Protein ubiquitination in auxin signaling and transport
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Chapter 5

COP9 signalosome association links the PINOID kinase to ubiquitination control

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Summary
The membrane-associated serine/threonine kinase PINOID (PID) has a crucial role in establishing polar distribution of the PIN auxin efflux carriers. PID directly phosphorylates PINs to regulate their polarity. In a screen for interacting partners of PID we identified the CSN subunit CSN8/COP9 as an interacting partner of PID, and found that the linked subunit CSN7/COP15 is phosphorylated by PID in vitro. The COP9 signalosome (CSN) is a protein complex found in eukaryotic cells that regulates many cellular processes linked to targeted protein degradation. In Arabidopsis, cop mutants display constitutive photomorphogenesis and expression of light-responsive genes in the dark. The interaction network of COP9 is broad and complex, influencing almost every aspect of plant development. PID-dependent phosphorylation of CSN7 appears not to be essential for CSN functions in planta, while the interaction of PID with CSN8 seems to regulate PID ubiquitination. In protoplasts CSN8 sequesters PID to the cytoplasm and nucleus and enhances PID ubiquitination. In addition from control of its own turn over, another possible role for CSN-association of PID could be to regulate the interaction between its phosphorylation targets BODENLOS/IAA12, PIN proteins and their corresponding ubiquitin E3 ligases. The identification of PID as CSN-associated kinase reveals an unexpected new aspect of PID signaling that links the action of this kinase to ubiquitination control.
INTRODUCTION

The COP9 signalosome (CSN) was initially identified through a series of Arabidopsis thaliana mutants that show a light-grown seedling phenotype when germinated in the dark and early seedling lethality (Wei and Deng, 1992; Wei et al., 1994; Kwok et al., 1996). Cloning of the CONSTITUTIVE PHOTOMORPHOGENIC 9 (COP9) gene in Arabidopsis was followed by the biochemical purification of a COP9-containing multiprotein complex from cauliflower, a species closely related to Arabidopsis (Wei et al., 1994; Chamovitz et al., 1996). The purified COP9 complex appeared to consist of eight subunits, and constitutive photomorphogenic/detiolated/fusca (cop/det/fus) mutants were found to carry mutations in genes encoding these CSN subunits (Staub et al., 1996; Serino et al., 1999; Karniol et al., 1999; Peng et al., 2001a; Serino et al., 2003). Common to all of these Arabidopsis mutants is the fact that loss of one subunit results in the de-stabilization of the CSN complex (Kwok et al., 1998; Serino et al., 1999; Peng et al., 2001a; Peng et al., 2001b; Wang et al., 2002; Lykke-Andersen et al., 2003; Yan et al., 2003; Gusmaroli et al., 2007).

Mutants in CSN subunit-encoding genes exhibit signal-independent expression of light-induced genes (Wei and Deng, 1999). Therefore the CSN was hypothesized to be a repressor of photomorphogenesis (Osterlund et al., 1999). Further research in various organisms has linked CSN function to ubiquitin-dependent protein degradation of for example the HY5 transcription factor that promotes transcription of light-induced genes (Osterlund et al., 2000), and the Aux/IAA proteins that repress auxin-responsive gene expression (Schwechheimer et al., 2001). The CSN regulates the activity of COP1 ring finger-like and CULLIN-ring E3 ubiquitin ligases. It controls the nuclear localization of COP1 in the dark (Chamovitz et al., 1996; Wang et al., 2009) and mediates the cyclic disassembly of CULLIN ring E3 ligases (CRLs) by deconjugation of RUB1/NEDD8 from the CULLIN subunit (Lyapina et al., 2001; Cope et al., 2002; Dohmann et al., 2005). The CSN interacts with CRLs (Lyapina et al., 2001; Schwechheimer et al., 2001; Schwechheimer et al., 2002)
and protects them from autcatalytic degradation (Cope and Deshaies, 2006; Stuttmann et al., 2009).

The CSN is evolutionary conserved in all eukaryotes and associates with many different proteins besides CRLs. Human CSN was found to co-purify with serine/threonine kinase activities (Seeger et al., 1998) that regulate the ubiquitination and degradation of well known CRL targets, such as p53 and c-Jun (Bech-Otschir et al., 2001; Uhle et al., 2003). The CSN-associated kinases responsible for these activities were identified as inositol 1,3,4- trisphosphate 5/6-kinase (5,6-kinase), casein kinase 2 (CK2), and protein kinase D (PKD) (Wilson et al., 2001; Sun et al., 2002; Uhle et al., 2003). In mammalian cells, the 5/6-kinase associates with CSN1 and CSN5 (Bech-Otschir et al., 2001), whereas CK2 and PKD bind CSN3, while CK2 also binds CSN7 (Uhle et al., 2003). In addition to its role in regulating kinases and/or kinase substrates, the CSN itself has been reported to be phosphorylated. Two dimensional gel electrophoresis of purified human CSN, followed by mass spectrometry, showed that especially subunits CSN2 and CSN7 are found in multiple forms with different pI's, suggesting different degrees of phosphorylation (Henke et al., 1999). CK2 and PKD were found to phosphorylate CSN7 and CK2 was also found to phosphorylate CSN2 (Uhle et al., 2003). Arabidopsis CSN7/FUS5/COP15 contains several putative phosphorylation sites and is phosphorylated in vitro by plant extracts (Karniol et al., 1999). The CSN-associated kinase activity and the post-translational phosphorylation of CSN subunits support the idea that CSN is a central component in signal transduction.

Genetic and molecular approaches have uncovered the serine/threonine kinase PINOID (PID) as a key component in the control of polar auxin transport (PAT) (Benjamins et al., 2001; Lee and Cho, 2006). Cellular levels of PID determine the apical-basal polarity of the PIN family of auxin efflux carriers via direct phosphorylation (Friml et al., 2004; Michniewicz et al., 2007). Recently, we found that PID is also able to phosphorylate the SCF_{TIR1} target, BDL/IAA12, thereby inhibiting its degradation by the proteasome (Chapter 4). In this chapter we reveal a new link between PID and protein ubiquitination. A screen for PID-
interacting proteins identified subunit 8 of the CSN, (CSN8) as PID binding protein, and \textit{in vitro} phosphorylation assays showed that PID is able to phosphorylate CSN7, indicating that PID might regulate CSN activity. We show that PID-dependent phosphorylation of CSN7 has no significant role in plant development, and provide evidence that CSN association might control the recently observed ubiquitination of PID itself (Chapter 3). An additional role of the CSN association of PID might be to bring the kinase in proximity of its phosphorylation targets PIN2 and BDL to inhibit their ubiquitination-dependent degradation (Abas \textit{et al.}, 2006) (Chapter 2). Our results add a new branch to the increasingly complex network of interactions in the PID signaling pathway that regulates many different aspects of auxin-dependent plant development.

**MATERIAL AND METHODS**

**Yeast two hybrid interaction, DNA cloning and constructs**

The Matchmaker yeast two-hybrid system (Clontech) was used to screen two \textit{Arabidopsis thaliana} cDNA libraries fused to the GAL4-activation domain (pACT2) with a PID-GAL4-DNA-binding domain (pAS2-1) fusion, as described previously (Benjamins \textit{et al.}, 2003). This led to the isolation of a single pACT2-CSN8/COP9 clone containing the complete \textit{CSN8} (AT4g14110) open reading frame. Interaction in the \textit{Saccharomyces cerevisiae} strain PJ69-4A (James \textit{et al.}, 1996) was performed using the same system with pACT2-CSN8 directly tested at 20°C with bait plasmids pAS-PID or pAS-PBP2. pAS2-PBP2 was obtained by cloning the \textit{PBP2} cDNA as \textit{PstI}/\textit{SalI}-blunted fragment from pSDM6014 into pAS2 digested with \textit{PstI}/\textit{XmaI}-blunted with Klenow DNA polymerase.

The \textit{CSN7} (AT1g02090) cDNA was amplified by PCR using primers 5’-ACGCAAGTCGACAAGATGGATATCGAGCAGAAGCAAGC-3’ and 5’-GATAGATCTAACAGAGGATCTTATACAAGTTG-3’, and subsequently digested with \textit{BglII} to be ligated into the pBluescript II SK+ plasmid cut with \textit{EcoRV}/\textit{BglII}. From this plasmid pBS-CSN7 a \textit{BamHI}/\textit{SalI} fragment was ligated into pET16B
(Novagen) digested with XhoI/BamHI, resulting in pHis-CSN7. The pHis-CSN8 construct was created by cloning a SalI fragment from pACT-CSN8 into pET16H cut with XhoI/Smal. The pHis-PID, pGST-PID (Benjamins et al., 2003) and pGST-PIN2HL (Michniewicz et al., 2007) constructs have been described previously. The mutations in the CSN7 cDNA were introduced into pHis-CSN7 with the Quickchange Mutagenesis Kit (Stratagene) using primers csn7ST-ATF 5'-GCTCGTGAAGCGAGCTTCTAGGTGCAAATCCGAGGC-3' and csn7ST-ATR 5'-GCCTCGGATTGACCTAGAAGCTGCCTTCACGAGC-3' for the ST>AT mutation, csn7ST-SAF 5'-GCTCGTGAAGCGAGCTAGGACTTGCAAATCCGAGGC-3' csn7ST-SAR 5'-GCCTCGGATTGCAATCATGCTGCCTTCACGAGC-3' for the ST>SA mutation, csn7ST-DDF 5'-GCTCGTGAAGCGAGCTGATGATTGCAAATCCGAGGC-3' csn7 ST-DD R 5'-GCCTCGGATTGCAATCATGCTGCCTTCACGAGC-3' for the SA>DD mutation. A PCR fragment containing a complete genomic sequence of CSN7 (AT1g02090) including 2 Kb upstream of the ATG was amplified from Arabidopsis ecotype Columbia total DNA using primers attB1CSN7promoterF 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAATTATACATGAAAGTTAGCCC-3' and CSN7attB2R 5'-GGGGACCACTTTGTACAAAAAAGCAGGCTTAGAAATTATACATGAAAGTTAGCCC-3' and used in a BP reaction with pDONR207 to create pDONR::gCSN7. This entry clone eliminates the stop codon in Exon IX allowing C-terminal fusions. The same mutations described above were introduced into this entry clone and the resulting plasmids were used in a LR reaction with pGreen0229 PL gateway mRFP1 (Carlos Galvan-Ampudia, unpublished) to obtain the T-DNA construct containing the CSN7::CSN7-mRFP fusion. This construct pCSN7-mRFP was used for mutagenesis as described above to generate pCSN7-mRFP AT, pCSN7-mRFP SA and pCSN7-mRFP DD. For expression in Arabidopsis protoplasts, EcoRI fragments from these plasmids containing the CSN7-mRFP fusion without the CSN7 promoter were cloned into pART7 (Gleave, 1992) in
The cDNA of *CSN8* was amplified from pHis-CSN8 with primers attB1 CSN8F 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGATCTTTCGCCCTGT-3’ and attB2 CSN8R 5’-GGGGACCACCTTTGTACAAAGAAAGCTGGGTCATGTCTTGAAGTGGAACCAC-3’. The *COP1* (AT2g32950) cDNA was amplified by RT-PCR from RNA isolated from 7 day-old wild type Columbia seedlings using primers attB1 COP1F 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGAAGATTTCGACG-3’ and attB2 COP1R 5’-GGGGACCACCTTTGTACAAAGAAAGCTGGGTCATGTCTTGAAGTGGAACCAC-3’. Each attB PCR fragment was used in a BP reaction with pDONR207 to create entry clones. These entry clones were used in LR reactions with pART7-YFP-HA (Carlos Galvan-Ampudia, unpublished) to generate p35S::CSN8-YFP-HA and p35S::COP1-YFP-HA. For construction of the p35S::5xHis-Ub construct, the cDNA of human ubiquitin was PCR amplified from His6-Ub (Stad et al., 2001) using primers 5’HisUb 5’-GGAATTCATGCATCATCATCATCAT-3’ and 3’Ub 5’-CCCTTACCCACCTCTGAGACGGAGGACC-3’ and cloned as a blunt fragment into pART7 cut with SmaI. Constructs p35S::PID-FLAG (Michniewicz et al., 2007), p35S::FLAG-GFP, p35S::HA-GFP, p35S::FLAG-GFP (Dos Santos Maraschin et al., 2009), p35S::BT1-YFP-HA (Robert et al., 2008) and p35S::PID-CFP (C.Galvan-Ampudia, unpublished) were also constructed in pART7.

**In vitro pull down experiments**

GST-tagged PID or the GST tag alone were used in *in vitro* pull down assays with histidine (His)-tagged COP9/CSN8. *E. coli* strain BL21 containing one of the constructs was grown in 50 ml LC cultures supplemented with antibiotics at 37°C to OD600 0.8. The cultures were then induced for 4 hours with 1 mM IPTG at 30°C, after which cells were harvested by centrifugation (10 min at 2,000 g in a tabletop centrifuge) and frozen overnight at -20°C. Precipitated cells were re-suspended in 2 ml Extraction Buffer (EB: 1x PBS, 2 mM EDTA, 2 mM DTT,
supplemented with 0.1 mM of the protease inhibitors PMSF (Phenylmethanesulfonyl Fluoride), Leupeptin and Aprotinin, all obtained from Sigma) for the GST-tagged proteins or in 2 ml Binding Buffer (BB: 50 mM Tris-HCl pH 6.8, 100 mM NaCl, 10 mM CaCl₂, supplemented with 0.1 mM PMSF, 0.1 mM Leupeptin and 0.1 mM Aprotinin 0.1 mM) for the His-tagged proteins and sonicated for 2 min on ice. From this point on, all steps were performed at 4°C. Eppendorf tubes containing the sonicated cells were centrifuged at full speed (20,000 g) for 20 min, and the supernatants were transferred to fresh 2 ml tubes. Supernatants containing H-proteins were left on ice, while 100 μl pre-equilibrated Glutathione Sepharose resin (pre-equilibration performed with three washes of 10 resin volumes of 1x PBS followed by three washes of 10 resin volumes of 1x BB at 500 g for 5 min) was added to the GST fusion protein containing supernatants. Resin suspensions were incubated with gentle agitation for 1 hour, subsequently centrifuged at 500 g for 3 min, and the precipitated resin was washed 3 times with 20 resin volumes EB. In between the washes, the resin was centrifuged for 5 min at 500 g. Next, the H-protein containing supernatants (approximately 2 ml per protein) were added to GST-fusion-containing resins, and the mixtures were incubated with gentle agitation for 1 hr. After incubation, the mixtures were centrifuged at 500 g for 3 min, the supernatants were discarded and the resins subsequently washed 3 times with 20 resin volumes EB. Elution was performed by addition of 50 μL 2X Laemmlli protein loading buffer to the resin samples, followed by denaturation by 5 min incubation at 95°C. Proteins were subsequently separated on a 12% polyacrylamide gel prior to transfer to a Immobilon™-P PVDF (Sigma) membrane. Western blots were hybridized with a horse radish peroxidase (HRP)-conjugated anti-pentahistidine antibodies (Qiagen), and detection followed the protocol described for the Phototope-HRP Western Blot Detection Kit (New England Biolabs).

**In vitro phosphorylation assays**

Cultures of *E. coli* strain BL21 containing one of the constructs were grown at 37°C to OD₆₀₀ 0.8 in 50 ml LC supplemented with antibiotics. The cultures were
then induced for 4 hr with 1 mM IPTG at 30°C, after which cells were harvested by centrifugation 10 min at 4,000 g and frozen at -20°C. Precipitated cells were re-suspended in 2 ml Extraction Buffer (EB: 1x PBS, 2 mM EDTA, 2 mM DTT, supplemented with 0.1 mM of the protease inhibitors PMSF, Leupeptin and Aprotinin) for the GST-tagged PID and WAG2 or in 2 ml Lysis Buffer (LB: 50 mM Tris-HCl pH 6.8, 100 mM NaCl, 10 mM CaCl₂, supplemented with 0.1 mM of the protease inhibitors PMSF, Leupeptin and Aprotinin) for the His-tagged CSN7, CSN7 AA, CSN7 AT and CSN7 SA proteins.

From this point on, all steps were performed at 4°C. To isolate the His-tagged proteins cells were sonicated for 2 min and centrifuged at 20,000 g for 20 min, the pellets were discarded, and supernatants from all aliquots of the same construct were transferred to a 15 ml tube containing 100 μl of pre-equilibrated Ni-NTA resin (pre-equilibration performed with three washes of 10 resin volumes of Lysis Buffer at 500 g for 5 min). Supernatant and resin were incubated with gentle agitation for 1 hr. After incubation, the mixture was centrifuged at 500 g for 3 min, the supernatant was discarded and the resin subsequently washed: 3 times with 20 resin volumes of Lysis Buffer, once with 20 resin volumes of Wash Buffer 1 (25 mM Tris.Cl pH 8.0; 500 mM NaCl; 40 mM Imidazole; 0.05% Tween-20) and once with 20 resin volumes of Wash Buffer 2 (25 mM Tris-HCl pH 8.0; 600 mM NaCl; 80 mM Imidazole). In between the washes, the resin was centrifuged for 5 min at 500 g. After the washing steps, 20 resin volumes of Elution Buffer (25 mM Tris.HCl pH 8.0; 500 mM NaCl; 500 mM Imidazole) was added to the resin and incubated for 15 min with gentle agitation. The resin was centrifuged for 3 min at 500 g, and the supernatant containing the desired protein was diluted a 1000-fold in Tris Buffer (25 mM Tris.HCl pH7.5; 1 mM DTT) and concentrated to a workable volume (usually 50 μl) using Vivaspin microconcentrators (10 kDa cut off, maximum capacity 600 μl, manufacturer: Vivascience). Glycerol was added as preservative to a final concentration of 10% and samples were stored at -80°C.

For the GST-tagged proteins, after sonication for 2 min, 100 μl of 20% Triton X-100 was added and the mixture was incubated for 5 min on ice, followed by centrifugation at 20,000 g for 20 min at 4°C. Supernatants were added to 400 μl
of pre-equilibrated 50% Glutathione Sepharose 4B beads (Amersham-Pharmacia) and incubated for 1.5 hrs. Beads were washed three times with 2 ml of Extraction Buffer and purified proteins were eluted in Elution Buffer (50 mM Tris-Cl pH 8.0, 10 mM reduced glutathione).

For the in vitro phosphorylation assays with plant extracts, one week-old seedlings were frozen in liquid nitrogen, except for pINTAM->>PID which was treated for 8 hrs in liquid MA with 0.1% DMSO (-) or 2 μM tamoxifen (+) before harvesting. For total protein extracts approximately 10 seedlings were ground in liquid nitrogen, 50 μL of cold extraction buffer (25 mM Tris-Cl pH 7.5, 1 mM DTT, 1X Roche Complete Protease Inhibitor Cocktail, 10% glycerol) was added and extracts were centrifuged for 10 min at 20,000 g at 4°C. The soluble fraction was transferred to a new tube and the protein concentration determined by the Bradford method.

In vitro kinase assays were performed in a final volume of 20 μl with 1X kinase buffer (25 mM Tris-Cl pH 7.5, 5 mM MgCl₂, 2 mM CaCl₂ and 1 mM DTT), 2 μg of purified GST-tagged kinase, 2 μg purified His-tagged CSN7 target protein, 2 μg GST-PIN2 HL, 100 μM ATP and 1 μCi [γ-³²P] ATP (3000 Ci/mmol) (GE Amersham). For the reactions using the seedling extracts 2 μg of total protein extract was used as kinase source and GST-PIN2 HL was omitted from the mix. Reactions were incubated at 30°C for 30 min and stopped by adding 5 μl of 5X SDS loading buffer (0.31 M Tris-Cl pH 6.8, 10% SDS, 50% glycerol, 7.5 M β-mercaptoethanol and 0.125% bromophenol blue) and boiled for 5 min. Samples were separated over 12.5% SDS-acrylamide gels, which were washed subsequently 3 times for 30 min with kinase gel wash buffer (5% Trichloroacetic Acid (TCA) and 1% Na₂H₂P₄O₇), coomassie stained, destained, dried and exposed to X-ray films (Fuji Super RX) for 24 to 48 hours at -80°C in a cassette with intensifier screens.

For the peptide assays, 1 μg of purified PID was incubated with 4 nmol of ⁹mer biotinylated peptides (Pepscan) in a phosphorylation reaction as described above. Reaction processing, spotting and washing of the SAM² Biotin Capture Membrane (Promega) were performed as described in the corresponding protocol. Following washing, the membranes were wrapped in plastic film and
exposed to X-ray films for 24 to 48 hrs at -80°C using intensifier screens. The phosphorylation intensities of each peptide were determined by densitometry analysis of the autoradiographs using ImageQuant software (Molecular Dynamics).

**Plant lines, transformation and growth**

Arabidopsis genotypes used for the *in vitro* phosphorylation assays were wild type Col-0, *pid-14* (SALK_049736) and *pid-14/wag1/wag2* loss-of-function mutants (Carlos Galvan-Ampudia, unpublished), and the 35S::PID-21 (Benjamins *et al.*, 2001) and the tamoxifen-inducible *pINTAM>>PID* (Friml *et al.*, 2004) overexpression lines. Flowering Arabidopsis (Col-0) plants were transformed by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991) for delivery of the pCSN7::CSN7-mRFP T-DNA constructs. Primary transformants were selected on medium supplemented with 30 mg/L phosphinothricin (PPT), 50 mg/L nystatin and 100 mg/L timentin to inhibit *Agrobacterium* growth. For further analysis, single locus insertion lines were selected by germination on 20 mg/L PPT and checked for transgene expression by epifluorescence microscopy to detect the mRFP signal.

To determine the functionality of the transgenes, the pollen from selected T2 plants were used in crosses with emasculated heterozygous *cop15-1* (TAIR/NASC #CS3833) plants. F1 seeds were selected on 20 mg/L PPT. Resistant plants were PCR genotyped by digestion of the 2125 bp fragment amplified with primers CSN7 fus5-1F 5’-AGGCCTTGGCCCAGAAACTACG-3’ and cop15-1 genomicR 5’-CACTGACCATTGCTCTCTTGC-3’ with *Ddel*. The *cop15-1/fus5-1* mutation in exon II of the *FUS5* gene creates an early stop codon and an extra *Ddel* restriction site. The reverse primer cop15-1 genomicR anneals in the 3’UTR of the genomic sequence so it does not hybridize with the transgene CSN7-mRFP. F2 seeds were sterilized and germinated on MA medium in the dark to score for *cop/fusca/det* seedling phenotypes. For analysis of RUB-modified CUL1 levels rabbit anti-AtCUL1 antibodies (kindly
donated by Claus Schwechheimer, Technische Universität München) were used.

**Protoplast isolation and transfection**

Protoplasts were isolated from Arabidopsis Col-0 cell suspension cultures and plasmid DNA was introduced by PEG-mediated transfection as described (Schirawski *et al.*, 2000; Dos Santos Maraschin *et al.*, 2009). Following transfection, the protoplasts were incubated for at least 16 hrs before treatments.

**Microscopy**

For imaging of transfected protoplasts a Leica DM IRBE confocal laser scanning microscope was used with a 63X water objective, digital zoom and 51% laser intensity. The fluorescence was visualized with an Argon laser for excitation at 514 nm (YFP) and 457 nm (CFP) with 522-532 nm and 471-481 nm band pass emission filters, respectively. For the mRFP red fluorescence we used the 568 nm (excitation) line of the krypton laser with an of 570-610 nm band pass emission filter. Image processing was performed with ImageJ (http://rsb.info.nih.gov/ij/). The *CSN*:CSN7-mRFP lines were analysed using a Leica stereomicroscope MZ16FA equipped with a dsRED filter set and a DFC 420C camera. Images were manipulated and assembled in Microsoft Powerpoint 2003.

**Purification of ubiquitinated proteins via Ni-affinity chromatography**

After isolation, 10⁶ protoplasts were transfected with 20 μg p35S::PID-FLAG and 20 μg of the p35S::HisUb plasmid. Where stated, 10 μg of p35S::CSN8-YFP-HA, p35S::COP1-YFP-HA, p35S::BT1-YFP-HA were co-transfected. All transfections also contained 5 μg p35S::HA-GFP as a transfection control and an empty pART7 plasmid for DNA equalization. Sixteen hours after transfection, cells were treated with 50 μM MG132 for 4h and harvested by centrifugation and frozen in liquid nitrogen. Pellets containing harvested cells were resuspended in 100 μL cold Extraction Buffer (TBS, 1% Triton X-100, 1X Roche
Complete Protease Inhibitor Cocktail, 10 mM NEM (N-Ethylmaleimide), vortexed and centrifuged for 10 min at 20,000 g at 4°C. Ten μL were mixed with an equal volume of 2X Laemmli Buffer to be further analyzed on gel (10% input), and the remaining volume was mixed with 910 μL of Buffer A (6 M guanidinium-HCl/0.1 M Na₂HPO₄/NaH₂PO₄, pH 8.0/10 mM imidazole) and used for Ni-affinity chromatography as described (Campanero and Flemington, 1997). PAGE-separated proteins were blotted onto PVDF membranes, blocked with nonfat dry milk and incubated with the HRP-conjugated antibodies anti-HA High Affinity 3F10 (Roche) and anti-FLAG M2 (Sigma). Detection of the HRP-conjugated antibody signal was performed with the LumiGLO Detection Kit (Cell Signalling). Signal intensity was measured on scanned X-ray films using the Genetools 3.07 (Synoptics Ltd.) software.

RESULTS

PINOID interacts with CSN8/COP9 and phosphorylates CSN7/COP15 in vitro

One of the PID-interacting proteins identified using the yeast two-hybrid system (Benjamins, 2004) was subunit 8 of the CSN (CSN8/COP9). This interaction was confirmed by re-transformation of the respective bait and prey vectors into the yeast strain PJ69-4A (Figure 1A) and by in vitro protein pull-down assays (Figure 1B).

CSN-associated kinases have up till now only been identified in animal cells, where for example CK2 and PKD bind CSN3 and phosphorylate CSN2 and CSN7 (Uhle et al., 2003). Based on this information, we hypothesized that PID might phosphorylate CSN8/COP9 or another subunit of the CSN complex. Our in vitro phosphorylation assays did not show any evidence that PID phosphorylates CSN8/COP9 (Figure 1C). Since it has been shown that CSN8/COP9 interacts with CSN7 (Bech-Otschir et al., 2002; Serino et al., 2003; Uhle et al., 2003), and that CSN7 was originally identified as a phospho-protein in Arabidopsis (Karniol et al., 1999), we tested whether CSN7 could be
phosphorylated by PID \textit{in vitro}. Indeed, CSN7 was efficiently phosphorylated by PID and in our \textit{in vitro} assays CSN7 phosphorylation occurred independently of CSN8/COP9 (Figure 1C). As observed before PID does not require a tight interaction with its phospho-targets, (Chapter 4, (Michniewicz \textit{et al.}, 2007)). We can not exclude however, that the excess of PID and CSN7 used in these experiments overruled the requirement for CSN8/COP9-mediated PID anchoring.

The NetPhos program identified eight potential CSN7 phosphorylation sites (Figure 1D), and these residues were tested using synthetic biotinylated nine amino acids peptides in \textit{in vitro} phosphorylation reactions with PID (Figure 1E). These assays showed that peptides 1 and 2, containing the amino acid sequence core KRASTCKS starting at position 16 in the CSN7 protein, were most efficiently phosphorylated by PID (Figure 1E). More detailed analysis of the KRASTCKS sequence in the ScanProsite database indicated that it has characteristics of phosphorylation substrates of cyclic AMP dependent Protein Kinase (PKA: R/K-R/K-X-S/T) and of Protein Kinase C (PKC: S/T-X-R/K). PepChip experiments showed that PID efficiently phosphorylates PKA and PKC substrates (Galvan and Offringa, unpublished data), and therefore we considered serine 19 or threonine 20 in CSN7 as putative PID phosphorylation targets.
Figure 1. PID interacts with CSN8/COP9 and phosphorylates CSN7/COP15. (A) Yeast two-hybrid assay with PID and PBP2 fused to the GAL4 binding domain (BD; pAS2.1 vector), and CSN8/COP9 fused to the GAL4 activation domain (AD) or the AD alone (pACT2 vector) in non-selective medium or in medium lacking either histidine or adenine. (B) *In vitro* pull-down of Histagged CSN8/COP9 with GST-tagged PID (lane 1) and or with GST (lane 2) followed by immunodetection with anti-His antibodies (top panel). The coomassie-stained gel is shown in the bottom panel. (C) Autoradiograph (right panel) and coomassie-stained gel (left panel) of *in vitro* phosphorylation assay using MBP (all lanes), His-CSN8 (lanes 1, 2, 5, 6, 7 and 10) and His-CSN7 (lanes 3, 4, 5, 8, 9 and 10) as substrates and PID (lanes 1, 3, 5, 6, 8 and 10) as protein kinase. (D) Amino acid sequence of CSN7, with all the putative phosphorylation sites identified by NetPhos as central residues within nine amino acid peptides indicated in bold. The peptides tested in the *in vitro* phosphorylation assay (E) are underlined and the putative PID phosphorylation sites are boxed in (D). BDL-derived peptide RSAESSSHQ (7) was used as a negative control (see chapter 4).
**PID and WAG2 phosphorylate CSN7 *in vitro* based on sequence recognition**

Based on the peptide phosphorylation results, we generated three mutant versions of the *p*His-CSN7 construct so that the ST aminoacid codons at positions 19 and 20 in the His-tagged protein were substituted by respectively AT, SA or AA. The three mutant CSN7 variants could not be phosphorylated by PID or the closely related AGC3 kinase WAG2 (*Figure 2A*) in vitro. WAG2 was shown to recognize PID phospho targets, and to be more active *in vitro* (*Figure 2A*, [Zegzouti *et al.*, 2006]; Galvan-Ampudia, C., Huang, F. and Offringa, R.; unpublished), and this result made us confident that there are no additional ACG-3 specific phosphorylation sites in CSN7. Unfortunately, these results did not allow us to unequivocally conclude whether the serine 19 or the threonine 20 is phosphorylated by PID, but based on the target sequences identified in the PIN auxin efflux carriers (Zago, 2006) and in the BDL protein (Chapter 4) it is most likely that the serine 19 is the phosphorylation target and that the threonine 20 is essential for proper substrate recognition by PID.

In order to establish the occurrence of PID-dependent phosphorylation *in planta*, we incubated purified His-CSN7 and the mutant variants in *in vitro* phosphorylation reactions with total protein extracts from Arabidopsis wild type seedlings. As shown in figure 2B, both wild type and the mutant His-CSN7 variants were equally phosphorylated by total protein extracts, indicating that the ST site has a minor influence on the overall phosphorylation status of CSN7 *in planta*, and that other phosphorylation sites are present in its primary sequence. In order to determine the contribution of PID and the WAG kinases to the phosphorylation of CSN7 *in planta*, we incubated His-CSN7 with total protein extracts from *pid* or *pidwag1wag2* loss-of-function mutants or from PID overexpression lines (*Figure 2C*). All extracts were able to phosphorylate the full length His-CSN7 at wild type levels, indicating that there is no direct correlation between PID/WAG expression levels and the capacity of total protein extracts to phosphorylate CSN7. These results suggest that the contribution of the PID and WAG kinases to the overall phosphorylation status...
of CSN7 is small, and that other phosphorylation sites are present in CSN7 that are recognized by other kinases.

PINOID does not control the CSN de-rubylolation activity

Subunit 7 is an essential component of the CSN. Arabidopsis csn7 loss-of-function mutants fail to assemble a functional CSN resulting in de-etiolated seedling phenotypes and seedling lethality (Dessau et al., 2008). One of the earliest discovered biochemical activities of the CSN was the control of SCF E3 ubiquitin ligases via de-rubylolation of CUL1 (Lyapina et al., 2001; Cope et al., 2002). RUB1 is an ubiquitin-like protein known to be covalently attached to
proteins of the CULLIN family. CSN mutants accumulate RUB1-conjugated CULLINs, a characteristic that can be used as a biochemical marker for CSN activity. To test if PID-dependent phosphorylation controls CSN activity, we used an Arabidopsis CUL1-specific antiserum to detected CUL1 modification in total protein extracts from different Arabidopsis mutant backgrounds (Figure 3). As expected, the csn7/cop15-1 loss-of-function mutant only showed the RUB1-conjugated form of CUL1 due to the lack of COP9 activity. Interestingly, rubylated CUL1 did not accumulate to higher levels in this background compared to wild type, suggesting that CUL1-RUB1 is turned over in the absence of the CSN (He et al., 2005; Wu et al., 2005; Cope and Deshaies, 2006; Gusmaroli et al., 2007). On the other hand, pid, 35S:PID and inducible plINTAM>PID showed a wild type CUL1 : CUL1-RUB1 ratio, demonstrating that PID-dependent phosphorylation of CSN7 does not control CSN de-rubylation activity.

Figure 3: PINOID does not control CSN de-rubylation activity. Twenty µg of total protein extracts from one week old seedlings were analyzed on a western blot probed with anti-AtCUL1 antibodies. Extracts were from wild type Columbia (WT Col) and Landsberg erecta (WT Ler), cop15-1 (in Ler background), and in the Columbia background pid-14, plINTAM>PID treated for 24h with DMSO (-) or with 400 nM tamoxifen (+) to induce PID expression (Friml et al., 2004), and the strong PID overexpression line 35S::PID-21 (Benjamins et al., 2001). The star indicates the size of the CUL1 free form and the arrow indicates RUB1-modified CUL1. The dot indicates a cross-reacting band that is used here as loading control.
PID-dependent phosphorylation of CSN7 is not required for CSN wild type function

Following the observation that PID is not involved in regulating CSN activity, we tested whether the CSN7 mutant versions lacking the PID phosphorylation site were still functional in planta. First, these CSN7 versions were translationally fused to mRFP and expressed in protoplasts under control of the 35S promoter. Besides CSN7-AT and -SA, also a mutant version was tested encoding CSN7 with ST substituted for DD to mimic phosphorylation at this position. All CSN7-mRFP variants showed identical subcellular localization patterns with strong nuclear accumulation and a weaker cytoplasmic signal (Figure 4). This is in accordance with previous reports on CSN7 and other CSN subunits (Tao et al., 2005; Wang et al., 2009). Also, co-expression of PID did not change the subcellular localization of CSN7 (Figure 4B), even when we induced nuclear localization of PID by co-expression of the PID interacting BTB-TAZ scaffold protein BT1 (Robert et al., 2008). These results indicate that PID-dependent phosphorylation of CSN7 at Ser19/Thr20 does not influence its cellular distribution and/or nuclear accumulation.

Next, we generated transgenic Arabidopsis plants carrying the wild type or the AT, SA or DD version of the genomic translational fusion pCSN7::CSN7-mRFP. Homozygous T2 plants with similar mRFP-fluorescence levels were crossed with cop15-1 heterozygous plants and after selfing of the F1 plants the F2 plants were assayed for the complementation of the cop/fus phenotype. Among the progeny of the heterozygous COP15/cop15-1 mutant plants not the expected 25%, but rather 8% of the seedlings showed the cop mutant phenotypes. This lower frequency of mutant seedlings has been observed before for csn mutants (Dessau et al., 2008), and can be explained by significant embryo lethality among the homozygous progeny. Table 1 shows the segregation frequencies of crossings performed with the different transgenic lines expressing the CSN7 variants. The result indicate that all variants of CSN7-mRFP were able to complement the csn7/cop15-1 loss-of-function phenotype at equal levels to the wild type version. For all constructs individuals homozygous for cop15-1 were recovered that displayed wild type phenotypes.
Together with our previous observations this indicates that phosphorylation of Ser19/Thr20 in CSN7 does not affect the functionality of the protein *in planta*.

**Table 1: PID-dependent phosphorylation of CSN7 does not have an obvious role in plant development**

<table>
<thead>
<tr>
<th></th>
<th>TOTAL</th>
<th>NON-cop</th>
<th>cop</th>
<th>% cop</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cop15-1/cop15-1</em></td>
<td>493</td>
<td>452</td>
<td>41</td>
<td>8.32</td>
</tr>
<tr>
<td>CSN7 WT#6</td>
<td>486</td>
<td>485</td>
<td>1</td>
<td>0.21*</td>
</tr>
<tr>
<td>CSN7 WT#8</td>
<td>559</td>
<td>553</td>
<td>6</td>
<td>1.07*</td>
</tr>
<tr>
<td>CSN7 AT#1</td>
<td>521</td>
<td>505</td>
<td>16</td>
<td>3.07*</td>
</tr>
<tr>
<td>CSN7 SA#7</td>
<td>500</td>
<td>500</td>
<td>0</td>
<td>0.00*</td>
</tr>
<tr>
<td>CSN7 SA#8</td>
<td>499</td>
<td>480</td>
<td>19</td>
<td>3.81*</td>
</tr>
<tr>
<td>CSN7 DD#1</td>
<td>492</td>
<td>472</td>
<td>20</td>
<td>4.07*</td>
</tr>
<tr>
<td>CSN7 DD#2</td>
<td>564</td>
<td>561</td>
<td>3</td>
<td>0.53*</td>
</tr>
</tbody>
</table>

*Significantly different from *Cop15-1/cop15-1* based on χ² test.

The expression pattern of the mRFP fusions based on fluorescence was similar for all constructs, with a high expression in meristematic and vascular tissues. The CSN7-mRFP signal was predominantly nuclear in all tissues, in agreement with the protoplast signal (Figure 5C, D). These results lead us to conclude that PID-dependent CSN7 phosphorylation does not affect its expression pattern, or its subcellular localization.

**CSN8 sequesters PID to the cytoplasm and the nucleus**

Our efforts to establish the function of CSN7 phosphorylation by PID indicated that the identified site is not crucial for its functionality. In fact, no function has been assigned to the phosphorylation of CSN2 and CSN7 by other kinases (Karniol *et al.*, 1999; Uhle *et al.*, 2003). It is therefore more likely that the association of PID with the CSN through its interaction with CSN8 is more relevant than the phosphorylation of CSN7. In a first assay to confirm this interaction in plant cells, we co-expressed PID-CFP with CSN8-YFP and CSN7-mRFP in Arabidopsis protoplasts. Indeed, CSN8 was able to sequester PID
Figure 4: PID-dependent phosphorylation does not affect CSN7 subcellular localization or expression.

(A) Confocal laser scanning microscopy images of representative Arabidopsis protoplasts transformed with 35S::CSN7-mRFP WT, AT, SA and DD. Bar represents 20 μm, cells in A and B are in the same scale. (B) Confocal laser scanning microscopy images of representative Arabidopsis protoplasts transformed with 35S::CSN7-mRFP alone (left column) or co-transfected with 35S::PID-CFP (middle column) or 35S::PID-CFP and 35S:BT1-YFP (right column). (C) Epifluorescence microscopy of 7-day old etiolated seedlings showing the expression pattern of the complementing construct pCSN7::CSN7-mRFP in the cop15-1 mutant background. (D) Comparison of expression patterns among the wild type and the ST to SA, AT or DD versions of the pCSN7::CSN7-mRFP construct. Etiolated homozygous plants of the T3 generation were imaged at 7 dag. The scale bar represents 0.2 cm in C and 0.5 cm in D.

from the plasma membrane to the cytoplasm and nucleus (Figure 5). Co-transfected CSN7 showed the same localization as in Figure 4 but curiously, when co-transfected, both CSN8 and CSN7 seemed to have stronger nuclear accumulation in the dark, indicating that the CSN subunits could have a light-
COP9 signalosome association links the PINOID kinase to ubiquitination control

sensitive subcellular distribution. These results provide the first evidence for the interaction between PID and CSN8 \textit{in vivo}, and indicate that CSN8 plays an active role in recruiting PID to become associated with the CSN in the cytoplasm and the nucleus.

Figure 5: CSN8 sequesters PID from the plasma membrane to the cytoplasm and the nucleus. Confocal laser scanning microscopy images of representative Arabidopsis protoplasts transformed with 35S::PID-CFP alone (left column) or co-transfected with 35S::CSN8-YFP (middle column) or 35S::CSN8-YFP and 35S:CSN7-mRFP (right column). Sixteen hours following transfection, cells were kept in continuous dark (DARK) or transferred to light 4 hours before imaging (LIGHT). The box shows protoplasts transfected with the control construct 35S::GFP and incubated under identical conditions. Scale bar represents 20 μm, all images are in the same scale.

CSN association enhances PID ubiquitination in the dark

The COP9 signalosome interacts with many elements of the ubiquitin/proteasome system. From previous studies we already had some indications that PID could be an ubiquitination target in Arabidopsis (Chapter 3). The nuclear localization and/or CSN association of PID could be an essential step for its ubiquitination. To test this we co-expressed PID with His-tagged ubiquitin and the nuclear proteins COP1, BT1 or CSN8 in protoplasts that were incubated in light or dark. PID ubiquitination was observed under all conditions, and whereas light had no clear effect when no other protein was co-expressed, 4 hours treatment with the 26S proteasome inhibitor MG132 significantly
enhanced the ubiquitinated pool of PID (Figure 6). Co-expression of CSN8, but not BT1, led to enhanced ubiquitination signals (Figure 6), especially in the dark, the condition that enhances nuclear localization of CSN8. The signal of CSN8-enhanced PID ubiquitination was similar to that of MG132 treatment in cells kept in the dark but not co-transfected with CNS8. Co-expression of COP1 also enhanced PID ubiquitination under dark conditions. In the dark the enhanced CSN-mediated nuclear localisation of COP1 (Chamovitz et al., 1996) possibly enhances nuclear localization of the CSN (Wang et al., 2009) and the associated PID kinase. MG132 treatment did not have a clear influence on the steady-state PID levels, indicating that turnover of this kinase in protoplasts is slow. This can be explained by the fact that even in the presence of BT1 or CSN8, the majority of the PID proteins remain cytoplasmic or plasma membrane associated, and therefore may not be ubiquitinated, since this process is likely to occur in the nucleus (Figure 5). Our results indicate that PID is associated with the COP9 signalosome via CSN8, and suggest that its abundance is regulated by ubiquitination and proteasomal degradation in the nucleus.

DISCUSSION

Recent advances in CSN research have attributed a biochemical activity to the CSN and have linked this complex to numerous biological processes involving E3 ubiquitin ligases. Additionally, substantial progress has been made in defining the specific role of the CSN in various aspects of cellular and physiological processes, using tools such as conditional knockdowns and subunit-specific knockouts in different model organisms. As a potent protease that can act on all rubylated cullins, cellular CSN activity must be under tight control. CSN can selectively de-rubylate specific SCFs through specific protein interactions. Besides this enzymatic role, the CSN is also emerging as a master docking station that controls the action of specific kinases, their phospho-substrates, E3 ubiquitin ligases and the proteasome by coordinated interactions.
COP9 signalosome association links the PINOID kinase to ubiquitination control

Figure 6: CSN association enhances PID ubiquitination in the dark. Western blot analysis of Ni-NTA purified samples (upper panel) or total extracts (INPUT and lower panels) of Arabidopsis protoplasts transfected with 35S::PID-FLAG and 35S:HA-GFP. His-Ub indicates co-transfection with the 35S:5xHis-Ub construct that allows the purification of ubiquitinated proteins via Ni-NTA affinity purification. Co-transfected 35S:BT1-YFPHA, 35S::COP1-YFPHA and 35S::CSN8-YFPHA are indicated at the top and expression levels in the total extracts are shown in the bottom panels. Sixteen hours after transfection, the cells were treated with 50 μM MG132 (+) or DMSO (-) for 4 hours in the dark (black horizontal bars) or 4 hours in the light (white bars). Numbers in bold indicate the most relevant signals. On the top panel mono-ubiquitinated PID is indicated by a white arrowhead, black arrowheads indicate poly-ubiquitinated forms of PID while the lower band is PID-FLAG which has a background affinity for the Ni-NTA beads as seen in the first lane not co-transfected with His-Ub, this band was used for correcting the intensity of the size-shifted Ub-signals between samples. Numbers below the upper panel indicate normalized Ub-signal relative to the sample in the fourth lane (PID, light - MG132). Underlined values indicate the most relevant differences.

Several kinases have been described to associate with CSN subunits in human and animal cells, controlling the ubiquitination and subsequent degradation of
E3 ubiquitin ligase substrates (Seeger et al., 1998; Bech-Otschir et al., 2001; Uhle et al., 2003) In this chapter we describe an unexpected new role for PINOID as the first CSN-associated kinase in plants. PID interacts with CSN8, and like other CSN-associated kinases, is able to phosphorylate CSN subunit 7 in vitro.

CSN7 has been described as a phosphorylated protein in Arabidopsis (Karniol et al., 1999), and we show here that PID dependent phosphorylation requires the Ser19/Thr20 motif in vitro. However, the contribution of these residues to the in vitro phosphorylation of CSN7 using Arabidopsis total protein extracts is negligible, and neither loss-of-function nor overexpression of PID affected the phosphorylation capacity of the extracts, indicating that PID does not provide a significant contribution to the overall in vitro kinase activity in total extracts towards CSN7. More importantly, we could not assign any in vivo function to the phosphorylation of the identified site, as the mutant CSN7 versions lacking the PID-specific phospho-residues were still able to fully complement the csn7 loss-of-function allele cop15-1. Previous reports on CSN subunit phosphorylation in animal cells did not map the phosphorylated residues, and did not report on a physiological function for this modification (Henke et al., 1999; Uhle et al., 2003). We therefore suspect that either this phosphorylation does not occur in vivo, or that this process has a more subtle effect than a complete loss of function.

Instead we identified a regulatory role for the CSN on PID activity. PID is a membrane-associated kinase (Galvan-Ampudia and Offringa, 2007) that controls apical-basal polar targeting of PIN proteins thereby regulating polar auxin transport (Friml et al., 2004). All PID binding proteins described until now appear to control its activity (Benjamins et al., 2003) or subcellular localization (Robert et al., in preparation; this chapter). Here we show that CSN8 in Arabidopsis protoplasts sequesters PID from the plasma membrane and enhances PID nuclear localization. Interestingly, we also observed that PID is ubiquitinated, and that this ubiquitination is enhanced by co-expression of CSN8 which enhances/induces nuclear localisation of PID. In animal cells, several proteins that are targets for ubiquitination and proteasomal degradation
are known to interact with CSN subunits (Schwechheimer, 2004; Wei et al., 2008). With our observation we provide the first evidence that PID levels are controlled by proteasomal degradation, and that the CSN seems to play an important role in regulating PID stability by recruiting this kinase to the nucleus for ubiquitination and subsequent proteasomal degradation.

Another role for PID as CSN-associated kinase may lie in the finding that in animal cells such kinases regulate the ubiquitination and degradation of key regulators, such as the central mammalian transcription factors, p53 and c-Jun. While CSN-dependent phosphorylation appears to stabilize c-Jun, it has an opposite effect on the tumor suppressor p53 resulting in its degradation. Similar to c-Jun in mammalian cells (Seeger et al., 1998; Naumann et al., 1999; Uhle et al., 2003), PID-dependent phosphorylation was found to prevent BDL ubiquitination and proteasomal degradation (Chapter 4). Moreover, the ubiquitin system has also been connected to the regulation of PIN2 protein cycling and turnover and, whereas light stimulates PIN2 localization at the plasma membrane, both the 26S proteasome and COP9 were found to be directly involved in PIN2 vacuolar targeting for its degradation in the dark (Abas et al., 2006; Laxmi et al., 2008). Interestingly, PID seems to stabilize the plasma membrane localization of PIN proteins (Huang, F. and Offringa R. unpublished observations) and one hypothesis could be that the effects observed of proteasomal inhibition and lack of a functional CSN would be explained by the presence of more active PID phosphorylating and stabilizing PINs on the plasma membrane. Alternatively, when associated with the CSN PID may prevent PIN2 ubiquitination by phosphorylation of the PIN2-HL.

The observation that the ubiquitin system controls different elements of auxin responses places PID as a central integrator of auxin transport and perception by acting both on the polarity of auxin transport and the downstream effects of auxin action. Association of PID with the CSN might be part of an interaction network of kinases and phosphorylation targets that tune specific auxin responses involving proteasomal degradation.
REFERENCES


Cope, G.A. and Deshaies, R.J. (2006) Targeted silencing of Jab1/Csn5 in human cells downregulates SCF activity through reduction of F-box protein levels. BMC. Biochem. 7, 1-
COP9 signalosome association links the PINOID kinase to ubiquitination control


Wang, X., Li, W., Piqueras, R., Cao, K., Deng, X.W. and Wei, N. (2009) Regulation of COP1 nuclear localization by the COP9 Signalosome via direct interaction with CSN1. *Plant J.*


