *EcoRI* - *XhoI* sites of pEG202 using the primers- 5' cgtcagcagagcttcaccattg 3' and 5' ggaactcgagaattggatc 3' to make in frame fusion with LexA to yield plasmid pEGeIF3e-N48. The C terminal 459 bp region of eIF3e/INT6, which contains the PCI domain, was cloned into the *EcoRI* - *Sall* sites of pEG 202 using the primers 5' caattatcgaattcctggc3' and 5' attccagtcgaccgaaggtg 3' to make an in frame fusion with LexA to yield plasmid pEGeIF3e-PCI. Yeast strain EGY48 was transformed with pEGeIF3e-PCI or pEGeIF3e-N48 and the resulting lines were established for re-transformation with a cDNA library in pJG4-5 constructed using Arabidopsis six day-old light-grown seedlings. Following transformation, leucine prototrophs were selected. In a screen of 1.6×10^6 and 6×10^9 yeast colonies for *eIF3-N48* and *eIF3-PCI* respectively, seven and 152 independent putative interacting clones were identified as leucine auxotrophs and showed β -galactosidase activity strictly dependent on galactose. Restriction analysis using *AluI* and *HaeIII* indicated that these clones represent two and three distinct cDNA classes for eIF3-Nterm and eIF3-PCI respectively. The longest representative of each class of genes was sequenced. The putative eIF3e interacting proteins were retransformed back into the original yeast strain to ensure that they do not activate the reporter genes by themselves. In addition, all the genes have been tested non-specific interactions against pRFHM-1. (Ausubel et al., 1995)

At4g30620 (*Pcip2*) was cloned into the *EcoRI* - *XhoI* sites of pEG202 to make an in frame fusion with LexA to make pEG:PCIP2. *Pcip2* was cloned as an in frame fusion with GST in pGEX-5x-3 (Pharmacia). For in vitro transcription and translation, *CSN7* and *eIF3e* were cloned in pCITE-3a (Novagen). pEG202:CSN1, pJG:CSN1, pJG:CSN5, pJG:CSN8; (Kwok et al., 1998) pJG:CSN4; (Serino et al., 1999) pJG:CSN6; (Peng et al., 2001a) pEG202:CSN7, pJG:CSN7; (Karniol et al., 1999) and pEG202:eIF3e (Yahalom et al., 2001) have been described.

Biochemical methods

GST-fusion proteins were isolated from *E. coli* lysates over a glutathione Sepharose 4B bead slurry (Pharmacia) according to standard procedure (Yahalom and Chamovitz, 2002). A TNT coupled reticulocyte lysate system (Promega) was used to radiolabel proteins according to manufacturer's instructions. 2 μ l of 35 S-labeled protein, out of a 50 μ l TNT labeling reaction (~0.3 μ g protein), was incubated with an increasing amount of GST fusion protein. Sample volumes were increased by adding 50% bed volume of Glutathione Sepharose 4B, and incubated for four hours at 4°C, washed 6 times with NETN (100mM NaCl, 1mM EDTA, 20 mM Tris HCl pH 8.0 and 0.5 % NP40) and suspended with sample buffer. The samples were separated on 12.5% SDS-PAGE, blotted on a PVDF (Immobilon TM-P, Millipore) membrane and exposed to film.

Phylogenetic and bioinformatics analysis

Pcip2 has been deposited in GenBank under accession number AY327123. Database searches were performed with a nonredundant data set constructed from current releases of SwissProt, TrEMBL, and GenPep. Generalized profile (Bucher et al., 1996) construction and searches were performed using the pftools package, version 2.1. (Philipp Bucher, available from <ftp://ftp.isrec.isb-sib.ch/sib-isrec/pftools/>). Profiles were constructed using the BLOSUM45 substitution matrix with default penalties of 2.1 for gap opening and 0.2 for gap extension. (Henikoff

and Henikoff, 1992) The statistical significance of profile matches was derived from the analysis of the score distribution of a randomized database as described. (Hofmann, 2000) Dendrogram were calculated by the neighbor joining method, (Saitou and Nei, 1987) using the PHYLIP program suite [J. Felsenstein, University of Washington, Seattle, WA]. Divergent and gap-rich regions were deleted from the alignment before dendrogram analysis

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RNR2a interacts with CSN7 in vitro

As the CSN has been implicated in cell cycle control, and regulates RNR2 in *S. pombe* (Liu et al, *Genes Dev* 2003), the CSN7-RNR2 connection was further studied. RNR2 is the regulatory subunit of the heterodimeric ribonucleotide reductase, which catalyzes the synthesis of ribonucleotides in response to DNA damage. To substantiate the interaction, an in-vitro pull-down assay was performed between RNR2a and CSN7. Binding assays were carried out using constant amounts of recombinant CSN7-His tag and increasing amounts of the recombinant RNR2a protein. The proteins pulled down by Ni-NTA-agarose beads were visualized by an anti-RNR2 antisera raised as part of this project. As can be seen in figure 3, an increasing RNR2 signal is visible as the amount of recombinant CSN7-His tag protein increases. These results corroborate the interaction detected in yeast.

Figure 3: Pull down assay between RNR2a and CSN7-His: Increasing amounts of purified recombinant RNR2a were incubated with 150µg of CSN7-HIS. Following incubation and washings, samples were separated on 12.5% SDS-PAGE, transferred to PVDF membrane and probed with αRNR2a.

Arabidopsis contains two similar RNR2 gene products

Analysis of the *Arabidopsis* genome reveals that it contains two RNR2 genes. The one isolated as a CSN7 interacting protein, At3g27060, was termed RNR2a, while a second gene, At3g23580, was

termed RNR2b. The two genes products are highly similar and share 70% identity. *Rnr2a* encodes a protein of 332 amino acids, while *Rnr2b* encodes a polypeptide of 341 amino acids.

The two *AtRNR2* genes products are highly similar to RNR small subunit proteins from other organisms, sharing between 44% to 64% amino acid identities between compared sequences. RNR2b is slightly more similar to the animal RNR2 proteins than RNR2a.

Arabidopsis RNR2b complements *S.cerevisiae* RNR4

While molecularly *AtRNRa* and *b* are highly similar to known RNR proteins from other organisms, their identity as ribonucleotide reductases has not been shown. To prove biological activity, and to check potential differences, the two *Arabidopsis* genes were assayed for their ability to complement the lethal phenotype of two yeast *rnr* mutants. The yeast mutants are kept as heterozygous due to the lethality caused by the knock out of either *rnr2* or *rnr4*. In the strains used, *rnr2* was knocked out by insertion of a *trp* gene, while *rnr4* was knocked out by insertion of a *leu* gene.

The *AtRNR2*'s were cloned to a pRS-316 vector which carries the uracil selection marker. An *ScRNR4* vector was used as a positive control and an empty vector and a pRS-CDC40 were used as negative controls for complementation. The plasmids were transformed into *RNR2/rnr2* and *RNR4/rnr4* heterozygous strains and plated on a *ura*⁻ media for selection. Sporulation was induced using liquid SPO media. In order to select the mutants spores, they were plated, after treatment to degrade the asci walls and to kill remaining diploid cells, on a selective media (Gal-UT or Gal-UL) and on YPD as reference for total cells (mutant haploids and wt haploids) number. Surviving colonies were counted 3 days following plating of set amounts of cells.

AtRnr2b was found to rescue *Scrnr4* while *AtRnr2a* was not able to rescue any of the yeast mutants in this assay. The fact that RNR2a was not able to rescue any of the mutants in yeast does not necessary means that it does not function as a ribonucleotide reductase small subunit in *Arabidopsis*. A bit surprising is that although RNR2a and RNR2b are more similar to *ScRNR2*, none of them were able to complement the *Scrnr2* mutant. As much as these proteins are evolutionary conserved in sequence and in structure, there might be plant specific post translation modifications or other plant proteins that are required for its activity.

CSN7 C-terminus interacts with RNR2a but not with RNR2b in a direct yeast two hybrid assay

In order to find potential differences between the two *AtRNR* genes, *Rnr2b* was cloned into a pJG 4-5 vector and its interaction with CSN7 and CSN7-deletion constructs were assessed in a direct yeast two hybrid assay. While both RNR2a and RNR2b interact with the full length CSN7, only RNR2a binds the C' terminus by itself. This may suggest that the interactions are differentially regulated and have different biological roles.

Figure 4: CSN7 C' terminus cannot bind RNR2b by itself. A direct yeast two-hybrid assay between the two RNR2's fused to the activation domain in pJG4-5 and CSN7 and its truncations fused to LexA DNA binding domain.

The two RNR2s have similar but not overlapping mRNA expression patterns

To learn about the two RNR2 mRNA expression patterns and regulations in *Arabidopsis*, data from the Stanford Microarray Database had been analyzed. Out of 209 experiments in which both

genes were present, 86% of the experiments showed both *Rnr* genes coregulated. However, out of these, in only two experiments were their expression co-regulated blue) (Figure 6) either positively or negatively, while in the remaining 85%, the expression of both genes was neither up or down regulated (Figure 6, grey). In 6% of the experiments *Rnr2a* is induced while *Rnr2b*'s level is constant (light green), and in only 2%, *RNR2b*'s level increase and *RNR2a*'s level remains constant (red). As for down regulation, in 5%, *RNR2a* (orange) and in 1%, *RNR2b* only are down regulated (pink). No cases were found in which one gene was up regulated and the other down regulated.

Figure 6: The two RNR2's have similar but not identical mRNA expression patterns. Summary of publicly available expression data from 209 gene expression experiments where both RNR2a and RNR2b were analyzed. Coregulation: grey – no change in expression levels for both genes; blue – both genes increase or decrease. Different regulation (14%): green – 2a up, 2b constant; red – 2b up, 2a constant; pink – 2b down and 2a constant; orange – 2a down and 2b constant.

To compare these different experiments, a pseudo reverse-cluster analysis was performed (Figure 7). Instead of examining a list of genes that are expressed differently upon treatments, experiments were compared between the two genes. Interestingly, eight different experiments that show differences in the regulation of the two RNR genes deal with the effects of cytosine methylation and histone deacetylation on gene expression and regulation. (no. 26630, 26631, 26634, 26629, 26635, 26632, 26644). Cysteine methylation and histone deacetylation play a role in gene silencing and are known to cause a reduction in transcription. It is difficult to link this information to RNR2's function. However what is clear from this analysis is that while RNR2a and 2b are highly similar genes, they are not simply duplicated genes with the same expression patterns, but rather the transcription of each gene may be differentially regulated a difference that can imply different cellular roles.

Figure 7: The two RNR2's have similar but not identical mRNA expression patterns. A "Reverse Cluster" overview of RNR2's expression by microarray analysis. For each gene the ratio Channel 2/Channel 1 was determined, and converted to log base 2. $\text{Log}_2 < -1$ (green colored) means at least two fold repression of the gene, $\text{Log}_2 > 1$ (red colored) is for at least two fold induction, and when $-1 < \text{Log}_2 < 1$ (in black), there is no statistically significant change on the mRNA levels

Isolation of a point mutation that disrupts RNR2a-CSN7 interaction using random mutagenesis PCR

To check the biological significance of CSN7-RNR2a interaction, we wanted to create a *csn7* mutant that contains a CSN7 protein, but that does not bind RNR2a. This mutated *csn7* will then be checked for complementation in *csn7/fus5* mutant plants. The method that was used is based on creating a library of mutated CSN7 proteins. The library was generated in two steps. Knowing that CSN7-RNR2a interaction maps to CSN7's C terminus (Figure 1), the first step is randomly mutating this region by mutagenic PCR. Using a pair of primers 80bp up/down-stream to the C' terminus, four PCR reactions were set, in each the concentration of one of the dNTP's was reduced five fold relative to normal PCR conditions. To further increase the mutagenic conditions, the following materials were added to the reaction mixtures: DMSO, MnCl₂, and β mercaptoethanol. The PCR conditions were set according to D. Leung *et al*, 1989. The PCR products are actually a population of different CSN7 C-terminus fragments that contain at least one point mutation (Figure 6A). The second step is inserting these PCR fragment into the two-hybrid vector so that a loss of interaction could be assayed. This was done using double homologous recombination *in yeast*. The next step is finding the colonies that do not interact with RNR2a using the LacZ assay.

Of the 300 colonies that were screened, 11 of them were white, out of which eight gave a specific PCR product using the C terminus primers. Only two of the eight showed homology to CSN7, they were named dATP6 and dATP2. dATP6 and dATP2 were sequenced and their interaction with other CSN7's C terminal interacting proteins (CSN6, Unknown) and with RNR2b was assayed. dATP2 causes a non-specific loss of interaction with all proteins checked, while dATP6 showed a specific loss of interaction with RNR2a (Figure 8).

ATP6 contained 3 point mutations: a change of A to G, changing glutamate to glycine (E214G); a change of C instead of G, leading to a change from serine to proline (S188P); and an A to G transversion leading to an arginine to become glycine (R164G). In order to see which mutation out of the three is responsible for this loss of interaction, the mutations were separated by PCR. A mutated *csn7* that contains the E214G mutation interacts with RNR2a as well as with all the others C7IPs that were checked, indicating that this mutation on its own has no effect on CSN7 binding to C7IPs. A mutated *csn7* that contains both S188P and R164G shows a specific loss of interaction with RNR2a and not with the other controls. (Figure 8).

Figure 8: Isolation of the RNR2a minimal binding site in CSN7: (Lac Z assay for dATP2, dATP6, E214G, S188P+R164G and their interacting proteins. AD – activation domain fusion clone. UNK – PCIP2.

Analysis of additional CSN subunits

When we submitted our original request for BARD funding over seven years ago, we had just succeeded in purifying the CSN, and none of its subunits had been analyzed. Now at the end of six years of funding, we have succeeded in analyzing all eight subunits. Bard funding supported the analysis of CSN7 (Karniol *et al.*, 1999; and above), CSN4 (Serrino *et al.*, 1999), and as presented below, subunits 2, 4 and 6: