Protein ubiquitination in auxin signaling and transport

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Regulation of auxin transport and signaling through protein ubiquitination

Felipe dos Santos Maraschin, Johan Memelink and Remko Offringa

Introduction

Charles Darwin's observations on bending of *Avena* coleoptiles towards the light at the end of the 19th century led him to conclude that some compound synthesized in the apical part of the coleoptile is transported to the lower part to regulate its directional growth (Darwin, 1880). Years later these initial findings led to isolation of the plant hormone auxin and its characterization as the compound indole-3-acetic acid (IAA) (Went, 1937). Since its discovery, many aspects of auxin biology have been extensively studied, from its biosynthesis and metabolism to its transport-driven asymmetric distribution and the elucidation of molecular components of downstream signaling. Based on these studies we know now that auxin plays a central role in diverse developmental processes throughout a plant's life cycle, by regulating cell division, growth and differentiation. The physiological effects of auxin are wide and complex. Application of exogenous auxin to plant cells leads to immediate responses, such as an increase in intracellular calcium levels, cell wall acidification, and changes in membrane potentials and enzyme activities, which are followed by changes in gene expression. In the context of the whole plant these changes regulate patterning processes, apical dominance, and root growth, and mediate lateral root- and fruit initiation, among others (Delker *et al.*, 2008; Benjamins and Scheres, 2008). In this chapter we shortly review the current knowledge on auxin transport, auxin response and protein ubiquitination.

Auxin transport

Using radioactively labeled auxin, it was observed that IAA is transported from cell to cell in a unidirectional manner. In the 1970s different hypotheses about the mechanism converged into the chemiosmotic model for polar auxin transport (PAT). This model postulates that due to the relatively acidic extracellular pH (5.5), a portion of the free IAA in the apoplast is in its protonated form (IAAH) that can pass the plasma membrane by import carriers, or freely by diffusion. In the more basic cytoplasmic environment (pH 7.0) auxin ionizes to form the anion IAA^- that cannot freely pass the plasma membrane, and becomes trapped inside the cell. The only way these IAA^- anions can exit

the cell is by auxin efflux carriers, and polar placement of such carriers in the plasma membrane will give directionality to the transport (Rubery and Sheldrake, 1973; Raven, 1975). In the past decade, the molecular elements in this model have been identified. The AUX1/LAX family of auxin permeases were shown to act as auxin import carriers (Swarup *et al.*, 2004; Yang *et al.*, 2006) that were first predicted and later shown to be important enhancers of PAT (Kramer, 2004; Swarup *et al.*, 2008; Bainbridge *et al.*, 2008). On the other hand, PIN FORMED (PIN) proteins were identified along with several ABC transporter-like phosphoglycoproteins (PGPs) to act as the auxin efflux carriers (Petrasek *et al.*, 2006; Bandyopadhyay *et al.*, 2007; Mravec *et al.*, 2008). The PIN proteins were named after mutants of the *PIN FORMED/PIN1* gene, which form pin-like inflorescence that develop only few or no flowers or other lateral organs (Okada *et al.*, 1991). The Arabidopsis PIN protein family comprises 8 members, six of which contain two transmembrane domain regions intervened by a large central hydrophilic loop (HL). The role of the HL-containing proteins PIN1, PIN2, PIN3, PIN4 and PIN7 in plant development has been well established, and apart from their specific function there is also considerable functional redundancy between the corresponding genes (Tanaka *et al.*, 2006; Vieten *et al.*, 2007). All five proteins show tissue-specific polar distribution at the plasma membrane (Tanaka *et al.*, 2006) that dictates of the direction of auxin flow through their asymmetric subcellular localization (Wisniewska *et al.*, 2006a). The function of PIN6 is still elusive, and also for PIN5 and PIN8 that lack a large HL no function has been reported.

Of all the Arabidopsis *pin* loss-of-function mutants, *pin1* is most severely affected in development with the needle-like inflorescences as most striking phenotype (Okada et al, 1991). This already indicated a crucial role for PIN1 in shoot development, and more detailed analysis has shown that PIN1-driven auxin transport in the epidermis of the shoot apical meristem generates auxin maxima that are responsible for the initiation of new organs and thus for phyllotactic patterning (Reinhardt et al., 2003; Heisler et al. 2005). An extensive screen for pin-formed mutants has revealed two allelic groups with a similar phenotype, and besides new *pin1* alleles the screen identified *pinoid (pid)*

mutants that all carry mutations in a gene coding for a protein serine-threonine kinase (Bennett *et al.*, 1995; Christensen *et al.*, 2000; Benjamins *et al.*, 2001). In *pid* loss-of-function plants PIN1 proteins were found at the basal, instead of apical, side of epidermal cells in the shoot meristem, explaining the defective organogenesis leading to the pin-formed phenotype (Friml *et al.*, 2004). The fact that in PID overexpressing roots PIN1, PIN2 and PIN4 were found at the apical side of cells, confirmed that PID is a central regulator of PIN polarity and auxin transport (Friml *et al.*, 2004). Recently, the PID kinase has been shown to directly phosphorylate the HL of PIN proteins, and to act antagonistically with the PP2A protein phosphatases on the phosphorylation status of PIN proteins (Michniewicz *et al.*, 2007). The current model defines that PID regulates polar auxin transport by controlling PIN localization, and thereby determines the direction of auxin flow (Benjamins *et al.*, 2001; Friml *et al.*, 2004). The role of calcium as a second messenger in modulating auxin responses and PAT is well established. Evidence that calcium is one of the early signals in auxin response came from experiments on maize coleoptile cells (Gehring *et al.*, 1990; Felle *et al.*, 1991), parsley cells, maize and pea roots (Gehring *et al.*, 1990). A rapid increase in the cytosolic calcium concentration is detected within minutes after auxin application. Early studies on sunflower stem sections showed that PAT was abolished by the presence of calcium chelators and restored by application of calcium solutions, which suggested an important role for calcium in the regulation of PAT (la Fuente and Leopold, 1973). A molecular link connecting calcium and PAT was found by the identification of the calcium-binding proteins PINOID BINDING PROTEIN1 (PBP1) and TOUCH3 (TCH3) as interacting proteins of PID (Benjamins *et al.*, 2003). Neither of the calcium-binding proteins is a phospho-target of PID but both regulate PID kinase activity. TCH3 is a negative regulator of PID activity, whereas PBP1 positively regulates the kinase *in vitro* (Benjamins *et al.*, 2003; Robert-Boisivon, 2008). TCH3 is a Calmodulin-like protein with six EF-hand domains encoded by a touch-responsive gene while PBP1 is a small protein with a single EF-hand (Braam and Davis, 1990; Sistrunk *et al.*, 1994). PBP1 was also named KRP2 (for KIC-related protein2), as it is part of a small protein family that includes KIC (KCBP-interacting

Calcium binding protein) and the close PBP1 homolog PBP1H/KRP1 (Reddy *et al.*, 2004). PID belongs in the AGC3 clade of the AGCVIII (cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C) kinase family together with three other members: WAG1 (WAVY ROOT GROWTH1), WAG2 and AGC3-4 (Galvan-Ampudia and Offringa, 2007). Functional analysis of WAG1 and WAG2 has indicated that these kinases play roles in root growth. The enhanced root growth sensitivity of *wag1wag2* loss-of-function seedlings to the PAT inhibitor NPA (Santner and Watson, 2006), together with the fact that WAG kinases, like PID, are membrane-associated suggests that they may also be involved in the regulation of polar targeting of PIN proteins (Galvan-Ampudia and Offringa, 2007). In fact WAG1 and WAG2 are able to phosphorylate PINs *in vitro* more efficiently than PID in some cases (Galvan-Ampudia, C.; unpublished). Many components have been identified regulating auxin transport and polarity maintenance in plants, from the auxin efflux carriers PINs and PGPs (Wisniewska *et al.*, 2006b; Mravec *et al.*, 2008), influx facilitators like AUX1 (Swarup *et al.*, 2001) to regulators of PIN polarity through phosphorylation/dephosphorylation like PID, WAGs, D6PK and RCN1 (Friml *et al.*, 2004; Michniewicz *et al.*, 2007; Galvan-Ampudia and Offringa, 2007; Zourelidou *et al.*, 2009) or cycling/stability processes regulated by GNOM, COP9 and the 26S proteasome (Geldner *et al.*, 2003; Abas *et al.*, 2006; Laxmi *et al.*, 2008). A complete understanding of phosphorylation- and cycling-dependent polarity maintenance explaining the way these processes proceed and interact *in planta* is still lacking The components discussed above are responsible for transporting auxin to the cells were it activates the responses essential for plant development. The way auxin is perceived by plant cells will be discussed below.

Perception: auxin-responsive gene expression

The polar transport-generated auxin maxima and gradients are instructive for plant cell growth and differentiation. At the cellular level, auxin concentrations are translated into a gene expression response by the complex and dynamic interaction between two large families of transcriptional regulators: the Auxin

Response Factors (ARFs) and the labile Aux/IAA proteins (Guilfoyle *et al.*, 1998b; Ulmasov *et al.*, 1999; Tiwari *et al.*, 2001).

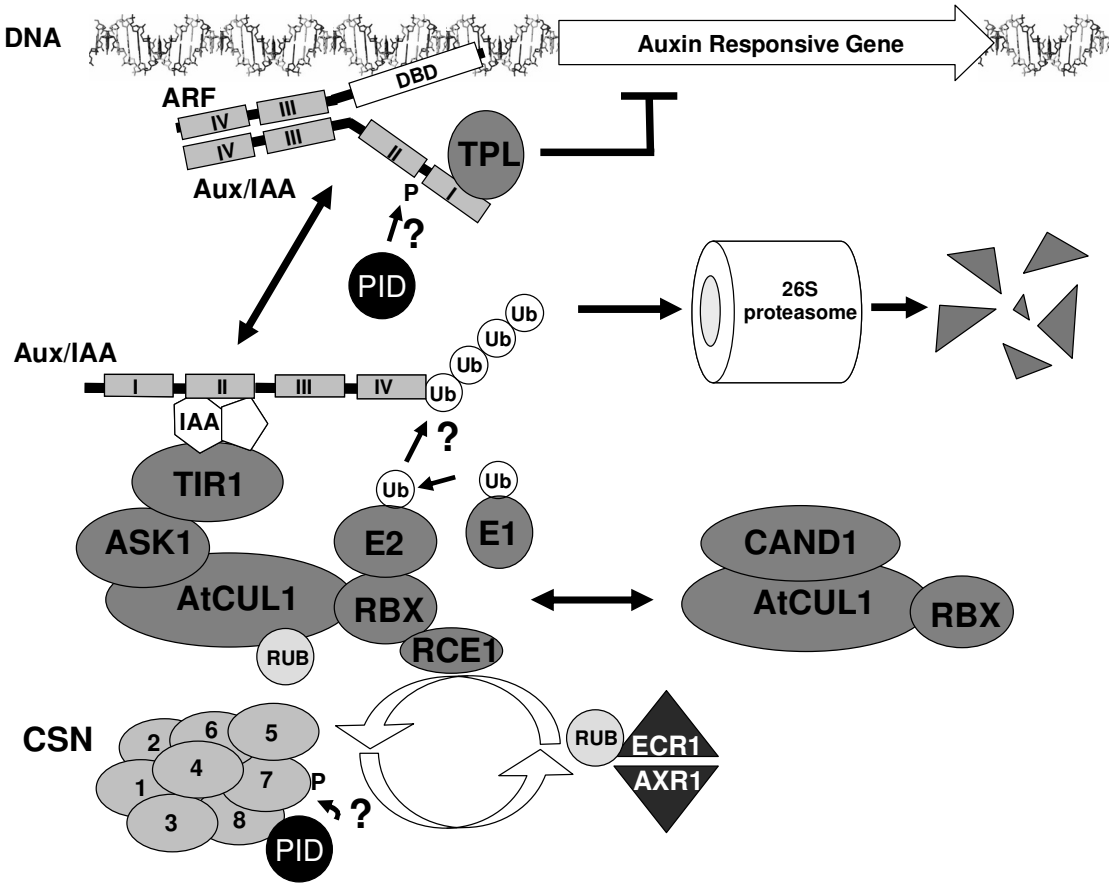

Figure 1: Mechanism of auxin perception by the SCF^{TIR1} E3 ubiquitin ligase in *Arabidopsis thaliana*. Aux/IAA proteins are labeled for proteolysis by ubiquitination. This process is mediated by the ubiquitin activating enzyme E1, the ubiquitin conjugating enzyme E2 and the ubiquitin ligase E3. Under low auxin concentrations Aux/IAAs proteins heterodimerize with the ARF transcription factors, thereby repressing auxin-inducible gene expression trough association with the co-repressor TPL. Auxin binding to TIR1 stimulates its interaction with the domain II of Aux/IAAs which leads to their proteasomal degradation, presumably preceded by Aux/IAA ubiquitination, releasing ARF-dependent transcription. PID-dependent phosphorylation of BDL/IAA12 close to domains I and II might impair TIR1 binding and/or TPL association. The CSN complex can cleave the RUB modifier from CUL1, thus facilitating CAND1 binding to CUL1 and SCF disassembly. PID interacts with the CSN8 subunit of COP9 and phosphorylates CSN7 *in vitro*. Conjugation of RUB to CUL1 by the AXR1-ECR1 and RCE1 enzymes might free CUL1 from CAND1, promoting re-assembly of the active complex. DBD, DNA-binding domain; Ub, ubiquitin; IAA, indole-3-acetic acid. For other abbreviations see text.

ARF proteins bind to specific sequences in the promoters of auxin-responsive genes through their N-terminal DNA-binding domain, and either activate or repress transcription. At the C-terminus they share the conserved domains III and IV with the Aux/IAA proteins, through which they homo- or heterodimerize with other ARFs or with the Aux/IAA proteins (Figure 1) (Guilfoyle *et al.*, 1998a; Guilfoyle *et al.*, 1998b). Several lines of evidence indicate that Aux/IAA proteins do not bind DNA directly, but function as transcriptional repressors by heterodimerizing with activating ARFs (Ulmasov *et al.*, 1997; Kim *et al.*, 1997; Guilfoyle *et al.*, 1998a). Most Aux/IAA proteins are short-lived and degradation of Aux/IAA proteins is essential for auxin signaling. Their half-lives and abundance are dramatically reduced by auxin as a primary response and this process can be blocked by treatment with proteasome inhibitors (Worley *et al.*, 2000; Ramos *et al.*, 2001). Aux/IAA proteins act as transcriptional repressors through the EAR motif present in the conserved domain I (Tiwari *et al.*, 2004) that was shown to mediate the interaction of BDL/IAA12 with the co-repressor TOPLESS (Szemenyei *et al.*, 2008). This interaction seems to be essential for the repressive activity of BDL/IAA12, as the *tlp-1* mutation is able to rescue the rootless *bdl* phenotype. BDL/IAA12 is known to interact and inhibit the activity of the MP/AFR5 transcriptional activator (Hamann *et al.*, 2002). A translational fusion of TOPLESS with domains III and IV of BDL/IAA12 resulted in *bdl/mp*-like phenotypes (Szemenyei *et al.*, 2008). These results indicate that one of the functions of BDL/IAA12 is to bridge the ARF-TLP interaction, which is disrupted upon BDL/IAA12 degradation (Figure 1).

It is clear now that transcriptional and developmental responses to auxin are sensitive to the levels of Aux/IAA proteins (Worley *et al.*, 2000; Ramos *et al.*, 2001; Zenser *et al.*, 2003; Dreher *et al.*, 2006). Several Arabidopsis mutants displaying diminished auxin responses were found to have gain-of-function mutations in *Aux/IAA* genes (Figure 2). Strikingly, all these mutations affect specific sites in domain II, and lead to extended protein half-life and presumably much greater abundance of the respective Aux/IAA proteins (Worley *et al.*, 2000; Ouellet *et al.*, 2001). The conserved domain II of Aux/IAA proteins contains a 13 amino acid sequence that functions as a transferable degradation

signal, and it is necessary and sufficient to define Aux/IAA protein stability (Ramos *et al.*, 2001). This motif was defined as QVVGWPPVRSYRK, underlined residues indicate those conserved among all the domain II-containing Arabidopsis Aux/IAAs.

Aux/IAA gene	Domain II mutations	References
AXR2/IAA7	PAKAQVV**GWPPV**RN	
arx2-1	**S**	Nagpal *et al.*, 2000
AXR3/IAA17	PAKAQVV**GWPPV**RS	
axr3-1	**L**	
axr3-3	**G**	Rouse *et al.*, 1998
axr3-101	**E**	Okushima *et al.*, unpub.
SHY2/IAA3	PPRKAIV**GWPPV**RS	
shy2-1, -2	**S**	
shy2-3	**E**	Tian and Reed, 1999
shy2-6	**L**	Fukaki *et al.*, unpub
SLR1/IAA14	PPAKAVV**GWPPV**RN	
slr1-1, -4	**S**	Fukaki *et al.*, 2002
slr-2	**S**	
slr-3	**A**	Fukaki *et at.* unpub
IAA28	VEVAPVV**GWPPV**RS	
iaa28-1	**L**	Rogg *et al.*, 2001
MSG2/IAA19	PAAKASV**GWPPV**CS	
msg2-1	**S**	
msg2-2	**R**	
msg2-3	**L**	
msg2-4	**L**	Tatematsu *et al.*, 2004
BDL/IAA12	PPRSSVV**GWPP**IGL	
bdl	**S**	Hamann *et al.,* 2002
IAA13	PPRSSVV**GWPP**IGL	
iaa13	**S**	Weijers *et al.*, 2005
IAA18	TAPGPVV**GWPPV**RS	
crane-1	**R**	
crane-2	**E**	Uehara *et al.* 2008
SHY1/IAA6	PVVKSAV**GWPPV**CS	
shy1-1	**R**	Reed, 2001
ARX5/IAA1	PPAKTQIV**GWPPV**R	
iaa1-GR	**L**	Park *et al.*, 2002
axr5-1	**S**	Yang *et al.*, 2004

Figure 2: Amino acid substitutions in domain II that stabilize Aux/IAA proteins

This domain interacts with the F-box-protein TIR1 and the interaction is promoted by auxin in a concentration-dependent manner leading to Aux/IAA proteasomal degradation (Gray *et al.*, 2001; Dharmasiri *et al.*, 2003). For a long time it was thought that the degron was modified upon auxin treatment. Recently, however it was uncovered that TIR1 binds auxin and this enhances the interaction with the Aux/IAA proteins (Figure 1). The Aux/IAA proteins bind

TIR1 in the absence of auxin, but with low affinity (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a). Crystallographic studies showed that the auxin molecule acts as "molecular glue" between TIR1 and its substrate, binding both proteins and facilitating hydrophobic packing between TIR1 and its substrate (Tan *et al.*, 2007). The conserved central GWPPV motif is the hallmark of the Aux/IAA degron. Two amino acids in the motif, tryptophan and the second proline, interact with the surrounding hydrophobic wall of the TIR1 pocket and stack against the auxin molecule lying underneath, packing against the auxin indole ring and the auxin side chain, respectively. In the structure, the glycine residue is located at a critical position, where flexibility of the peptide is required for the N-terminal region of the substrate peptide to take a sharp turn and continue interacting with TIR1, indicating that the integrity and hydrophobicity of domain II is crucial for TIR1 recognition. Aux/IAA domain II mutants were identified with these core amino acids changed into the acidic residue glutamic acid (Tian and Reed, 1999; Uehara *et al.*, 2008; Ploense *et al.*, 2009), indicating that the acidic modification (i.e. phosphorylation) of domain II is a plausible mechanism for reducing TIR1-Aux/IAA interaction (Figure 2).

TIR1 is the first true auxin receptor described, acting alongside other members of the AFB (Auxin F-box protein) family to form $SCF^{TIR1/AFB}$ complexes that control auxin-dependent degradation of Aux/IAA proteins (Dharmasiri *et al.*, 2005b). The dependence on SCF^{TIR1} and the 26S proteasome suggests that Aux/IAA proteins are degraded via the ubiquitin-dependent pathway, although direct evidence for this post-translational modification is lacking. Ubiquitin dependency of proteasomal degradation will be discussed in more detail below.

Proteasomal degradation and ubiquitination.

Much of cellular physiology, growth, and development are controlled by the selective removal of regulatory proteins. Like all macromolecular components of an organism, the proteome is in a dynamic state of synthesis and degradation. In eukaryotic organisms, ubiquitin conjugation to target proteins and

subsequent degradation by the proteasome plays an important role in diverse cellular processes.

Ubiquitin is a highly conserved 76 amino acid (~9 kDa) protein that is abundantly expressed in all eukaryotic cells. Protein ubiquitination is a multistep process, involving at least three types of enzymes and generally results in the covalent attachment of poly-ubiquitin chains to target proteins. A prominent role of poly-ubiquitin chains is that they label proteins for degradation by the proteasome (Figure 1). As a first step in the ubiquitination process, an ubiquitin-activating enzyme (also known as E1) forms a thiol-ester bond with the carboxy-terminal glycine of ubiquitin in an ATP-dependent process. Then, a ubiquitin-conjugating enzyme or ubiquitin-carrier enzyme (UBC, also known as E2) accepts ubiquitin from the E1 by a *trans*-thiolation reaction, again involving the glycine at the carboxy-terminus of ubiquitin. Finally, an ubiquitin protein ligase (E3) catalyses the transfer of ubiquitin from the E2 enzyme to the ε-amino group of a lysine residue on the substrate (Glickman and Ciechanover, 2002). Chains containing at least four glycine-76 to lysine- 48 isopeptide-linked ubiquitins are necessary for efficient binding to the component S5a/Rpn10 of the proteasome (Baboshina and Haas, 1996; Thrower *et al.*, 2000). The quaternary structure of ubiquitin polymers and the exact spatial relationship between each ubiquitin molecule is also critical for their ability to target substrates for degradation by the proteasome.

The 26S proteasome is a 2.5-MDa ATP-dependent proteolytic complex that mostly degrades ubiquitin conjugates (Voges *et al.*, 1999). It contains 32 principal subunits arranged into two subcomplexes, the 20S core protease (CP) and the 19S regulatory particle (RP). The 20S core subunit bears a broad spectrum ATP- and Ub-independent protease activity. The active sites of the CP are very sensitive to the proteasome inhibitors MG115, MG132, lactacystin, and epoxomycin (Yang *et al.*, 2004). The 19S RP associates with one or both ends of the CP and confers both ATP-dependence and specificity for Lys48-linked polyubiquitin chains to the particle. ATP-ase and de-ubiquitinase (DUB) activities associated to 19S RP subunits are responsible for protein unfolding and directing the unfolded de-ubiquitinated polypeptides into the lumen of the

CP for breakdown. Posttranslational modification of proteins by covalent attachment of ubiquitin is a reversible process and the processed ubiquitins are recycled to be re-used by the cell. All known deubiquitinating enzymes (DUBs) are cysteine proteases that specifically hydrolyze the amide bond immediately after the COOH-terminal residue. Based on their molecular size, sequence homology, and active site residues, DUBs are categorized as UCHs (ubiquitin COOH-terminal hydrolases) or UBPs (ubiquitin-specific proteases) (Nijman *et al.*, 2005). UCHs are generally small enzymes (20–30 kDa) that remove short or flexible peptide chains from the COOH terminus of ubiquitin. UBPs on the other hand belong to a larger and a more diverse group of enzymes and have a larger molecular mass, typically in the range of ~100 kDa. UBPs can cleave the isopeptide bond linking Ub-Ub or Ub-protein. Despite the common active site residues, the UBP and UCH families do not share sequence homologies with one another or with cysteine proteases (D'Andrea and Pellman, 1998; Chung and Baek, 1999). DUBs play several roles, both in maintaining the levels of free ubiquitin and in regulating the stability of Ub-conjugated proteins (Nijman *et al.*, 2005), including Aux/IAAs in Arabidopsis (Yang *et al.*, 2007).

Ubiquitin was first identified as a covalently attached signal to proteins targeted for degradation (Hershko *et al.*, 1982). Polyubiquitin chains linked *via* Lys-48 are the principal signals recognized and degraded by the proteasome. It has, however, now been realized that ubiquitination not only regulates intracellular proteolysis, but also diverse processes such as transcription, receptor-mediated signal transduction and endocytotic sorting (Mukhopadhyay and Riezman, 2007). Ubiquitination on Lys-63 of ubiquitin appears to play a role in a variety of processes not involving proteolysis including endocytosis of cell surface receptors (Hicke, 1999), post-replicative DNA repair (Spence *et al.*, 1995), stress response (Arnason and Ellison, 1994), mitochondrial DNA inheritance (Fisk and Yaffe, 1999), ribosomal function (Spence *et al.*, 2000), and activation of the IκBα signaling complex (Wang *et al.*, 2001). Mono-ubiquitination also plays important non-proteolytic roles such as endocytosis (Terrell *et al.*, 1998) and control of gene transcription (Pham and Sauer, 2000).

The necessity of ubiquitin modification as a signal for proteasomal degradation was challenged by the discovery of ornithine decarboxylase (ODC) which is degraded via an ubiquitin-independent process (Figure 3). ODC is a rate-limiting enzyme in polyamine biosynthesis and accumulation of polyamines stimulates the synthesis of the antizyme protein. Antizyme in turn was found to negatively regulate ODC by binding, which is sufficient to trigger ODC degradation by the 26S proteasome (Coffino, 2001). Non-ubiquitinated ODC monomers are degraded by 20S proteasomes in a process regulated by NAD(P)H Quinone Oxidoreductase1 (NQO1) (Asher *et al.*, 2005a). The REGγ alternative lid complex of the proteasome was shown to mediate the ubiquitin-independent degradation of the mammalian cell cycle regulator $p21^{CIP21}$ (Chen *et al.*, 2007). Moreover, the tumor suppressor proteins p53 and p73 are degraded by the proteasome in an ubiquitination-independent manner, and association with the 20S proteasome gatekeeper NQO1 blocks this degradation (Asher *et al.*, 2005b). The regulation of inherently unstable proteins like ODC, p53 and p73 was proposed to follow a "degradation by default" mechanism (Asher *et al.*, 2006) where degradation occurs unless specific intervention with NQO1 or homodimerization, in the case of ODC, prevents it. What is clear from these examples from the animal research field is that proteasomes have diverse ways for target recognition (Figure 3).

Considering the amount of examples of different ubiquitin modifications and the downstream effects of these processes, it is now clear that ubiquitination is much more than a proteasomal targeting signal. How it mediates responses to DNA damage, facilitates endosomal transport, and increases the efficiency of translation are all open questions. The genome of *Arabidopsis* encodes more than 1400 (or >5% of the proteome) ubiquitin pathway components, illustrating the importance of the ubiquitin pathway in the regulatory plasticity of plants (Lechner *et al.*, 2006).

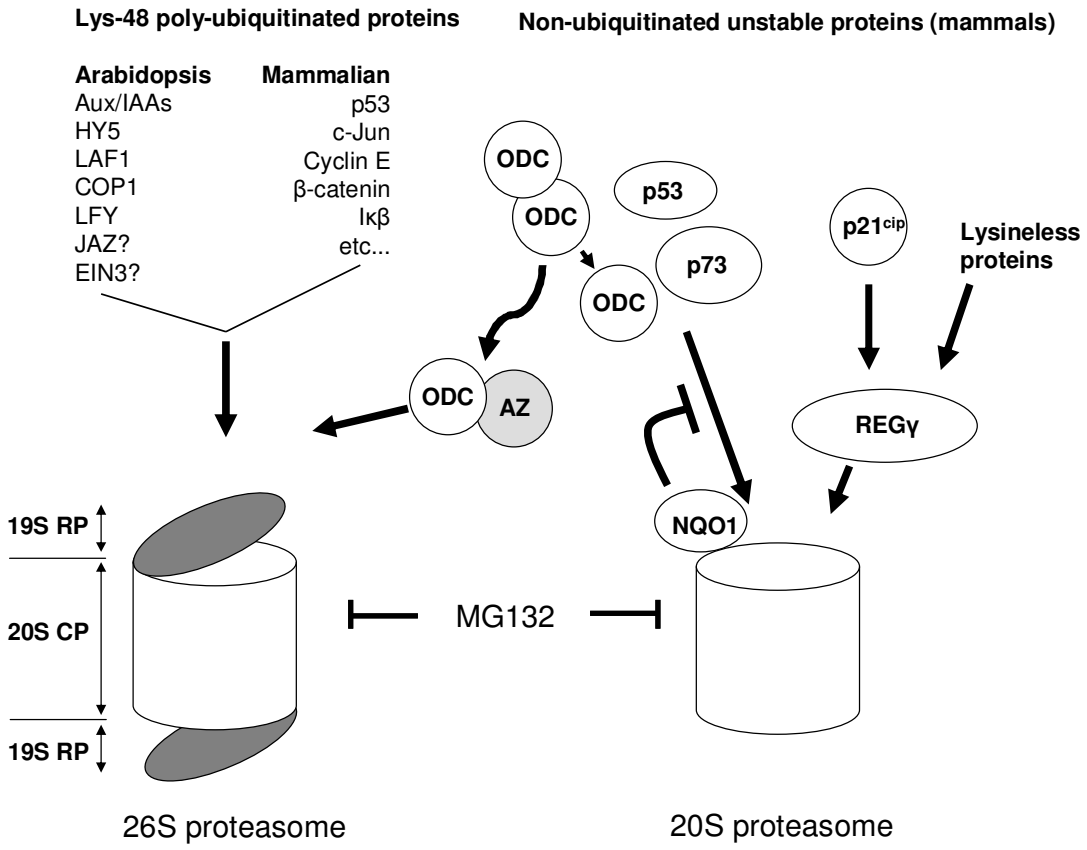

Figure 3: Schematic representation of ubiquitin-dependent and -independent pathways of proteasomal degradation. Proteins containing Lys-48 linked poly-ubiquitin chains are recognized by the 19S regulatory particle (RP) of 26S proteasomes and degraded in the proteolytically active 20S core particle (CP). Examples of *Arabidopsis* proteins experimentally demonstrated to be ubiquitinated are shown together with the presumably Ub-modified JAZ and EIN3 proteins. Classical examples of mammalian ubiquitinated proteins degraded by the 26S proteasome are shown. Some unstable proteins are degraded via ubiquitin-independent processes in mammals. Binding of antizyme (AZ) to ODC disrupts ODC homodimers and induces ubiquitin-independent 26S proteasomal degradation of ODC. Free ODC monomers are also degraded by 20S proteasomes without ubiquitination. NQO1 functions as a gatekeeper of 20S proteasomes and interacts with p53, p73 and ODC in a NADH-dependent manner to protect them from 20S proteasomal degradation. The REGγ complex acts as an alternative lid of 20S proteasomes and controls the degradation of $p21^{cip}$ and other lysine-less proteins. Both 20S and 26S degradation pathways are inhibited by 20S CP proteasome inhibitors like MG132.

CULLIN1 containing (SCF) E3 ligases and hormone responses in plants

Ubiquitin is a widespread cellular signal and, as described above, ubiquitin conjugation is achieved through an ATP-dependent reaction cascade involving the sequential action of three enzymes, E1, E2s, and E3s. E1 activates

ubiquitin, E2s catalyze covalent attachment of ubiquitin to target proteins which are recognized by associated E3s. As the final enzyme in the cascade, the E3s or Ubiquitin-protein ligases are responsible for recognizing the substrate and facilitating Ub transfer, determining the specificity of the response. Different types of E3s have been identified that differ according to their subunit organization and/or mechanism of Ub transfer (Deshaies, 1999). One important E3 type comprises the SCF complexes which are composed of four major subunits: CULLIN 1 (CUL1), SUPPRESSOR OF KINETOCHORE PROTEIN 1 (SKP1), RING-BOX 1 (RBX1)/REGULATOR OF CULLINS 1 (ROC1) and an F-box protein (Figure 1). Structure–function studies in yeast and mammals have demonstrated that CUL1 functions as a scaffold in assembling the different subunits of the complex. CUL1 interacts at its carboxyl terminus with the RING-domain protein RBX1 (forming the core catalytic domain) and, at its amino terminus, with the adaptor protein SKP1, which links to one of multiple F-box proteins. F-box proteins, in addition to the loosely conserved F-box motif that binds to SKP1, usually carry one of a variety of typical protein–protein interaction domains that confer substrate recognition specificity to the SCF complexes. The large number of F-box proteins in plant genomes, nearly 700 in Arabidopsis (Gagne *et al.*, 2002), is thought to allow for the specific ubiquitination of a large number of functionally and structurally diverse substrates.

Besides the role of the $SCF^{TIR1/AFB}$ E3 ligases in auxin responses, SCF complexes also regulate other phytohormone signaling pathways, including those for jasmonate, gibberellin and ethylene. The jasmonate perception mechanism involves the F-box protein, COI1. Since its discovery, the *coi1* mutant was regarded as the strongest jasmonate-insensitive mutant (Feys *et al.*, 1994; Xie *et al.*, 1998). The F-box protein COI1 was shown to assemble into an active SCF complex (Devoto *et al.*, 2002; Xu *et al.*, 2002) but its targets remained unknown until the discovery of JAZ repressors. JAZ (Jasmonate-ZIM domain) proteins represent a family of labile proteins which are postulated to negatively regulate the expression of jasmonate-responsive genes via their interaction with the activator MYC2. (Chini *et al.*, 2007). Their stability is

regulated by SCF^{COI1} and the 26S proteasome (Thines *et al.*, 2007; Chini *et al.*, 2007). Jasmonate-Isoleucine (JA-Ile) was shown to stimulate the interaction of certain members of the JAZ family with COI1 *in vitro* and in yeast (Thines *et al.*, 2007; Melotto *et al.*, 2008). This is postulated to lead to JAZ proteasomal degradation supposedly via ubiquitination. COI1 is the closest relative to the TIR1/AFB clade of F-box proteins and their mechanism of action is very similar since COI1 (or the COI1-JAZ complexes) was found to be the receptor for Jasmonate-Isoleucine/Coronatine analogous to TIR1 for auxin (Spartz and Gray, 2008; Katsir *et al.*, 2008a; Katsir *et al.*, 2008b). The gibberellin (GA) signaling pathway is regulated in Arabidopsis by the F-box proteins SLEEPY1 (SLY1) and SNEEZY (SNE) (McGinnis *et al.*, 2003; Strader *et al.*, 2004) and in rice by the F-box protein GID2 (Sasaki *et al.*, 2003). Like TIR1 and COI1, these F-box proteins are involved in the degradation of negative regulators, which in the case of GA responses are the DELLA proteins which belong to the GRAS superfamily of putative transcriptional regulators. DELLA proteins directly or indirectly repress the expression of GA-induced genes (Feng *et al.*, 2008; Daviere *et al.*, 2008). In Arabidopsis, the gibberellin molecule is recognized by the soluble receptor GID1. The interaction leads to a conformational change in the GID1 protein that traps the GA molecule inside a receptor pocket forming a closing lid. This induced modification allows DELLA proteins to interact with the upper surface of the lid, and it is hypothesized that this interaction may cause a change in the shape of the DELLA protein that allows it to associate with the ubiquitin ligase $SCF^{SLY/SNE}$. Thus, GA functions as an allosteric activator of GID1, causing structural changes that allow the receptor to associate with DELLA proteins but GA does not interact directly with DELLAs (Murase *et al.*, 2008; Shimada *et al.*, 2008).This is significantly different from the TIR1-Aux/IAA and COI1-JAZ interaction, where the signaling molecule does not induce conformational changes but rather acts as a molecular glue, in the case of TIR1, between F-box protein and the repressor. SCF-dependent degradation of transcriptional regulators is emerging as a common feature in plant developmental and adaptive responses with several examples described of interactors for the many Arabidopsis F-box proteins. Despite the emerging

importance of SCF complexes and protein degradation in plants the demonstration that the interacting partners of SCF complexes are actually ubiquitinated is missing for the vast majority of these targets.

Regulation of SCF E3 ubiquitin ligases by RUB: a role for the COP9 signalosome.

Given their importance in cellular functions, it is not surprising that SCF assembly and activity are highly regulated. So far, three proteins or protein complexes have been implicated in SCF regulation. These are the ubiquitin-related protein RUB/Nedd8 (for Related to Ubiquitin1 or Neural precursor cell expressed developmentally down-regulated 8), the COP9 signalosome (CSN), and CAND1 (for Cullin Associated Neddylation Dissociated1) (Figure 1). RUB conjugation to CUL1 is achieved by the activity of the ECR1 and AXR1/RCE1 complexes (del Pozo *et al.*, 2002) and phenotypes of different mutants indicate that rather than working as an on/off switch, the RUB cycling is essential for the assembly of SCF complexes in cooperation with CAND1 (Chuang *et al.*, 2004). CAND1 binds de-rubylated CUL1 and inhibits CUL1/RBX1 binding to SKP1 (Figure 1), thus preventing the formation of an active SCF complex (Feng *et al.*, 2004). Reducing the amount of CAND1 in cells leads to an increase in the number of complexes containing CUL1 and SKP1. The regulation of SCF E3 ubiquitin ligases is dependent on the activity of the COP9 (CSN) signalosome that cleaves RUB from the CUL1 subunit of SCF (Cope *et al.*, 2002b) releasing CAND1 from the CUL1/RBX1 complex allowing the formation of new SCF complexes (Zhang *et al.*, 2008). Based on these results, it has been proposed that CAND1, the RUB conjugation pathway, and the CSN together regulate a cycle of SCF assembly and disassembly (Cope and Deshaies, 2003; Pintard *et al.*, 2003; Parry and Estelle, 2006). One of the strongest auxin-resistant mutants is *axr1*, which is unable to conjugate RUB to CUL1 impairing the activity of the SCF^{TIR1} (del Pozo *et al.*, 2002). Modification of SCF^{TIR1} by RUB has been implicated as a central step in the response to the plant hormone auxin.

The COP9 signalosome (CSN) is a large nuclear-enriched multiprotein complex identified in genetic screens for *constitutive*

photomorphogenic/deetiolated/fusca (*cop/det/fus*) mutants in *Arabidopsis*. The COP9 signalosome consists of eight subunits, and shows intriguing structural and sequence homology to the 19S regulatory particle (RP) of the proteasome. A mutation in a single CSN subunit can destabilize the entire complex (Schwechheimer *et al.*, 2002; Serino *et al.*, 2003). All strong *cop/det/fus* mutations lead to seedling lethality shortly after germination, indicating that besides controlling light-dependent processes the integrity of the CSN complex is central to plant development (Kwok *et al.*, 1996). CSN-dependent RUB de-conjugation from CUL1 is accomplished by the CSN5 subunit which bears a metalloprotease activity necessary for RUB cleavage (Cope *et al.*, 2002a). CSN and SCF complexes are known to physically interact and it was shown that CSN5 reduction-of-function lines display a phenotype similar to that of the *axr1* mutant and slower degradation rates of Aux/IAA proteins (Schwechheimer *et al.*, 2001). Besides auxin, SCF-CSN processes are tightly connected to many cellular and developmental responses such as light, jasmonate, gibberellins, ethylene, floral organ formation, circadian rhythms, shoot branching and many others (Chamovitz *et al.*, 1996; Karniol and Chamovitz, 2000; Feng *et al.*, 2003; Wang *et al.*, 2003; Guo and Ecker, 2003; Han *et al.*, 2004; Cheng *et al.*, 2004; Stirnberg *et al.*, 2007). Considering the wide repertoire of SCF complexes that can be formed by the Arabidopsis proteome, the CSN is emerging as central regulator of E3 ubiquitin ligases in plant biology.

Thesis outline

Auxin biology is among the oldest fields of experimental plant research. Nowadays, auxin is one of the most extensively studied plant hormone. Most of its effects on regulating cell division, growth and differentiation are dependent on its transport driven asymmetric distribution. At the cellular level, the molecular components of downstream signaling still demand further investigation. Auxin action was found to be dependent on dynamic gradients generated by PIN efflux carriers' asymmetric distribution. Only recently, the F-box protein TIR1 was identified as a receptor for auxin and the link between synthesis, transport, perception and effects of auxin gained a molecular

framework to be explored. Being part of a SCF E3 ubiquitin ligase complex, the TIR1 receptor uses the ubiquitin system to control levels of auxin-dependent gene responses via degradation of the Aux/IAA transcriptional repressors. **Chapter 2** uses an Arabidopsis protoplast system to show the ubiquitination of the SHY2/IAA3 and BDL/IAA12 proteins by the SCF^{TIR1} E3 ubiquitin ligase complex. The mechanism of auxin perception based on auxin-induced Aux/IAA binding to the SCF^{TIR1} receptor was based on the supposition that this interaction would lead to Aux/IAA ubiquitination followed by 26S proteasomal degradation. Our results confirm that Aux/IAAs are ubiquitinated and the process is stimulated by TIR1 overexpression, whose protein levels determine the sensitivity of cells towards auxin and leads to Aux/IAA degradation even in the absence of auxin treatment. It is known that Aux/IAAs display differential activities on auxin-responsive gene expression and our experiments indicate that BDL/IAA12 acts as a stronger and less stable protein than SHY2/IAA3. It is likely that these differences observed might be linked to differential affinity of these proteins to the TIR1 receptor which reinforces our hypothesis on phosphorylation-dependent regulation of the BDL protein (Chapter 4).

Our concern on demonstrating the ubiquitination of Aux/IAAs was stimulated after the findings described on **Chapter 3** with the characterization of a calcium binding protein PBP1, first identified as an interactor of the serine-threonine kinase PINOID, a regulator of auxin transport. When expressed in Arabidopsis cell suspensions protoplasts, PBP1 was found to be highly unstable protein that is poly-ubiquitinated and degraded by the proteasome. Mutation of all the lysines on the PBP1 primary sequence abolishes ubiquitin attachment but does not affect the proteasomal degradation of the protein. All known biochemical functions are maintained in the lysine-less versions of PBP1. We believe that PBP1 is controlled by ubiquitin-independent proteasomal degradation confirming observations from the animal field that not all targets of proteasomal degradation are necessarily ubiquitinated proteins.

Chapter 4 describes the Aux/IAA protein BODENLOS (BDL/IAA12) as a putative *in vivo* phosphorylation target. From *in vitro* an *in vivo* studies there was indication that the two proteins could interact during plant development. *In*

vitro phosphorylation assays identified a PRSS motif as the site of phosphate modification by PID. Mutation of the two Serines on PRSS to PRKA abolishes *in vitro* phosphorylation of BDL/IAA12 by PID and analysis of transgenic plants carrying a phosphorylation-insensitive version of the gain-of-function *bdl* protein indicated that the phosphorylation site identified is essential for the function of the *bdl* protein *in planta.* Although the identified site seems to be important on the regulation of the BDL protein, the contribution of PID to the process is still hypothetical and the *in vivo* phosphorylation of this site still remains to be shown.

Chapter 5 describes the identification of CSN subunit CSN8/COP9 as an interacting partner of the PINOID kinase. PID phosphorylates the neighboring subunit CSN7/*COP15 in vitro* but further analysis showed that this phosphorylation is not relevant for CSN activity *in planta*. On the other hand, PID is ubiquitinated *in vivo* and overexpression of CSN8 induces PID nuclear localization and enhances its ubiquitination. Is discussed the possibility of PID to act as a CSN-associated kinase regulated by ubiquitination and that this interaction would control the association of PID with phosphorylation targets also controlled via ubiquitination such as BDL/IAA12 and PIN2.

The results presented on this thesis illustrate the wide spectrum of cellular processes in plants regulated though protein ubiquitination and proteasomal degradation. The confirmation of the TIR1-Aux/IAA model to be ubiquitin-dependent contrasts with the PBP1 ubiquitin-independent degradation and rises the discussion of how many proteasome targets are ubiquitinated. The large representation of F-box proteins in the Arabidopsis proteome and the many processes regulated by ubiquitin modification will demand careful interpretation of experimental results. The well-established TIR1 recognition of Aux/IAAs has to be challenged with other members of the diverse Aux/IAA family to assess the particularities of each protein as well as for the other TIR1/AFB proteins. Regulation of polar auxin transport through ubiquitin- and COP9-related processes seem to involve PINs and PID via a integrated mechanism using both phosphorylation and ubiquitination as targeting signals. During a long time

PID functions were analyzed based on the effects it has on its targets and now the regulators of PID, such as CSN, open field for a new exploration.

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Chapter 2

Auxin-induced, SCF^{TIR1}-mediated poly-ubiquitination marks AUX/IAA proteins for degradation

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Summary

The plant hormone auxin (indole-3-acetic acid or IAA) regulates plant development by inducing rapid cellular responses and changes in gene expression. Auxin promotes the degradation of Aux/IAA transcriptional repressors, thereby allowing auxin response factors (ARFs) to activate the transcription of auxin-responsive genes. Auxin enhances binding of Aux/IAA proteins to the receptor TIR1, which is an F-box protein that is part of the E3 ubiquitin ligase complex SCF^{TIR1}. Binding of Aux/IAA proteins leads to degradation via the 26S proteasome, but evidence for SCF^{TIR1}-mediated poly-ubiquitination of Aux/IAA proteins is lacking.

Here we used an *Arabidopsis* cell suspension-based protoplast system to find evidence for SCF^{TIR1}-mediated ubiquitination of the Aux/IAA proteins SHY2/IAA3 and BDL/IAA12. Each of these proteins showed a distinct abundance and repressor activity when expressed in this cell system. Moreover, the amount of endogenous TIR1 protein appeared to be rate-limiting for a proper auxin response measured by the co-transfected *DR5::GUS* reporter construct. Co-transfection with *35S::TIR1* led to auxin-dependent degradation, and excess of *35S::TIR1* even led to degradation of Aux/IAAs in the absence of auxin treatment. Expression of the mutant tir1-1 protein or the related F-box protein COI1, which is involved in jasmonate signaling, had no effect on Aux/IAA degradation. Our results show that SHY2/IAA3 and BDL/IAA12 are poly-ubiquitinated and degraded in response to increased auxin or TIR1 levels. In conclusion, our data provide experimental support for the model that SCF^{TIR1}-dependent poly-ubiquitination of Aux/IAA proteins marks these proteins for degradation by the 26S proteasome, leading to activation of auxin-responsive gene expression.

Introduction

The plant hormone auxin (indole-3-acetic acid or IAA) plays an essential role in a large variety of developmental processes throughout a plant's life cycle. Auxin is transported in a polar cell-to-cell manner, and this transport directs cell division and growth by generating dynamic auxin gradients in tissues and organs. At the cellular level, auxin concentrations are translated into a gene expression response by the complex and dynamic interaction between two major families of transcriptional regulators: the Auxin Response Factors (ARFs) and the labile Aux/IAA proteins (Guilfoyle *et al.*, 1998; Ulmasov *et al.*, 1999; Tiwari *et al.*, 2001). ARF proteins bind to specific sequences in the promoters of auxin-responsive genes through their N-terminal DNA-binding domain, and either activate or repress transcription. At the C-terminus they share the conserved domains III and IV with the Aux/IAA proteins, through which they homo- or heterodimerize with other ARFs or with the Aux/IAA proteins (Guilfoyle *et al.*, 1998) .

Aux/IAA proteins are short-lived transcriptional regulators that repress transcription controlled by auxin-responsive elements (AuxREs) by heterodimerizing with ARFs. The repressor activity of these proteins is located in the N-terminal domain I, whereas their stability is regulated by the central domain II. Auxin binds to the receptor TIR1 or the related Auxin signalling F-Box (AFB) proteins that are part of the E3 ubiquitin ligase complexes $SCF^{TIR1/AFB}$. Auxin enhances the affinity of TIR/AFB for domain II of Aux/IAAs (Gray *et al.*, 2001; Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b; Tan *et al.*, 2007). Mutations in either the Aux/IAA domain II or in one of the SCF components lead to auxin-resistant phenotypes that are mostly due to the stabilization of the Aux/IAA repressors (Worley *et al.*, 2000; Ouellet *et al.*, 2001). Moreover, treatment of plants with proteasome inhibitors leads to the accumulation of these proteins, indicating that Aux/IAA protein levels are controlled by the 26S proteasome (Gray *et al.*, 2001; Ramos *et al.*, 2001; Tian *et al.*, 2003). Taken together, this information leads to a model in which auxin-enhanced binding of TIR1/AFB to domain II of the Aux/IAAs results in

ubiquitination of these proteins, which marks them for degradation by the 26S proteasome. However, there is no direct experimental evidence for the $SCF^{TIR1/AFB}$-mediated ubiquitination of Aux/IAA proteins. Recent proteome-wide screens using a multi-dimensional protein identification technology were not able to detect Aux/IAAs among the ubiquitinated proteins in *Arabidopsis* (Maor *et al.*, 2007; Manzano *et al.*, 2008), suggesting that more direct methods may be necessary to detect the ubiquitinated Aux/IAAs.

Although implicated in a large variety of cellular responses, protein ubiquitination of plant transcriptional regulators has been demonstrated for only a few targets, including SLENDER RICE1 (SLR1) by SCF^{GID2} in gibberellin responses (Sasaki *et al.*, 2003), LONG HYPOCOTYL IN FAR RED (HRF1), LONG AFTER FAR-RED LIGHT1 (LAF1) and LONG HYPOCOTYL 5 (HY5) by CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) during photomorphogenesis (Xie *et al.*, 2002; Saijo *et al.*, 2003; Jang *et al.*, 2005), and LEAFY (LFY) by SCF^{UFO} (Chae *et al.*, 2008) in floral development. Targeted proteolysis by SCF-mediated ubiquitination has been implied for the transcription factor ETHYLENE INSENSITIVE3 (EIN3) by $SCF^{EBF1/EBF2}$ in ethylene signaling (Potuschak *et al.*, 2003; Guo and Ecker, 2003), for JASMONATE-ZIM-DOMAIN (JAZ) repressors by SCF^{COI1} in jasmonate signaling (Thines *et al.*, 2007; Chini *et al.*, 2007), and for many other proteins in diverse processes, but experimental evidence for ubiquitination of these proteins and for the specificity of the SCF-ligases for their targets is lacking.

SHORT HYPOCOTYL 2 (SHY2/IAA3) and BODENLOS (BDL/IAA12) are distantly related Aux/IAA proteins that regulate auxin responses in different stages of a plant's life cycle. Both have been shown to interact with TIR1 and to be stabilized by treatment with proteasome inhibitors. The mutant proteins shy2-2 and bdl carry the same Proline to Serine mutations in their domain II, leading to stabilized products show no or only residual interaction with TIR1/AFB (Tian *et al.*, 2003; Dharmasiri *et al.*, 2005b). BDL/IAA12 has been described as an interactor/regulator of the ARF MONOPTEROS (MP/ARF5) acting on embryo patterning (Hamann *et al.*, 2002). SHY2/IAA3 is also able to interact with MP/ARF5 and to inhibit its activity, but the expression patterns of

these two proteins do not overlap *in planta*. Recent studies have connected SHY2/IAA3 action to modulation of NON-PHOTOTROPHIC HYPOCOTYL4 (NPH4/ARF7)- and ARF19-regulated hypocotyl growth (Weijers *et al*., 2005).

Aux/IAAs are short-lived proteins with hardly detectable endogenous levels (Abel *et al*., 1994; Ramos *et al*., 2001; Tian *et al*., 2003). This instability and rareness makes the study of their targeted degradation difficult to perform *in planta*. For a long time now, plant cell protoplasts have been used to assess hormone responses due to their amenability for transformation and their responsiveness to diverse stimuli (Abel and Theologis, 1998; Sheen, 2001). Much of the information gathered on the mechanisms of regulation of Aux/IAA stability has been performed using transiently transformed protoplasts (Tiwari *et al*., 2001; Ramos *et al*., 2001; Tiwari *et al*., 2004), and other important components of the auxin signaling pathway have been functionally characterized in this system including ARFs, SCF^{TIR1} and RAC GTPases (Guilfoyle *et al*., 1998; Tao *et al*., 2005; Wang *et al*., 2005).

In this study, we used *Arabidopsis* cell suspension protoplasts to demonstrate that auxin-enhanced TIR1-mediated ubiquitination of SHY2/IAA3 and BDL/IAA12 marks these proteins for degradation and leads to auxin-responsive gene expression. Our results show that auxin sensitivity of *Arabidopsis* protoplasts is strictly connected to the relative abundance of the TIR1 protein and Aux/IAA proteins, and that an excess of TIR1 leads to depletion of Aux/IAAs even in the absence of exogenous auxin. The mutant protein tir1-1 and the related F-box protein COI1, which is involved in jasmonate signaling, had no effect on Aux/IAA stability or ubiquitination, corroborating the specificity and importance of TIR1 in the process.

EXPERIMENTAL PROCEDURES

Protoplast isolation and transformation

Arabidopsis thaliana Col-0 cell suspension cultures were used for protoplast preparations. Culture maintenance, protoplast isolation and transfections were performed as previously described (Schirawski *et al.*, 2000) with minor modifications. Four-to-six days old cultures were diluted 5-fold in auxin-free Cell Medium (30 g /L saccharose, 3.2 g/L Gamborg's

B5 basal medium with mineral organics, adjusted to pH 5.8 with KOH and sterilized by autoclaving), incubated overnight and used for protoplast isolation in auxin-free solutions. Transfected cells were kept at 25°C in the dark for 16 hours before treatments. Where necessary, additional DNA of plasmid pART7 (Gleave, 1992) was added, to equalize the amount of DNA for each transformation.

DNA constructs

For the auxin-responsive GUS assays, a *DR5::GUS* reporter construct with 7 copies of the DR5 sequence cloned in the plasmid GusXX-47 (Pasquali *et al.*, 1994) was used. A plasmid carrying the *Renilla reniformis* luciferase (*LUC*) gene under the control of the *CaMV 35S* promoter was co-transfected as a control for transformation efficiency (De Sutter *et al.*, 2005). All effector plasmids are based on pART7 carrying the *CaMV 35S* promoter and *OCS* terminator. GATEWAY® (Invitrogen, www.invitrogen.com) destination cassettes derived from pEarleyGate 201 and 202 (Earley *et al.*, 2006) were transferred into pART7 to generate plasmids pART7-HA and pART7-FLAG for the expression of respectively N-terminally HA- or FLAG- tagged proteins in plant cells.

N-terminally HA-tagged cDNAs of *SHY2/IAA3* and *shy2*-2 (P69→S) were cloned from *pACT2-SHY2* and *pACT2-shy2-2* (kindly provided by Jason Reed, (University of North Carlolina, Chapel Hill, North Carolina) using *Xho*I/*Xba*I sites into *pART7*, generating *35S::HA-SHY2/IAA3* and *35S::HA-shy2-2*. The *BDL*/*IAA12* cDNA was excised with *BamH*I/*Xba*I from pET16H-BDL (Weijers *et*

al., 2006) and introduced into pENTR 3C (Invitrogen), and the resulting entry clone was used to create *35S::HA-BDL/IAA12* via LR recombination in pART7-HA (C.S Galvan-Ampudia and Offringa, unpublished) to generate *35S::HA-BDL/IAA12*. The *bodenlos* (P75→S) mutation (Hamann *et al.*, 2002) was introduced in this plasmid using the Quickchange Site-directed Mutagenesis kit (Stratagene) resulting in *35S::HA:bdl*.

The entry clone for *TIR1myc* (Gray *et al.*, 1999) was used for generating the *tir1-1*(G147→D) mutation by Quickchange site-directed mutagenesis. The deletions of the F-box motif in *TIR1* and *tir1-1*, which removes the first 50 amino acids from the original sequence substituting I50 for an alternative M as a start codon, were generated via PCR with primers: 5'-GAATTCATGGGGAACTGCTACGCCGTGAG-3' and 5'-GCGGATCCCTAAAACCTCATTGTTGAGTC-3'.The *COI1* cDNA was amplified from a leaf cDNA library with the primers 5'-CGAGCTCAAAATGGAGGATCCTGATATCAAG-3'and 5'-GGGGTACCGACTGACTCTATGTAATCTCC-3' and cloned into pENTR2B. Entry clones were used in an LR reaction with pART7-FLAG, generating *35S::FLAG-GFP*, *35S::FLAG-TIR1myc*, *35S::FLAG-tir1-1myc* and *35S::FLAG-COI1*.

GUS and LUC assays

In the *DR5::GUS* transactivation assays 10^6 protoplasts were transfected with 10 µg of the *DR5::GUS* reporter construct and 2 µg of *35S:Rluc* (p2rL7 (De Sutter *et al.*, 2005)) for experimental normalization. The DNA amounts of the effector constructs varied per experiment and are indicated in the figure legends. All transformations contained 10 µg of *35S::FLAG-GFP* as a control for transformation efficiency, and were split in 2 portions containing 5 x 10^5 protoplasts in a total volume of 2.5 mL of protoplast medium. After 16 h the samples were treated for 4 h either with 1 µM IAA or the same volume of the solvent DMSO. Treated cells were collected by centrifugation at 80 g for 1 minute and the pellets were frozen in liquid nitrogen for GUS (van der Fits and Memelink, 1997) and LUC measurements (Dyer *et al.*, 2000). Triplicate

transfections were assayed and mean GUS/LUC relative activities were analyzed by One-way ANOVA using SPSS 15.0 software.

Immunoblotting and Immunoprecipitation

For the Aux/IAA degradation/ubiquitination assays, 10^6 protoplasts were transfected with 20 µg *35S::HA-Aux/IAA* construct and 10 µg of *35S::FLAG-GFP*. Depending on the experiment, plasmids encoding FLAG-tagged TIR1myc, tir1-1myc or COI1 were co-transfected in the amounts indicated in the figure legends. Treated protoplasts were resuspended by vortexing in cold Extraction Buffer (PBS, 1x Roche Complete Protease Inhibitor Cocktail) containing 1% Triton X-100, and the lysate was cleared by centrifugation at 20.000 g for 10 min. Total protein was quantified by Bradford assay (Bio-Rad) and 20 µg was mixed with sample buffer and separated on 15% SDS-PAGE minigels. PAGE-separated proteins were blotted onto nitrocellulose membranes, blocked with nonfat dry milk and incubated with the HRP-conjugated antibodies anti-HA High Affinity 3F10 (Roche) and anti-FLAG M2 (Sigma). For detection of ubiquitinated proteins, 10^6 transformed cells were resuspended in 100 µL Extraction Buffer containing 1% Triton X-100, 5 mM EDTA, 10 mM OPA (1,10-Phenanthroline monohydrate, Sigma), and 10 µM MG132 (Sigma). The lysate was cleared by centrifugation at 20.000 g for 10 min, and 5 µL was Western-analyzed as 5 % input control. The remaining total extract was diluted to a final volume of 900 µL with Extraction Buffer without Triton X-100 to bring the Triton concentration to 0.1%. This extract was then mixed with 40 µL of Anti-HA Affinity Matrix (Roche) and incubated for 2 h at 4^oC. The matrix was pelleted and washed 3x in Extraction Buffer, mixed with sample buffer, and the eluted proteins were separated on 12 % SDS-PAGE minigels. PVDF membranes containing transferred proteins were blocked with Qiagen Blocking Reagent and probed with 1000-fold diluted HRP-Anti-Ub P4D1 antibodies (Santa Cruz). After chemiluminescent detection (LumiGLO, Cell Signalling) the blots were stripped and reprobed with anti-HA High Affinity 3F10 antibodies (Roche). When necessary, quantification of the signal was

performed on scanned x-ray films using a BioRad™ GS-800 calibrated densitometer.

RESULTS AND DISCUSSION

Aux/IAAs repress auxin-responsive gene expression in Arabidopsis cell suspension protoplasts.

Leaf protoplasts have extensively been used to study auxin signaling and the regulation of auxin-responsive gene expression by ARF and Aux/IAA proteins (Ulmasov *et al.*, 1997; Kovtun *et al.*, 1998; Abel and Theologis, 1998; Ulmasov *et al.*, 1999). For our experiments we obtained protoplasts from *Arabidopsis* cell suspension cultures because of their continuous availability, the easy isolation procedure, and the high transformation efficiencies obtained. To establish the experimental conditions under which the repressive effect of Aux/IAA proteins on auxin-responsive gene expression could be detected in these cells, we transfected the auxin-responsive reporter construct *DR5::GUS* alone or in the presence of plasmids encoding HA-tagged Aux/IAA proteins. BDL/IAA12 or SHY2/IAA3 were selected for our studies, because they are well-characterized, but distantly related, and they are involved in different developmental processes and therefore representative for the other Aux/IAAs (Tian and Reed, 1999; Tian *et al.*, 2002; Tian *et al.*, 2003; Weijers *et al.*, 2005).

In order to identify working parameters for assaying transcriptional responses to auxin in protoplasts, we transfected one million cells with 10 µg of the auxin-responsive *DR5::GUS* reporter plasmid alone. GUS expression was induced by auxin in a concentration-dependent manner (Figure 1a), and a maximum response was obtained with 1 µM IAA. Co-transfection with 1 µg of the *35S::HA-BDL/IAA12* effector plasmid led to a significant reduction of this response, and when 5 or 10 µg effector plasmid was cotransfected the repression of the *DR5* promoter was saturated in that its activity remained at 30%, even when the cells were treated with 1 or 10 µM IAA (Figure 1a). Similar results were obtained with NAA (data not shown). Based on these results (Figure 1a), a reporter:effector plasmid ratio of 10 : 1 (in µg) and an auxin concentration of 1 µM IAA were used to study the repression activity on the

DR5 promoter. SHY2/IAA3 and BDL/IAA12 both showed repression activity on the DR5 promoter. The transfected amount of BDL/IAA12 plasmid resulted in a stronger repression than transfection with the same amount of SHY2/IAA3 plasmid. The auxin response was almost completely repressed by the stabilized mutant versions shy2-2 and bdl (Figure 1b).

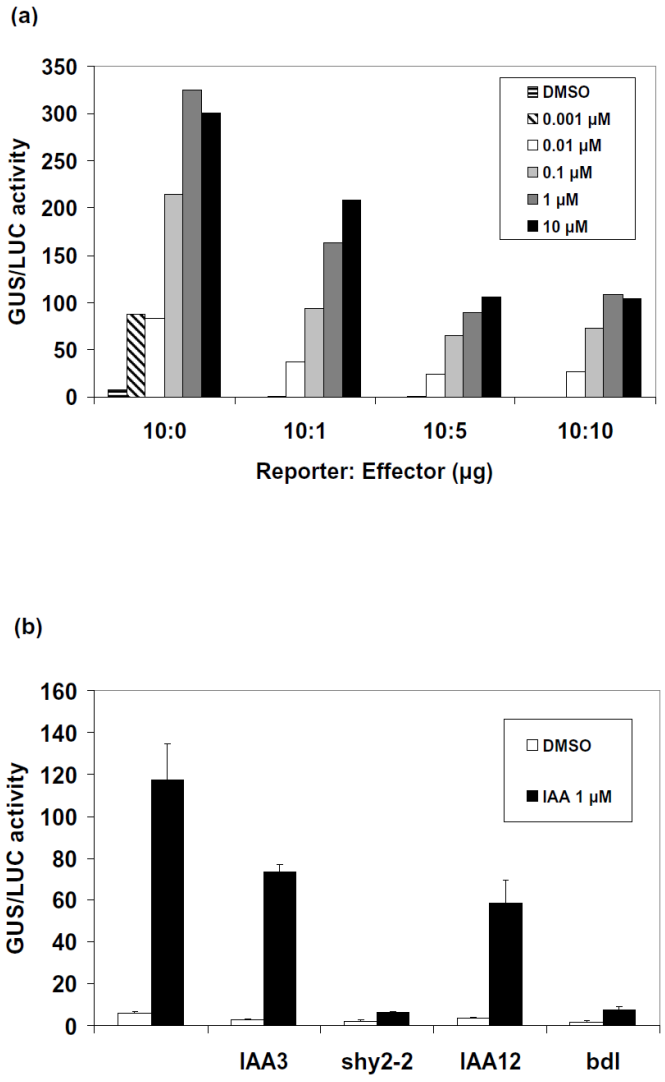

Figure 1. Auxin-responsive *DR5::GUS* reporter gene expression in Arabidopsis protoplasts is repressed by the Aux/IAA proteins BDL/IAA12 and SHY2/IAA3.

(a) Relative GUS/LUC activity (arbitrary units) in transfected Arabidopsis protoplasts treated for 8 hours with increasing concentrations of auxin (IAA). The numbers on the X-axis indicate the reporter (*DR5::GUS*) to effector (*35S::HA-BDL/IAA12*) ratio in µg plasmid DNA per transformation.
(b) Effect of co-transfecting Aux/IAA effector plasmids expressing SHY2/IAA3, BDL/IAA12 or the stabilized mutant proteins shy2-2 or bdl on the activity of the *DR5::GUS* reporter. Reporter and effector plasmids were co-transformed in a reporter to effector ratio of 10:1 µg plasmid DNA. Protoplasts were treated for 8 hours with DMSO or 1 µM IAA. Letters indicate significantly different groups based on One-way ANOVA ($p<0.05$).

Cellular auxin and TIR1 levels are interdependent parameters in Aux/IAA degradation

Although clearly active in the repression of transcription, the transiently expressed Aux/IAA proteins in the previous experiments could not be detected on Western blots (data not shown). To be able to correlate the level of transcriptional inhibition caused by the HA-tagged Aux/IAA proteins with the degree of protein turn over, we designed a different experimental set-up. Protoplasts were transfected with 20 µg of the effector plasmids *35S::HA-SHY2/IAA3* or *35S::HA-BDL/IAA12* and after auxin treatment total protein extracts were analyzed on Western blots using antibodies against the HA-epitope. HA-BDL/IAA12 and HA-SHY2/IAA3 were clearly detectable, but under these conditions, the latter failed to show a clear enhanced turn over after auxin treatment (Figure 2a, left panel). In fact, IAA3 protein levels increased during incubation, indicating that the *de novo* production was higher than the turn over rate. It is interesting to note that although the HA-SHY2/IAA3 protein was more abundant in figure 2a, BDL/IAA12 had higher repression activity in the *DR5* promoter assays in figure 1b. The facts that excessive amounts of repressor construct led to saturated repression of the *DR5* promoter, which could not be overcome by the addition of higher auxin concentrations (Figure 1a), and that the increased amount of SHY2/IAA3 protein failed to be degraded following auxin treatment (Figure 2a, left panel), led us to hypothesize that some component of the auxin-responsive protein degradation machinery in protoplasts was rate-limiting. To test if the amount of auxin receptor TIR1 was rate-limiting, we co-transfected the cells with 20 µg Aux/IAA effector construct and increasing amounts of *35S::FLAG-TIR1-c-Myc*. Co-transfection of 4 µg

TIR1 plasmid led to enhanced auxin-dependent turn over of SHY2/IAA3 (Figure 2a, IAA3/TIR panels). When 10 or 20 µg of the *TIR1* plasmid was added, the basal levels of SHY2/IAA3 became very low while at 20 µg the effect of auxin could not be visualized due to detection limitations (Figure 2a, IAA3 / TIR 20 µg panel). The stability of the mutant HA-shy2-2 protein was not affected by auxin without co-transfected *TIR1*, but with high *TIR1* amounts even the turn over of the stabilized HA-shy2-2 started to become evident (Figure 2a shy2-2 / TIR1 panels). For shy2-2 it has been reported that the mutant protein retains part of its TIR1 binding activity in the presence of auxin (Tian *et al.*, 2003). This may explain the enhanced turn over of the shy2-2 protein in the presence of additional TIR1 and exogenous auxin.

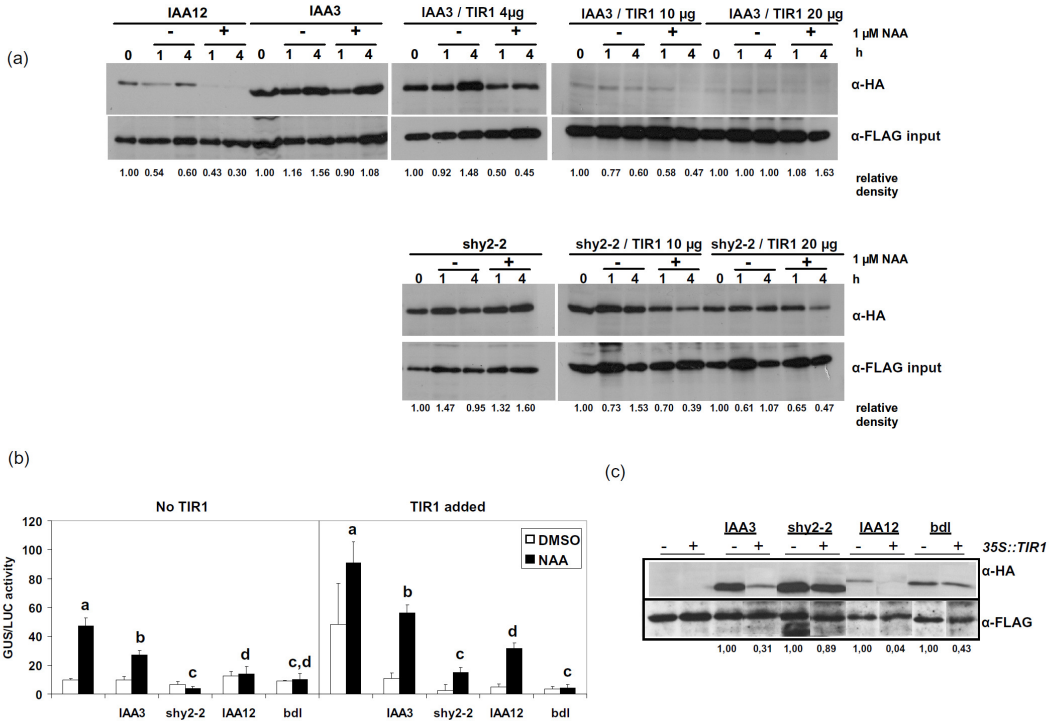

Figure 2. Auxin-induced TIR1-dependent degradation of Aux/IAA proteins leads to auxin-responsive gene expression in *Arabidopsis* protoplasts.

(a) Western blot detection of transiently expressed HA-BDL/IAA12, HA-SHY2/IAA3, HA-bdl and HA-shy2-2 in *Arabidopsis* protoplasts co-transfected with control plasmid *35S::FLAG-GFP* and increasing amounts of plasmid *35S::FLAG-TIR1c-myc*. One million transfected protoplasts were were split in two halves and each was treated either with 0.1% DMSO (-) or 1 µM NAA (+) and samples were harvested on the indicated time points (in hours). The labels on top indicate the Aux/IAA construct and the TIR1 plasmid amount. The panels indicated with "α-HA" represent the

detection of HA-tagged Aux/IAA with anti-HA antibodies and "α-FLAG input" indicates detection of FLAG-GFP with anti-FLAG antibodies. Numbers on the bottom of each lane indicate the HA versus FLAG signal density ratio relative to time point zero in each transformation.

(b, c) Correlation between auxin-responsive *DR5::GUS* expression and TIR1-dependent Aux/IAA degradation. Protoplasts were transfected with 10 µg *DR5::GUS*, 10 µg *35S::RLuc*, 10 µg *35S*::*FLAG-GFP* and where indicated with 20 µg *35S::HA-Aux/IAA* effector plasmid. The graph in (b) shows relative GUS/LUC activity (in arbitrary units) in protoplasts after 4 hours treatment with DMSO or 1 µM NAA, in the absence (No TIR1) or presence of 10 µg *35S::FLAG-TIR1-c-Myc* (TIR1 added). Letters indicate significantly different groups based on One-way ANOVA ($p<0.05$). (c) Western blot analysis showing the level of HA-tagged Aux/IAA protein in the NAA-treated samples in (b). Addition of *35S::TIR1* is indicated (-/+). Detection of the FLAG-GFP control (α-FLAG) is used to demonstrate comparable transformation efficiencies and loading.

TIR1-dependent Aux/IAA degradation coincides with auxin-responsive gene expression

To correlate the TIR1-dependent turn over of Aux/IAAs with auxin-responsive gene expression, we repeated the *DR5::GUS* trans-activation experiments, but now using 20 µg of effector plasmid, and measuring both GUS activity and protein levels. Again, in these experiments the BDL/IAA12 protein showed equal or even stronger repression activity on *DR5::GUS* expression as SHY2/IAA3 (Figure 2b), whereas it accumulated to a much lower level than SHY2/IAA3 (Figure 2c). In the absence of additional TIR1, auxin treatment led to a weak activation of the *DR5* promoter (Figure 2b, "No TIR1" panel and Figure 2c). Co-transfection of 10 µg *TIR1* plasmid enhanced the responsiveness of the *DR5* element (Figure 2b, "TIR1 added" panel) and this effect correlated with an increased turn over of the Aux/IAA repressors (Figure 2c). TIR1 alone resulted in an increase of GUS activity in DMSO-treated cells without co-transfected Aux/IAAs (Figure 2b, first bar in "TIR1 added" panel). This effect is probably due to the degradation of the endogenous pool of Aux/IAAs. As expected, the levels of the mutant shy2-2 and bdl proteins were less sensitive to overexpression of TIR1. The slight increase in *DR5::GUS* activity observed after auxin treatment in the samples co-transfected with *shy2-2* and *TIR1* might reflect the residual binding of the mutant shy2-2 protein to TIR1 (Tian *et al.*, 2003) and its enhanced turn over rate at higher TIR1 levels

(compare to Figure 2a, shy2-2 / TIR1 20 µg panel). Additionally, comparative analysis of BDL/IAA12 and SHY2/IAA3 indicated that optimized ARF and Aux/IAA interaction pairs are active in specific auxin-regulated developmental processes (Weijers *et al.*, 2005). It is therefore likely that the specific ARFs that are responsible for the activation of the *DR5* element in cell suspension protoplasts interact more efficiently with BDL/IAA12 or endogenous Aux/IAAs than with SHY2/IAA3. This may also explain the stronger repressor activity of BDL/IAA12.

The observation that excessive amounts of TIR1 led to reduction of detectable Aux/IAAs even in the absence of auxin treatment is in agreement with the effect of overexpressing TIR1 in plants, which mimics auxin treatment and causes auxin hypersensitivity (Gray *et al.*, 1999). It also corroborates the finding that in *in vitro* pull-down experiments Aux/IAA proteins do interact with TIR1 in the absence of auxin, albeit at low efficiency (Dharmasiri *et al.*, 2003; Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b; Tan *et al.*, 2007), and that Aux/IAA degradation and auxin-responsive gene expression are severely affected in the *tir afb2 afb3* triple mutant (Dharmasiri *et al.*, 2005b). All these data indicate that TIR1 and AFB protein levels are important determinants in the cellular auxin responsiveness.

TIR1 differentially regulates SHY2/IAA3 and BDL/IAA12 abundance

Interestingly, there is a clear and significant difference in abundance and repressor activity of the two wild type Aux/IAA proteins, with BDL/IAA12 being the stronger but less abundant repressor (Figure 2b). To assess the roles of auxin and TIR1 in this different behavior of the two Aux/IAA proteins, we tested the auxin-induced degradation of SHY2/IAA3 and BDL/IAA12 with increasing TIR1 levels (Figure 3a). HA-BDL/IAA12 levels were auxin-sensitive when cells were transfected with 20 µg of *35S::HA-BDL/IAA12* effector plasmid alone. The observed variation between experiments in the effect of auxin treatment on IAA12 turn over (compare Figures 2a and 3b) possibly relates to differences in endogenous TIR1 levels. In the same experiment, auxin treatment did not lead to a clear reduction in HA-SHY2/IAA3 levels, corroborating our observation that

SHY2/IAA3 has a longer half-life than BDL/IAA12 (Figure 2b). Co-transfection of the effector plasmids with increasing amounts of *35S::FLAG-TIR1-c-Myc* made the levels of both proteins more sensitive to auxin treatment (Figure 3a). As a control, effector plasmids were also transfected with plasmids expressing mutant versions of TIR1 (tir1-1 [G147→D]), ΔF-TIR1 lacking the F-box, or ΔF-tir1-1 carrying both mutations (Figure 3b), or the related F-box protein COI1, which is involved in jasmonate signaling (Figure 3c). Neither the mutant versions of TIR1 (Figure 3b) nor COI1 (Figure 3c) affected the abundance of the Aux/IAA proteins (compare to 20/10 HA-IAA/TIR1 treatments in Figure 3a), corroborating the specificity of TIR1 in Aux/IAA degradation.

In these experiments TIR1, tir1-1 and COI1 were not detectable by Western blot analysis of total protein extracts using the anti-FLAG antibody, even though their expression was driven by the strong *35S* promoter. This observation suggests that TIR1 and COI1 themselves are short-lived proteins, which is in line with the observation that several F-box proteins, including TIR1 and COI1, are targets for ubiquitination (Maor *et al.*, 2007; Jurado *et al.*, 2008; Stuttmann *et al.*, 2009). To demonstrate that the F-box proteins were expressed to similar levels in our model system, we transfected 5 times more cells than usual (5 x 10^6) and immunoprecipitated the FLAG-tagged F-box proteins from total cell extracts. Western blot analysis of the concentrated eluates showed that the FLAG-tagged versions of TIR1, tir1-1 and COI1 were expressed at similar levels (Figure 3d). As expected, transfection with 2 times more plasmid led to the production of more TIR1 protein (Figure 3e). Treatment with MG132 for four hours did not lead to elevated TIR1 levels nor to the appearance of additional modified bands (Figure 3e), which is in contrast to the conclusion by Stuttmann and coworkers (2009) that the protein is a target of the 26S proteasome.

TIR1 was shown to act as an auxin receptor together with other F-box family members ABF1, ABF2 and ABF3 (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b). Interestingly, a *tir1 afb1 afb2 afb3* quadruple loss-of-function mutant shows a variable phenotype, but several of these mutant plants are able to flower and set seed, suggesting further functional redundancy. We have not tested the effect of AFB1, AFB2 or AFB3 in our

system, but it is likely that they will show similar effects as TIR1 overexpression, since they were found to physically interact with GST-tagged BDL/IAA12 in an auxin-dependent manner in pull-down assays, and the BDL/IAA12 protein was stabilized in the *tir1-1 afb2 afb3* triple mutant (Dharmasiri *et al.*, 2005b).
The related F-box proteins AFB5 and AFB4 have not been studied in detail yet, but the specific resistance of *afb5* mutants to picolinate auxin analogs indicates that AFB5 is involved in the response pathway to these herbicides (Walsh *et al.*, 2006). COI1 is the closest relative of the TIR/AFB auxin receptors in the F-box family tree. The similarity in sequence and the ability of different proteins from this clade to associate with the same SCF components (Gray *et al.*, 1999; Xu *et al.*, 2002) raised the possibility that there is cross-recognition of targets among related F-box proteins and that COI1 may also be actively involved in Aux/IAA protein degradation. The results in Figure 3c clearly show that the presence of overexpressed FLAG-COI1 did not affect the stability of either SHY2/IAA3 or BDL/IAA12. Based on our results, we conclude that COI1 is not involved in the process of Aux/IAA proteolysis.

SCF^{TIR1}-mediated ubiquitination marks Aux/IAA proteins for degradation

The previous experiments demonstrated that the cell suspension protoplast system reproduces the *in planta* action of Aux/IAA proteins and the receptor F box protein TIR1 in auxin responses. The same system was used to establish whether auxin- and TIR1-enhanced degradation of Aux/IAA proteins is connected to SCF^{TIR1}-mediated ubiquitination. A plasmid encoding HA-tagged versions of BDL/IAA12 or SHY2/IAA3 together with a plasmid carrying TIR1 or its mutant version *tir1-1* were transfected into cells. Transformations of one million protoplasts were performed in triplicate and one of the samples was pre-treated for 1 h with 50 µM of the proteasome inhibitor MG132. Samples were subsequently treated for one additional hour with either DMSO or 1 µM NAA. Five percent of the protein input was analyzed by Western blotting using anti-HA antibodies, and the remaining sample was immunoprecipitated with anti-HA antibodies conjugated to agarose beads. The recovered proteins were analyzed by Western blotting using anti-Ubiquitin and anti-HA antibodies.

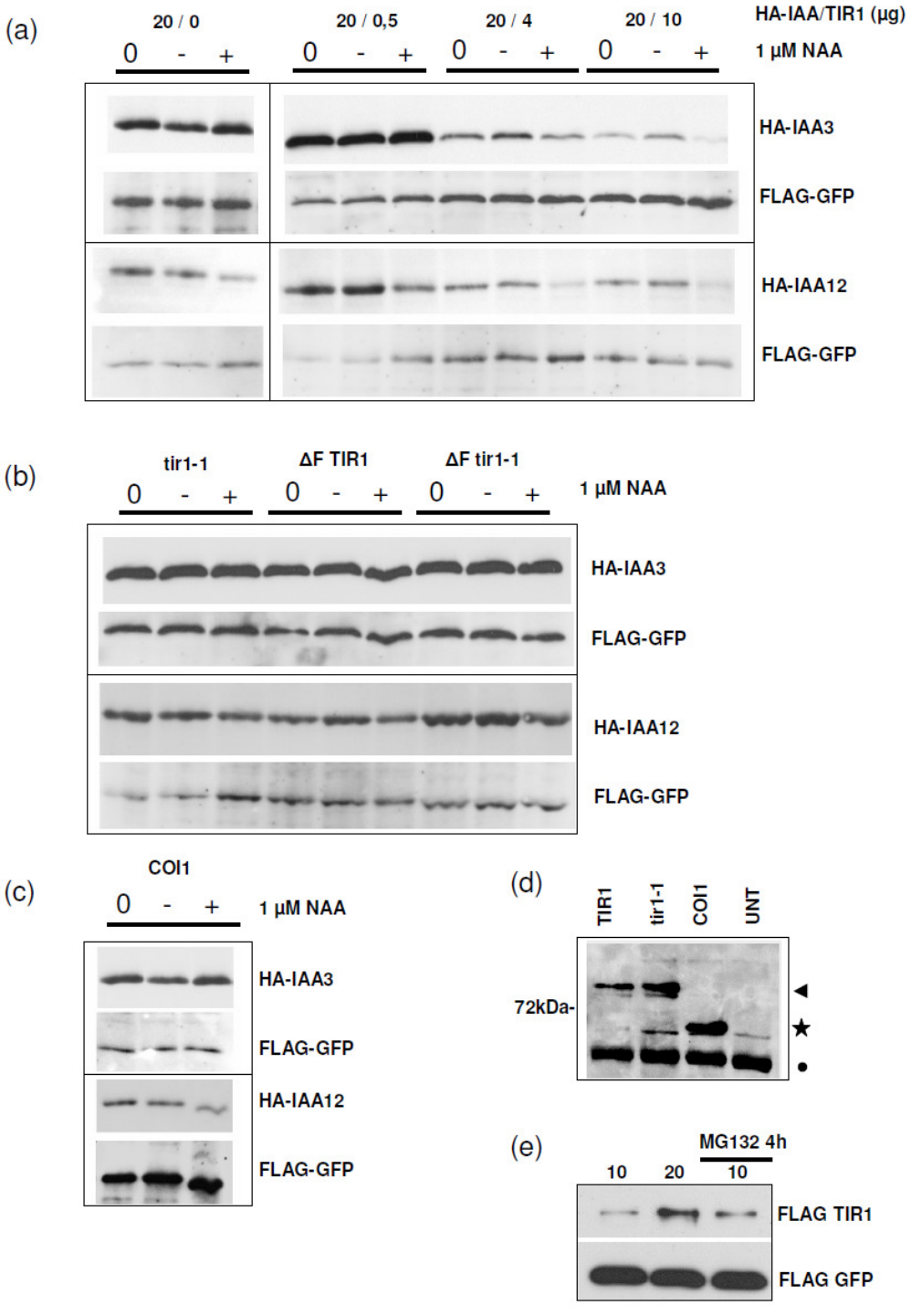
(a)
20 / 0
20 / 0,5
20 / 4
20 / 10
HA-IAA/TIR1 (µg)
0 - +
1 µM NAA
HA-IAA3
FLAG-GFP
HA-IAA12
FLAG-GFP
(b)
tir1-1
ΔF TIR1
ΔF tir1-1
1 µM NAA
HA-IAA3
FLAG-GFP
HA-IAA12
FLAG-GFP
(c)
COI1
1 µM NAA
HA-IAA3
FLAG-GFP
HA-IAA12
FLAG-GFP
(d)
TIR1
tir1-1
COI1
UNT
72kDa-
(e)
MG132 4h
10
20
10
FLAG TIR1
FLAG GFP

Figure 3. TIR1-dependent degradation of transiently expressed SHY2/IAA3 and BDL/IAA12 in *Arabidopsis* protoplasts.

(a) Western blot analysis of total extracts from protoplasts co-transfected with increasing amounts of *35S::FLAG-TIR1-c-Myc*, *35S::FLAG-GFP* control plasmid and *35S::HA-SHY2/IAA3* (upper panel) or *35S::HA-BDL/IAA12* (lower panel). Transfected protoplasts were either untreated (0), or treated for 1 hour with DMSO (-) or 1 µM NAA (+). The numbers on top indicate the *35S::HA-AuxIAA* to *35S::FLAG-TIR1-c-Myc* plasmid ratio in µg DNA. Blots were probed with anti-HA or anti-FLAG antibodies.

(b) As in (a), following co-transfection with 10 µg *35S::FLAG-tir1-1-c-Myc*, *35S::FLAG-ΔF-TIR1-c-Myc* or *35S::FLAG-ΔF-tir1-1-c-Myc*.

(c) As in (a) , following co-transfection with 10 µg *35S::FLAG-COI1*.

(d) Western blot detection of FLAG-tagged TIR1, tir1-1 and COI1 immunoprecipitated using anti-FLAG antibodies from pooled extracts from five transformations of 10^6 protoplasts with 20 µg DNA of *35S::FLAG-TIR1-c-Myc*, *35S::FLAG-tir1-1-c-Myc* or *35S::FLAG-COI1*, respectively. The position of the FLAG-tagged TIR1-c-Myc and *tir1-1*-c-Myc proteins (79 kDa) is indicated by an arrowhead, the position of FLAG-COI1 (70 kDa) with a star. The lane marked UNT contained protein from untransformed protoplasts. An unspecific band recognized by the anti-FLAG antibody in all samples (circle) shows equal loading.

(e) Increasing amounts of 35S:FLAG-TIR1myc plasmid leads to higher expression of the recombinant protein and TIR1 is not stabilized by inhibition of the 26S proteasome. Anti-FLAG western blot of FLAG-immunoprecipitated samples from one million protoplasts transfected with 1 µg of 35S:FLAG GFP and 10 or 20 µg of 35S:FLAG-TIR1myc as indicated over each lane. One sample transfected with 10 µg was treated for 4 h with 10 µM MG132. Total protein was immunoprecipitated with anti-FLAG affinity matrix and analyzed by western blot. FLAG TIR1 and FLAG GFP bands are indicated.

The relative amounts of Aux/IAA proteins recovered by immunoprecipitation correlated well with those present in the input extracts (Figure 4, α-HA: 5% input versus IP-HA). Samples from protoplasts that were treated with auxin and MG132 showed additional anti-HA reactive bands migrating slower than the unmodified SHY2/IAA3 and BDL/IAA12 monomers (Figure 4a and b, IP-HA α-HA, +/+ lanes). Anti-Ubiquitin antibodies detected bands of sizes larger than 40 kDa (IP-HA α-Ub panels) that overlapped with the additional bands detected with anti-HA antibodies (marked with black arrow heads), and thus represent poly-ubiquitinated versions of Aux/IAA proteins. The combined auxin and MG132 treatment enhanced the amount of detectable ubiquitinated Aux/IAA proteins. Interestingly, when TIR1 was co-expressed,

auxin treatment resulted in an increase of the ubiquitinated signal for both HA-SHY/IAA3 and HA-BDL/IAA12 (Fig 4a and b, +/+ lanes), corroborating our previous observation that SCF^{TIR1}-directed degradation of Aux/IAA proteins is dependent on a fine balance between auxin and TIR1 levels (Figure 3a and b). As expected, the co-expression of the mutant tir1-1 protein had no effect on the turn over rate or the ubiquitination level of SHY2/IAA3 (Figure 4a, tir1-1 lanes).

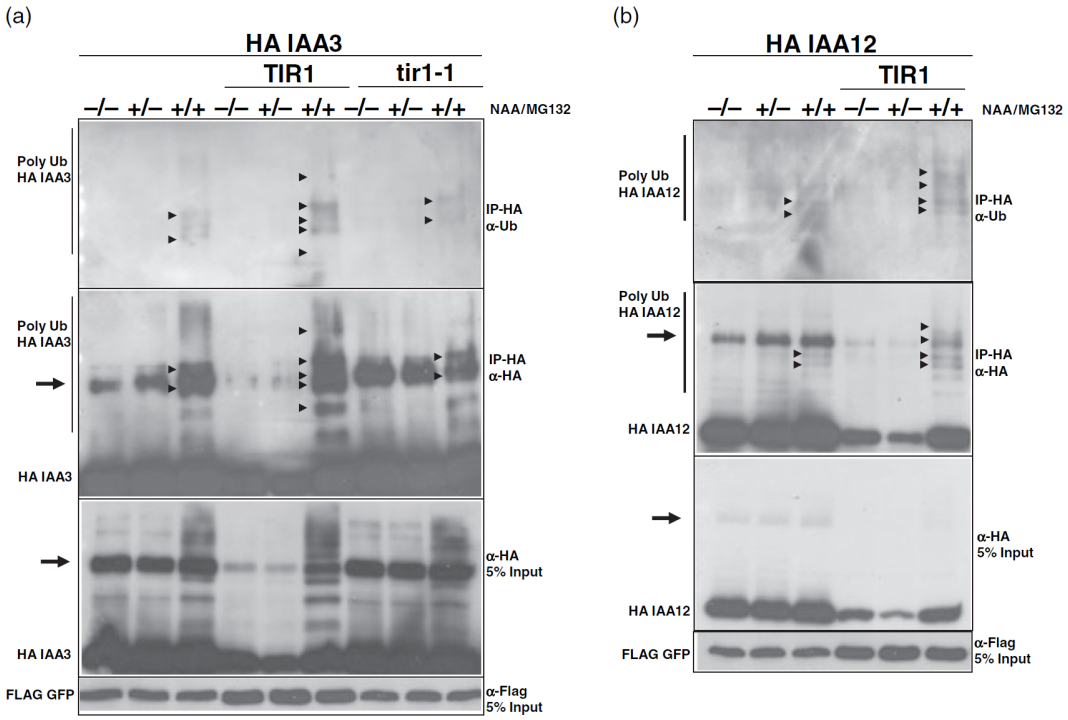

Figure 4. Aux/IAA proteins are poly-ubiquitinated and degraded in an auxin- and TIR1-dependent manner.

(a-b) Western blot analysis of anti-HA immunoprecipitations (IP-HA) or total extracts (5% input) from *Arabidopsis* protoplasts transfected with:

(a) *35S::FLAG-GFP* and *35S::HA-SHY2/IAA3* with either 10 µg of *35S::FLAG-TIR1-c-Myc* (TIR1) or *35S::FLAG-tir1-1-c-Myc* (tir1-1).

(b) *35S::HA-BDL/IAA12 and 35S::FLAG-GFP* co-transfected with 10 µg *35S::FLAG-TIR1-c-Myc*.

Each transformation was performed in triplicate. One sample was treated for 1 h with 0.1% DMSO (-/-), the second sample for 1 h with 1 µM NAA (+/-), and the third replicate was pre-treated for 1 h with 50 µM MG132 followed by 1 h treatment with 1 µM NAA (+/+). Five percent of the total protein extract of each transfection was analysed as input (5% input). The remaining sample was used in an immunoprecipitation with anti-HA antibodies conjugated to agarose beads and these samples

(IP-HA) were first probed with anti-Ubiquitin antibodies (α-Ub) to detect ubiquinated proteins, and subsequently with anti-HA antibodies (α-HA) to detect HA-tagged proteins. The bands corresponding to unmodified HA-IAA3 and HA-IAA12 monomers are indicated. The poly-ubiquitinated forms of IAA12 and IAA3, visible as higher molecular size bands/smears in the α-Ub and α-HA blots, are indicated by Poly-Ub. Arrow heads mark the bands overlapping in the α-Ub and α-HA blots. The arrow indicates the position of a putative homo-dimer of the HA-tagged Aux/IAA proteins.

As the goal of this experiment was to detect the rare ubiquitinated Aux/IAAs forms, significantly higher protein amounts were loaded on gel, and this prevented us to observe the auxin-induced turn over of Aux/IAA proteins in the absence of cotransfected *35S::FLAG-TIR1-c-Myc*.

One constant observation in the Western blots with anti-HA antibodies was the presence of two distinct bands: one at ~30 kDa corresponding to the size of unmodified Aux/IAA monomers (indicated in Figure 4 on the left of each panel by HA IAA3 or HA IAA12) and a fainter band around 60 kDa size (Figure 4, indicated by arrows, α-HA panels). This band was not detected with the anti-Ub antibodies, and although we can not exclude other pos-translational modifications such as phosphorylation or sumoylation, based on the size shift we believe this band to represent Aux/IAA homo-dimers. The 60kDa band was also detected even when protein samples were prepared and gel separated under strong denaturing conditions (boiled in Laemmli loading buffer prior to Urea-SDS-PAGE, data not shown), suggesting a covalent coupling, or a denaturation-resistant association of the Aux/IAA proteins. One possibility is that the dimers are stabilized by intermolecular disulfide bonds, which is a common mechanism in redox control of transcription factor activity (Benezra, 1994; Zheng *et al.*, 1998; Mou *et al.*, 2003). However, the fact that the band is not dissolved by the thiol reducing agent 2-mercaptoethanol in the Leammli buffer suggests that the Aux/IAA dimers are stabilized by another mechanism. The 60 kDa band putatively representing Aux/IAA homodimers almost disappeared in TIR1 co-transfected samples, suggesting that an additional consequence of Aux/IAA degradation is the dissolution of Aux/IAA dimers. Hypothetically, the dimerized forms might be more accessible for interaction

with SCF-^{TIR1} than the DNA-ARFs associated ones and hence be more easily degraded.

Repressor poly-ubiquitination: a paradigm for plant hormone signaling pathways

Our results indicate that auxin-responsive gene expression in *Arabidopsis* protoplasts depends on a fine tuning of the intracellular concentrations of different elements that participate in the auxin perception pathway. Auxin sensitivity and Aux/IAA stability are directly correlated with intracellular levels of TIR1. Previous reports showed that auxin responses in seedlings are enhanced by *TIR1* overexpression (Gray *et al.*, 1999) and repressed by TIR1/AFB loss-of-function (Dharmasiri *et al.*, 2005b). In addition, our experiments directly correlate the TIR1-enhanced auxin response with the increased turn over of Aux/IAA proteins, and suggest that TIR1, when present at sufficiently high levels, sensitizes the protoplast cells to endogenous auxin levels and can mediate Aux/IAA degradation without the need for exogenous auxin application. We demonstrate that the SHY2/IAA3 and BDL/IAA12 proteins behave differently in protoplasts, the latter being less stable but more active in the repression of the auxin response in this system. The simplest explanation is that IAA12 is a more efficient repressor than IAA3, but we can not rule out that the effect is indirect through the efficient interaction of overexpressed IAA12 with the SCF$^{TIR1/AFB}$ complexes, which sequesters these complexes and thereby stabilizes endogenous Aux/IAAs.

It is well established that several plant hormonal signaling pathways act through proteasomal degradation of transcriptional repressors, and that the hormones show similar roles in enhancing the association of SCF E3 ligase complexes with their targets (e.g. auxin/TIR1/Aux/IAA, JA-Ile/COI1/JAZ and GA/GID/DELLA.) (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Griffiths *et al.*, 2006; Thines *et al.*, 2007). Our data provide experimental support for the model that hormone-responsive gene expression is mediated by hormone-enhanced poly-ubiquitination and subsequent proteolytic degradation of repressor proteins by the 26S proteasome.

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Chapter 3

Ubiquitination-independent proteasomal degradation of the calcium binding protein PBP1

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Summary

In order to regulate physiological functions, eukaryotic cells have developed many control mechanisms for fine-tuning the levels of intracellular proteins. Generally, targeted protein degradation occurs through the specific recognition by the proteasome of proteins that have been marked with ubiquitin chains. Protein labeling with ubiquitin is however emerging as a much more complex signal not only targeting proteins for degradation, but also with roles in the control of subcellular trafficking of proteins. The Arabidopsis calcium binding protein PINOID BINDING PROTEIN 1 (PBP1) was originally identified as an interactor of the PINOID (PID) kinase, which was shown to control its activity in vitro and subcellular localization in vivo. Using an Arabidopsis cell suspension protoplast system we show that PBP1 is a highly unstable, poly-ubiquitinated protein that can be stabilized by inhibition of proteasomal degradation. Co-expression of PID did not affect PBP1 poly-ubiquitination. Surprisingly, substitution of all the lysines in PBP1 blocked poly-ubiquitination, but did not affect its proteasomal degradation. The mutant protein retained all tested wild type functions, such as its interaction with PID, and its subcellular localization. Translational fusions of the lysine-less PBP1 with YELLOW FLUORESCENT PROTEIN (YFP) were ubiquitinated, which indicates that the PBP1 sequence contains a cis-acting motif that is recognized as an ubiquitination signal but not as a degron, since the YFP fusions were not unstable. PBP1 is the first example of a plant protein that is degraded by the proteasome in an ubiquitination-independent pathway. Although the functions of PBP1 ubiquitination remain to be elucidated, our results show that its proteasomal degradation is not dependent on ubiquitination .

INTRODUCTION

In order to regulate physiological functions, eukaryotic cells have developed many control mechanisms for fine-tuning the levels of intracellular proteins. One of these involves post-translational modification by ubiquitination. Ubiquitin (Ub) is a highly conserved 76 amino acid protein that is present in all eukaryotic cells. It is conjugated to cellular proteins through a conserved mechanism involving Ub activating (E1), -conjugating (E2) and -ligating (E3) enzymes. This process, termed ubiquitination, couples the C-terminal glycine residue of Ub to a target lysine residue of the protein substrate, forming an isopeptide bond (Glickman and Ciechanover, 2002). Protein ubiquitination controls many basic cellular processes, such as cell division, signal transduction and DNA repair, by regulating the activity, subcellular localization, and proteasome-mediated degradation of proteins (Pickart and Fushman, 2004). The ubiquitin protein itself contains 7 lysine residues that serve as ubiquitin-linking sites. The 19S regulatory lid of the 26S proteasome recognizes proteins containing a Lys-48 Ub polymer as substrates for degradation by the 20S catalytic core (Baboshina and Haas, 1996; Thrower *et al.*, 2000). Proteins that are polyubiquitinated at Lys-48 are usually unstable and treatment of cells with proteasome inhibitors blocks their degradation enriching the pool of Ub-modified molecules (Lee and Goldberg, 1998). Other ubiquitin conjugations such as mono-ubiquitination, or polymers attached at Lys-11 or Lys-63 are believed to regulate processes such as sub-cellular localization and/or protein activity (Weissman, 2001). About 5% of the Arabidopsis proteome comprises elements of the ubiquitination pathway with a high representation of Ubiquitin Ligases. In plants ubiquitinaiton plays a key role in signal transduction of several phytohormones (Gray *et al.*, 1999; Xu *et al.*, 2002; Guo and Ecker, 2003; Gomi *et al.*, 2004; Jang *et al.*, 2005; Dos Santos *et al.*, 2009) and although all these processes seem to involve the activity of specific ubiquitin ligases, the ubiquitination signal itself has been neglected in scientific studies. Ubiquitin was first discovered as a degradation signal with the best studied examples including mammalian proteins such as the Cdk-inhibitor p27Kip1 which is ubiquitinated by SCF^{SKP2} (Tsvetkov *et al.*, 1999), Sic1 which is ubiquitinated by SCF^{Cdc4} (Petroski and Deshaies, 2003), β-

catenin and Ikβ which are ubiquitinated by $SCF^{\beta\text{-}TrCP}$ (Winston *et al.*, 1999) and p53 which can be ubiquitinated by several E3 ligases (Scheffner *et al.*, 1993; Fang *et al.*, 2000; Xia *et al.*, 2009). Proteasomal p53 degradation occurs via both ubiquitin-dependent and independent ways (Asher and Shaul, 2006), indicating that proteasomal degradation does not necessarily occur via an ubiquitinated intermediate. The removal of the ubiquitin attachment sites by mutation of lysines in a target protein has been shown as an efficient way to assess the relevance of the ubiquitin signal for many unstable proteins degraded independently of ubiquitination, such as p21cip1 (Sheaff *et al.*, 2000) and KLF5 (Chen *et al.*, 2007a) revealing the existence, at least in mammalian cells of both ubiquitin-dependent and independent degradation pathways.

Previously, we identified a single EF-hand calcium-binding protein as an interacting protein of the *Arabidopsis thaliana* AGC kinase PINOID (PID), and named it PINOID BINDING PROTEIN 1 (PBP1). PBP1 binds to PID in a calcium-dependent manner and positively regulates PID activity *in vitro*, while inhibiting the auxin-induced calcium-dependent sequestration of PID from the plasma membrane *in vivo*. (Benjamins *et al.*, 2003; Robert-Boisivon, 2008). In our further study on the role of PBP1 as regulator in PID signaling, we tested the biochemical properties of PBP1 by expressing the protein in Arabidopsis cell suspension protoplasts. Here we provide evidence that PBP1 is an unstable protein that is poly-ubiquitinated However, PBP1 degradation by the proteasome is an ubiquitination-independent process. Our results indicate that poly-ubiquitination is not an obligatory signal for PBP1 proteasomal degradation suggesting that plant proteasomes also have diverse mechanisms for recognizing their targets.

MATERIAL AND METHODS

Molecular cloning and constructs

Molecular cloning was performed following standard procedures. The *HA-PBP1* coding region was amplified by PCR from *pET16H-PBP1* (Benjamins *et al.*, 2003) using primers *HAadd PBP1 HindIII* 5'-

GGAAGCTTG**ATGTACCCATACGATGTTCCAGATTACGCT**ATGGCATCTCC TAAAT-CCTC-3' and *PBP1XbaIR* 5'-GGTCTAGATCAATGCCGGTAAAACTCTTCC-3' (HA-tag in bold and restriction sites underlined), and the *Hin*dIII and *Xba*I sites in the primers were used to clone the fragment into pART7 (Gleave, 1992), to obtain *p35S::HA-PBP1*. *p35S::HA-PBP1(-K)* was obtained by cloning a synthetic *HindIII/BamHI* fragment containing the *HA-PBP1* coding region with the lysine K5, K29, K41, K49 and K51 codons replaced by the arginine codon (AGA) via Gene Synthesis (http://www.baseclear.com/) into pART7. To generate His-HA-PBP1(-K), the synthetic *HA-PBP1(-K)* coding region was ligated as a *Bam*HI/*Hind*III fragment in frame to the His-tag coding region of pET16b (Promega, www.promega.com). To create C-terminal YFP-HA fusions, both HA-PBP1 and HA-PBP1(-K) were PCR amplified with *attB* Gateway™ (Invitrogen, www.invitrogen.com) primers *attB1FHA* 5'-GGGGACAA-GTTTGTACAAAAAAGCAGGCTTAATGTACCCATACGATGTTCCA-3' and *attB2PBP1R* 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCATGCCGGTAAAACTCTTCCT C-3', removing the original stop codon. Each of the *attB* PCR fragments was cloned into *pDONR207* via BP recombination following the manufacturer's instructions, and the resulting entry clones were recombined via an LR reaction into *pART7-YFP-HA* (C. Galvan Ampudia, unpublished) to generate *p35S::PBP1-YFP-HA* and *p35S::PBP1(-K)-YFP-HA.* For construction of the *p35S::5xHis-Ub* vector, the cDNA of human ubiquitin was PCR amplified from His_6-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 *Sma*I. Plasmids *p35S::PID-FLAG* (Michniewicz et al., 2007) *p35S::FLAG-GFP, p35S::GFP* (Dos Santos *et al.*, 2009) *p35S::PID-CFP* (C.Galvan-Ampudia, unpublished) were also constructed in pART7.

Protoplast isolation and transformation

Four to six days old *Arabidopsis thaliana* Col-0 cell suspension cultures were diluted 5-fold in auxin-free Cell Medium and incubated at 25°C with shaking (150 rpm) overnight and used for protoplast isolation and transfection, performed as previously described (Schirawski *et al.*, 2000) with minor modifications (Dos Santos *et al.*, 2009). Transfections were performed with 10^6 cells and 20 µg of the test plasmid DNA. Amounts of the other plasmids are indicated in the figure legends. After transfection the cells were incubated at 25ºC in darkness for at least 16h prior to treatments or observation using confocal laser scanning microscopy. Cells were incubated for 1h with 0.1% DMSO (-) or 50 µM MG132 (+) where indicated.

Western blot analysis and immunoprecipitation

Total protein was extracted from pelleted transfected protoplasts with 50 µL cold Extraction Buffer (Tris Buffered Saline,TBS, 1% Triton X-100, 1x Roche Complete Protease Inhibitor Cocktail), and centrifuged for 10 minutes at 20.000 g at 4°C. The 40 µL extract was mixed with 10 µL 5X Laemmli sample buffer and boiled for 5 minutes. Proteins were separated on a 15% SDS-PAGE gel, blotted into a PVDF membrane using semi-dry electrotransfer (BioRad), blocked for 1h with 5 % low-fat dry milk in TBST (TBS, 0.05% Tween20) and probed with HRP-conjugated anti-HA antibodies (1/2000, Roche) for 16h at 4°C. Detection was performed using LumiGLO Detection reagent (Cell Signalling) following the manufacturer's instructions.

For immunoprecipitation, cells were extracted in 500 µL of Extraction Buffer, centrifuged and 50 µL was analysed as 10% input. The remaining volume was mixed end-over-end with 50 µL 50% slurry of anti-HA agarose beads (Roche) for 2 h at 4°C, washed 4x and mixed with 50 µL 2X Laemmli sample buffer for SDS-PAGE and subsequent western blot analysis (see above) using anti-HA antibodies (1/2000, Roche) and P4D1 anti-UB antibodies (1/400, Santa Cruz).

Purification of ubiquitinated proteins via Ni-NTA affinity chromatography

In addition to the indicated test constructs, the protoplasts were co-transfected with 20 µg of the *p35S:His-Ub* plasmid and 16h after transfection the cells were

treated with 50 µM MG132 for 1h, harvested by centrifugation and frozen in liquid nitrogen. Pellets containing 10^6 harvested cells were resuspended in 100 µL cold Extraction Buffer (TBS, 1% Triton 100-X, 1x Roche Complete Protease Inhibitor Cocktail, 10 mM N-Ethylmaleimide NEM), vortexed and centrifuged 10 min at 20.000 g at 4°C. Ten microliters of the supernatant was mixed with an equal volume of 2x Laemmli sample buffer, and used as INPUT for Western analysis. The remaining 90 µL was mixed with 910 µL of Buffer A (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 pH 8.0, 10 mM imidazole) and used for Ni-affinity chromatography, using Ni-NTA agarose beads (Qiagen) as previously described (Campanero and Flemington, 1997). The eluted proteins were analyzed by western blotting as described above.

In vitro pull down experiments

Crude extracts of *E.coli* cells expressing recombinant proteins were used for *in vitro* GST pull down experiments of His-PBP1 and His-HA-PBP1(-K) with GST alone and GST-PID and GST-WAG2 (C. Gavlan-Ampudia, unpublished) as described (Benjamins *et al.*, 2003).

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. Image processing was performed with ImageJ (http://rsb.info.nih.gov/ij/).

RESULTS

PBP1 is degraded by the proteasome in a ubiquitination-independent manner

PBP1 was first identified in Arabidopsis as an interactor of the PID kinase (Benjamins *et al.*, 2003). PBP1 was also named KRP2 (for KIC-related protein

2) and contains one calcium binding EF-hand motif in common with other related members of a small protein family that includes KIC (KCBP-interacting Calcium binding protein) and the close PBP1 homolog PBP1H/KRP1 (Reddy *et al.*, 2004). The alignment of the three proteins (Figure 1A) shows the high degree of similarity between PBP1 and PBP1H. From the 5 lysines present in the primary PBP1 sequence only one is conserved in all members and another two are present in KIC.

As part of our analysis of the biochemical properties of PBP1 in relation to its interaction with PID, we transfected *Arabidopsis thaliana* cell suspension protoplasts with plasmid *35S::HA-PBP1*. Total protein was extracted from the transfected cells and analyzed by Western blot with a specific antibody against the HA-tag (Figure 1B). The very weak signal observed in untreated cells is significantly enhanced when cells were treated for 1h with the proteasome inhibitor MG132, suggesting that PBP1 is a target for degradation by the proteasome. In addition to the stronger signal representing the full length protein (~16 kDa), a ladder of discrete larger molecular mass bands becomes apparent following MG132 treatment. Immunoprecipitation (IP) with anti-HA and detection with either anti-HA or anti-Ub antibodies shows that these larger molecular mass bands represent ubiquitinated versions of PBP1 (Figure 1C).

Ubiquitin attachment occurs on lysine residues. PBP1 is a relatively small protein with only 5 lysine residues, To confirm that the additional bands observed are due to lysine ubiquitination, we generated a construct encoding a PBP1 variant in which all five lysine residues were substituted by arginines (Figure 1A, arrows). Transfection of the resulting *35S::HA-PBP1-K* construct into protoplasts shows that PBP1-K is still an unstable protein stabilized by MG132 treatment (Figure 2A), but without showing additional higher molecular weight bands representing ubiquitinated forms. These observations indicate that ubiquitination of PBP1 is not a signal for its degradation by the proteasome. PBP1 ubiquitination may serve some function other than signaling proteolysis. Regardless, the example of PBP1 illustrates that observation of poly-ubiquitination and proteasome sensitivity *in vivo* forms insufficient evidence to

conclude that proteasomal degradation of a protein must proceed through an ubiquitinated intermediate.

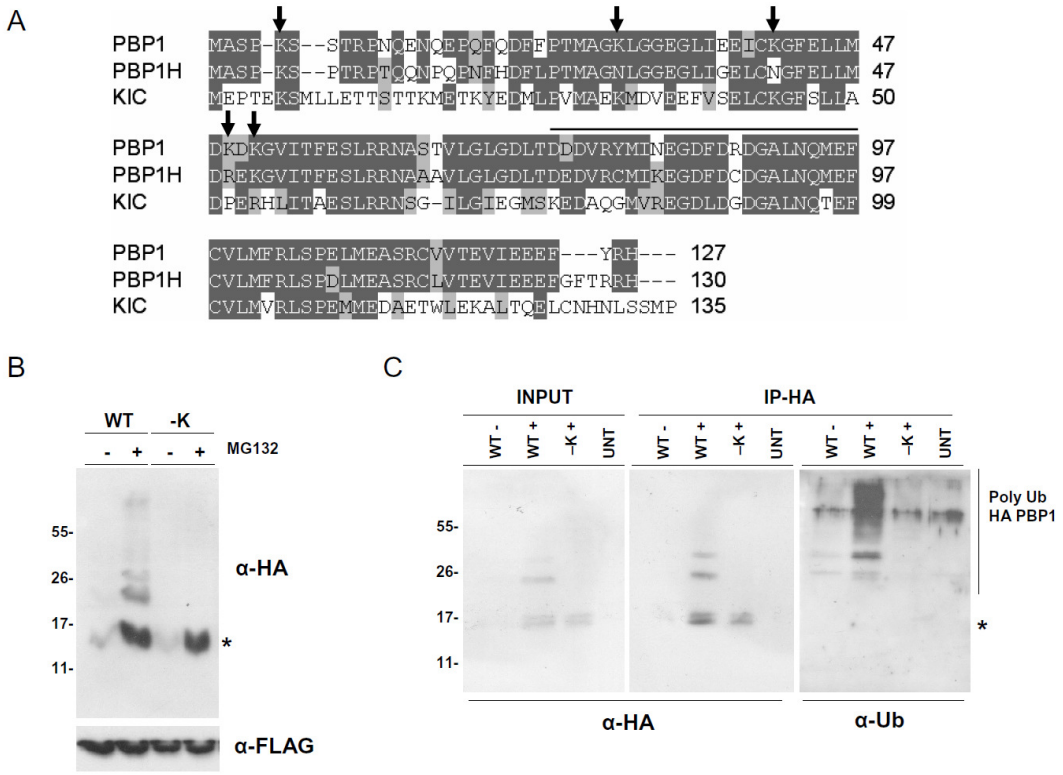

Figure 1: PBP1 is degraded by the proteasome in a ubiquitination-independent manner. (A) Alignment of PBP1, PBP1H and KIC. The lysine (K) residues are indicated by black arrows. The position of the EF-hand calcium binding pocket is indicated with a line. (B) Arabidopsis protoplasts transfected with either *35S::HA-PBP1* (WT) or *35S:HA-PBP1-K* (-K) together with 5 µg *35S::FLAG-GFP*. Sixteen hours after transfection, cells were incubated for 1h with 0.1% DMSO (-) or 50 µM MG132 (+). Total protein extracts (20 µg) were analyzed on western blots using anti-HA (α-HA) or anti-FLAG (α-FLAG) antibodies. (C) Western blot of protein extracts from protoplasts transfected as in B, using anti-HA (α-HA) or anti-Ub (α-Ub) antibodies. Ten percent of the total extract was analyzed as INPUT. The rest was used for immunoprecipitation with anti-HA affinity matrix (IP-HA) prior to Western blot analysis. UNT indicates untrasfected control. The position of the poly-ubiquitinated HA-PBP1 is indicated on the right. The asterisk indicates the position of the full length HA-PBP1 protein. Numbers on the left indicate the molecular mass of marker proteins in kDa.

Ubiquitination of PBP1 does not change its functional properties

The interaction of calcium-binding proteins with Ca^{2+} results in a reduced mobility, and therefore these proteins show a double band on a protein gel (Ling

and Zielinski, 1993). Proteins extracted from protoplasts expressing HA-PBP1 also show a double band recognized by anti-HA antibodies (Figure 1C), and interestingly, we found both free and calcium-bound PBP1 to be ubiquitinated, since similar double bands were identified with anti-Ub antibodies. Moreover, PBP1-K retains its ability to bind calcium, since a double band could still be observed (Figure 1C). In order to analyze if ubiquitination of PBP1 is essential for its functionality, we tested binding of PBP1 and PBP1-K to PID, and to another AGC3 kinase, WAG2 (Galvan Robert and Offringa, in prep) in an *in vitro* pull-down assay. GST-PID or GST-WAG2 containing glutathione beads were used to pull down His-PBP1 or His-PBP1-K from total *E.coli* protein extracts. After several washes, the beads were analyzed for bound proteins on a Western blot using anti-His antibodies. Both the wild type and the -K version of PBP1 were found to bind PID and WAG2 with similar affinities (Figure 2A).

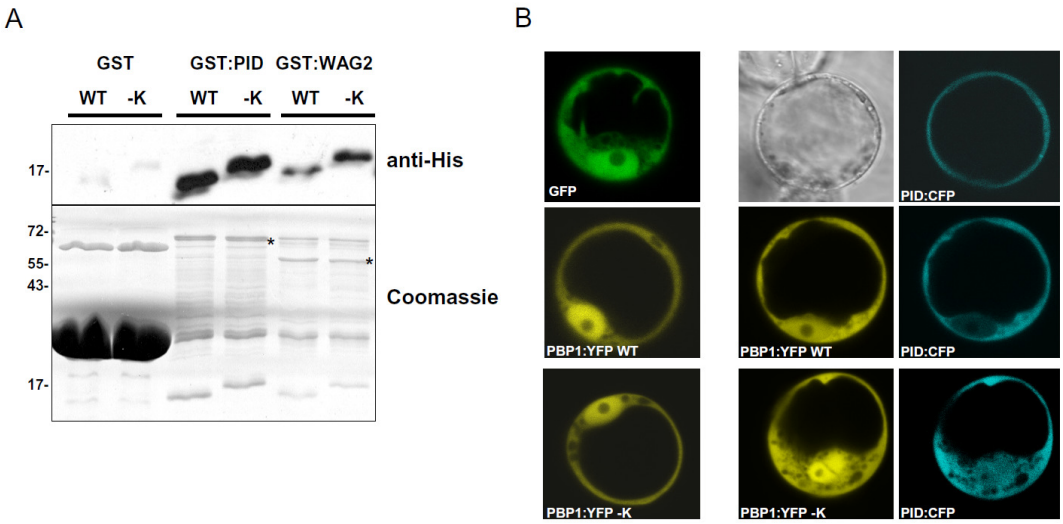

Figure 2: Ubiquitination of PBP1 does not alter its functional properties. (A) *In vitro* pulldown of His-PBP1 (WT) and His-HA-PBP1(-K) with GST, GST:PID or GST:WAG2 bound to glutathione agarose beads. W-His indicates a western blot of the eluates probed with anti-His antibodies. A coomassie stained SDS-PAGE gel of the same samples ran in parallel is depicted below. The asterisks indicate the GST:PID and GST:WAG2 bands. Molecular mass of marker proteins is indicated on the left in kDa. (B, C) Confocal laser scanning microscopy images of Arabidopsis protoplasts transfected with *35S::PID:CFP* (B, upper panel), *35S::GFP* (C, upper image), *35S::PBP1:YFP-HA* (C, middle image) or *35S::PBP1-K:YFP-HA* (C, lower image), or co-transfected with *35S::PID:CFP* and *35S::PBP1:YFP-HA* (B middle panel) or with *35S::PID:CFP* and *35S::PBP1-*

K:YFP-HA (B, lower panel). In the latter two panels the YFP and CFP signal are shown separately in the left and right images, respectively.

To further test the interaction between PBP1-K and PID *in vivo*, we co-expressed these proteins as respectively YFP and CFP fusions in Arabidopsis protoplasts. Previously, it was observed that PBP1:YFP co-transfection induces the membrane-associated PID:CFP to localize in the cytoplasm (Galvan, Robert, Offringa, in prep). Both the wild type and the -K version of PBP1 are able to sequester PID to the cytoplasm (Figure 2B), indicating that they both are able to bind PID *in vivo*. Our results indicate that the lysine-less PBP1 still retains the tested functional properties of the wild type protein, and that PBP1 ubiquitination is not essential for the protein to bind calcium or PID.

PID and PBP1 are both ubiquitinated and do not affect each other's poly-ubiquitination

We also tested whether the co-expression of PID would affect the ubiquitination status of PBP1 using the modified His-tagged ubiquitin (His-Ub) method for detection of protein ubiquitination (Campanero and Flemington, 1997) which allows identification of *in vivo* ubiquitinated proteins via Nickel-affinity purification. While this assay clearly showed that PID does not have any effect on the ubiquitination of HA-PBP1 (Figure 3, Ni-NTA α-HA panel), we found the PID kinase to be poly-ubiquitinated in Arabidopsis protoplasts (Figure 3, Ni-NTA α-FLAG panel). A single band representing the full length PID-FLAG was detected with the anti-FLAG antibody in the absence of cotransfected His-Ub, indicating basal binding of the abundantly expressed PID-FLAG protein to the Ni-NTA beads during affinity purification. However, only in cells co-expressing His-Ub, an additional smear of slower migrating FLAG tagged protein was detected, representing poly-ubiquitinated PID-FLAG proteins. Co-transfection with *35S::HA-PBP1* does not affect the ubiquitination of PID-FLAG (last lane Ni-NTA α-FLAG panel). These results indicate that PID is ubiquitinated *in vivo* independently of the co-expression of PBP1, and that co-expression of PID does not affect the ubiquitination of PBP1.

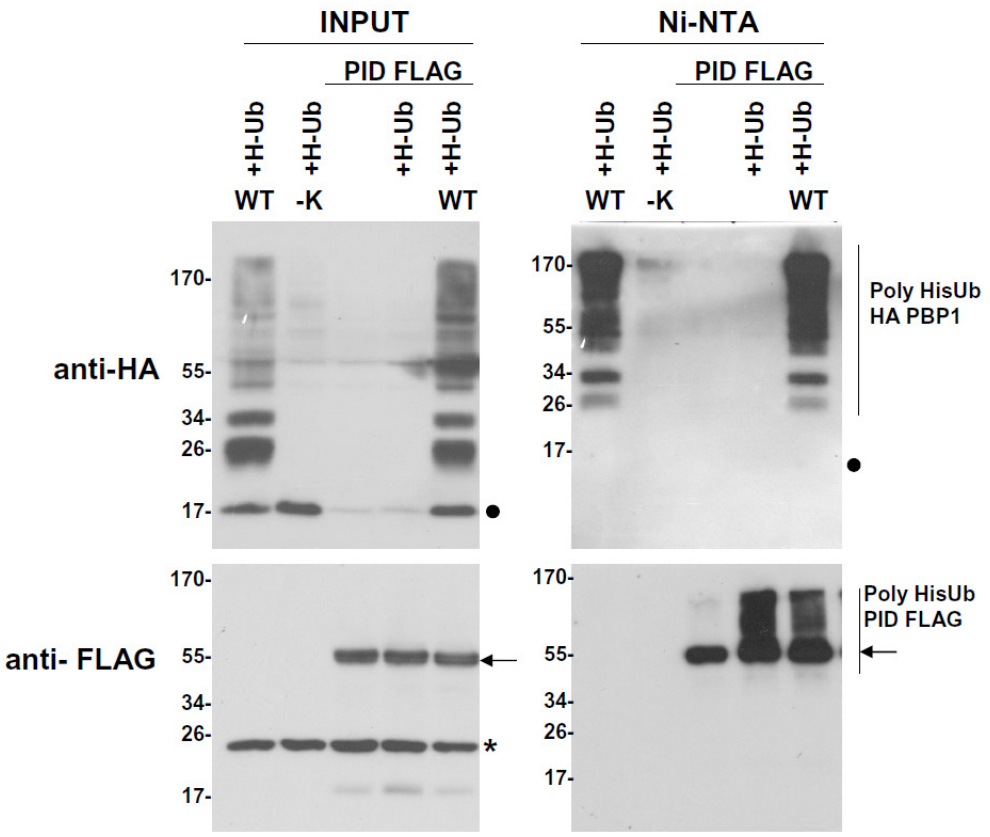

Figure 3: PID and PBP1 are ubiquitinated proteins that do not affect each other's poly-ubiquitination..(A) Western blot analysis of total protein extracts from transfected protoplasts before (INPUT) and after Ni-NTA (Ni-NTA) affinity purification, using anti-HA (upper panels) or anti-FLAG (lower panels) antibodies. Protoplasts were transfected with 20 µg of plasmid DNA of either *35S::HA-PBP1* (WT) or *35S:HA-PBP1-K* (-K) and *35S::PID-FLAG* (PID-FLAG) or *35S::5xHis-Ub* (H-Ub) where indicated. In all samples 5 µg *35S::FLAG-GFP* was cotransfected as an internal standard. Sixteen hours after transfection, cells were incubated for 1h with 50 µM MG132 and harvested in liquid nitrogen. Ten percent of the total extract was analyzed as INPUT and the remaining sample was used to affinity-purify His-Ub tagged proteins with Ni-NTA agarose beads. Poly-ubiquitinated HA-PBP1 and PID-FLAG are indicated. The black dot indicates the full length HA-PBP1, the arrow PID-FLAG, and the asterisk FLAG-GFP.

PBP1 contains a transferable ubiquitination signal

The HA-PBP1 fusion protein is very unstable in protoplasts and is significantly stabilized following treatment with the proteasome inhibitor MG132 (Figure 1A and B). However, when the HA-PBP1-YFP-HA fusion protein was expressed in protoplasts, the fluorescent signal was very strong (Figure 2B and C), and the

fusion protein was produced at high levels without MG132 treatment (Figure 4A). Substitution of the lysines in PBP1 for arginines prevented ubiquitination of HA-PBP1, but did not affect its tested physiological properties, or those of HA-PBP1-YFP-HA (Figure 2B and C). When we used the His-Ub method to analyze the ubiquitination of the PBP1-YFP-HA fusion protein, both HA-PBP1-YFP-HA and HA-PBP1(-K)-YFP-HA appeared as multiple bands before affinity purification (INPUT), with a different pattern of high molecular mass bands in the two samples. Interestingly, after Ni-NTA purification, the HA-PBP1(-K)-YFP-HA version was also recovered in the ubiquitinated pool. These results indicate that one or more of the lysines present in the YFP portion of this fusion protein now serve as substrates for ubiquitination, and that the PBP1(-K) protein can still interact with a E3 ubiquitin ligase and cause ubiquitination of a fused polypeptide. The FLAG-GFP present in all samples was not recovered in the His-Ub tagged fraction, which indicates that GFP (which differs in only one amino acid from YFP) is not ubiquitinated itself. The different patterns of bands observed in the input of the anti-HA blot indicates that different ubiquitin chains are attached to the different proteins due to the difference in the number of Ub attachment sites. These observations suggest that PBP1 contains a transferable ubiquitination signal that can lead to ubiquitination of a lysine-containing amino acid sequence present in *cis*. However, the ubiquitination signal does not function as a degron, since it does not lead an unstable fusion protein.

DISCUSSION

The paradigm in targeted protein degradation is that substrates of the eukaryotic 26S proteasome are ubiquitinated as a prelude to their destruction, and that the primary function of these poly-ubiquitin chains is substrate recognition by the 19S regulatory lid of the proteasome. Several plant proteins were shown to be recruited by E3 ubiquitin ligases, and to be substrates for proteasomal degradation (Gray *et al.*, 2001; Gomi *et al.*, 2004; Thines *et al.*, 2007). For some proteins poly-ubiquitination has been demonstrated (Xie *et al.*, 2002; Saijo *et al.*, 2003; Sasaki *et al.*, 2003; Jang *et al.*, 2005; Dos Santos *et*

al., 2009), and in addition recent high throughput MS analyses have identified multiple plant proteins that are labeled by poly-ubiquitin chains (Maor *et al*., 2007; Manzano *et al*., 2008). However, many of these studies lack a detailed analysis of the actual function of the poly-ubiquitin chains.

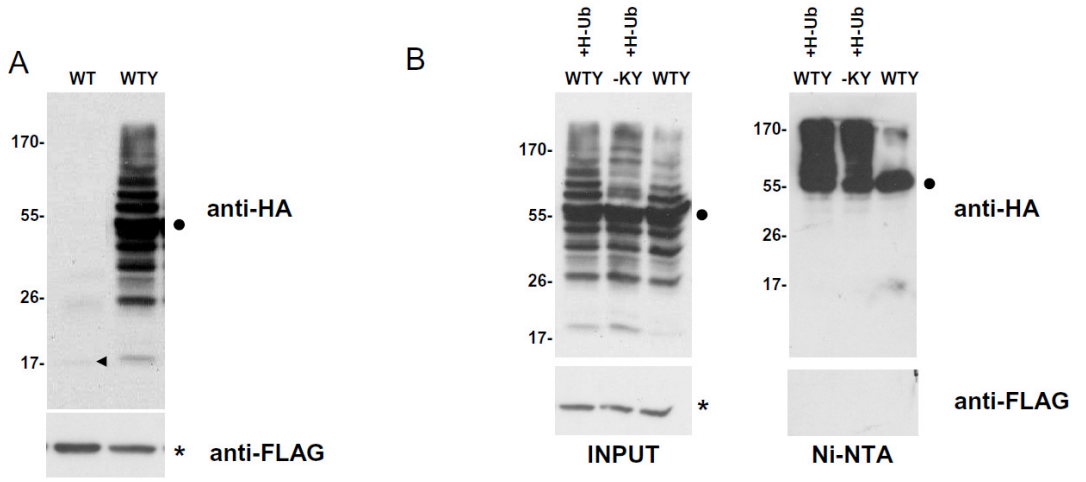

Figure 4: PBP1 contains a transferable ubiquitination signal that does not function as a degron. (A) PBP1 has increased stability when fused to YFP-HA. The arrow indicates the position of HA-PBP1 (WT) and the dot indicates the position of the full length HA-PBP1-YFP-HA (WTY). (B) HA-PBP1(-K) is ubiquitinated when fused to YFP-HA. Arabidopsis protoplasts were transfected with, *35S::HA-PBP1-YFP-HA* (WTY) or *35S::HA-PBP1(-K)-YFP-HA* (-KY), and *p35S::5xHis-Ub* (H-Ub) was co-transfected where indicated. Five micrograms of the plasmid *35S:FLAG-GFP* was added to all transfections as an efficiency control. Western blot analysis of total protein extracts before (INPUT) and after affinity purification (Ni-NTA) was performed with anti-HA (upper panels) or anti-FLAG (lower panels) antibodies. The black dot indicates the position of the full length HA-PBP-YFP-HA and the asterisk that of FLAG-GFP.

Here we show that the Arabidopsis calcium-binding protein PBP1 is an unstable protein exhibiting proteasome-sensitive turnover and ubiquitination *in vivo*. However, after substituting the lysines for arginines PBP1 remains unstable and its degradation is proteasome-dependent even though it cannot be ubiquitinated. Therefore, PBP1-ubiquitin conjugates are not obligatory intermediates in proteasome-dependent PBP1 turnover. These data do not exclude the possibility that its turnover may be mediated by ubiquitination in some physiological contexts. Alternatively, PBP1 ubiquitination may serve some

function other than signaling proteolysis. Regardless, this example illustrates that observing poly-ubiquitination and proteasome-mediated degradation *in vivo* are insufficient to conclude that protein turnover must proceed through a ubiquitinated intermediate.

Currently, the concept of a linear relationship between ubiquitination and proteasomal degradation knows many exceptions involving other mechanisms than ubiquitination to target proteins to the proteasome (Asher and Shaul, 2005). The ubiquitin-proteasome pathway can be regulated at the level of ubiquitination or at the level of proteasome activity (Glickman and Ciechanover, 2002). Ubiquitination is emerging as an additional regulatory step for the different way proteasomes use to recognize its targets. The proteasome can assume ubiquitin independent recognition of targets via alternative lid configurations such as the REGγ complex (Chen *et al.*, 2007b) showing that proteasome recognition is also a variable and dynamic process. Nonubiquitinated proteins have been reported to be directly recognized by the proteasome in a “degradation by default” mechanism (Asher *et al.*, 2006), via which the degradation occurs unless specific intervention prevents it. The existence of this mechanism suggests that the 20S catalytic core of proteasomes is able to recognize and degrade nonubiquitinated proteins. Examples of proteins degraded via this alternative proteasome pathway are ODC (Ornithine Decarboxylase) (Murakami *et al.*, 1992), p21cip1 (Chen *et al.*, 2007b) and p53/p73 (Asher *et al.*, 2005) for mammalian cells.

To our knowledge, PBP1 is the first plant protein shown to be degraded via a proteasomal ubiquitin-independent process. The presence of ubiquitinated PBP1 suggests that it is a substrate for an E3 ubiquitin ligase. An interesting possibility is that PBP1 may be recruited to the proteasome by being bound to a protein that is itself targeted to the proteasome following ubiquitination. In this case proteasome inhibition may indirectly stabilize PBP1 by affecting the stability of this other protein. Our results indicate that PID is also ubiquitinated, but we have no indications that PID affects the stability or ubiquitination state of PBP1.

The observation that PID is ubiquitinated *in vivo* indicates that the control of protein abundance is an additional step of regulation for the activity of this kinase, which is known to phosphorylate proteins that are themselves ubiquitinated and targeted to the 26S proteasome such as PIN2 (Abas *et al.*, 2006) and BDL (Chapters 2 and 4). Furthermore, the ubiquitination of PID may be regulated through its association with the CSN complex (Chapter 5), something that has been observed for other proteasome targets.

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Chapter 4

A phosphorylation site for the PINOID kinase is important for BODENLOS/IAA12 stability and activity

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Summary

The protein serine/threonine kinase PINOID (PID) is a signaling component in the control of polar auxin transport (PAT), as it determines the apico-basal polarity of many members of the PIN family of auxin efflux carriers. The polar transport of auxin results in differential distribution of this hormone, and the cellular auxin concentrations are subsequently translated into a primary gene expression response by the complex and cell-specific interactions between ARF transcription factors and labile Aux/IAA repressors. The abundance of Aux/IAA repressors is controlled by auxin-induced, ubiquitination by the E3 ligase SCF^{TIR1}. We identified the labile auxin response repressor BODENLOS (BDL/IAA12) as *in vitro* phosphorylation target of PID. The observation that PID-mediated phosphorylation possibly occurs in the PRSS motif close to the SCF^{TIR1}-interacting domain II of BDL/IAA12 suggests that this event plays a role in the stability of this repressor protein. Blockage of the identified phosphorylation site has minor negative effects on the repressor activity of the BDL protein in protoplasts and *in planta*, but plants carrying a phosphorylation insensitive version of the gain-of-function bdl protein fail to reproduce the *bodenlos* phenotype. Additionally, the phosphorylation-insensitive bdl protein is much less stable and has a more restricted tissue distribution in the root tip. This indicates that the control of BDL via phosphorylation might be an important mechanism regulating Arabidopsis root development. Although the mechanisms and roles of PID-mediated regulation of BDL require further elucidation, our data suggest that the PID protein kinase provides a direct link between auxin transport and signaling.

INTRODUCTION

The plant hormone auxin affects gene expression through the action of two types of transcriptional regulators: the Auxin Response Factors (ARFs) and the Aux/IAA transcriptional repressor proteins. ARFs bind with their amino(N)-terminal DNA-binding domain to promoters containing Auxin Responsive (AuxRE) elements, and can either activate or repress transcription, depending on the structure of their middle region {Tiwari, 2003 69 /id}. Aux/IAA proteins are short-lived nuclear proteins that function as repressors of auxin-responsive gene expression. Aux/IAA proteins form a family of twenty-nine members in Arabidopsis that mostly share four conserved domains (Liscum and Reed, 2002). From N- to carboxy(C)-terminus, domain I has been shown to have transcription repression activity (Tiwari *et al.*, 2004) and to interact through an EAR motif with the transcriptional co-repressor TOPLESS (TPL) (Shemenyei et al., 2008), domain II is involved in destabilization of Aux/IAA proteins (Ramos *et al.*, 2001), and domains III and IV allow Aux/IAA proteins to dimerize with ARFs or with other Aux/IAA proteins (Ulmasov *et al.*, 1999). Domain II of Aux/IAAs interacts with the auxin receptors TRANSPORT INHIBITOR RESISTANT 1/ AUXIN SIGNALING F BOX (TIR1/AFB), which are part of a Skp1/cullin/F-box protein (SCF) E3 ubiquitin ligase complex (Gray *et al.*, 2001; Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a). Auxin stabilizes this interaction, leading to the proteasomal degradation of Aux/IAAs, which subsequently allows ARFs to initiate transcription. Screening for auxin-insensitive mutants in Arabidopsis has identified specific mutations in domain II of Aux/IAAs that disrupt the interaction with the TIR1 protein family, thereby abolishing their auxin-induced degradation. Such gain-of-function mutations lead to reduced auxin response and related semi-dominant phenotypes, such as the lack of a primary root meristem, reduced hypocotyl growth and curled cotyledon phenotypes that are typical for the *bodenlos* (*bdl*) mutant (Hamann *et al.*, 1999). The *bdl* mutant seedling phenotypes imply that the BDL/IAA12 protein is involved in auxin-mediated apical-basal patterning of the Arabidopsis embryo. BDL/IAA12 physically interacts with MONOPTEROS/AUXIN RESPONSE FACTOR5

(MP/ARF5) to control its activity (Hamann *et al.*, 2002). Consistent with this, both *bdl* mutants and *mp/arf5* loss-of-function alleles display reduced vasculature and form a "basal peg" instead of a root and a hypocotyl. The function of MP-BDL in embryo patterning is to control an auxin-responsive gene expression response in specific embryonic cells. The polarization of the embryo occurs after the first cell division of the zygote that already responds to auxin transported in a polar manner due to the action of the auxin efflux carriers PIN1 and PIN7 (Friml *et al.*, 2003). The polar localization of these two proteins was shown to be controlled by the serine/threonine (Ser/Thr) kinase PINOID (PID) (Friml *et al.*, 2004) Loss of *pid* function causes an apical-to-basal shift in PIN polarity, correlating with defects in embryo and shoot organogenesis (Friml *et al.*, 2004). PID is known to phosphorylate PIN proteins controlling their polarity inside the cells (Michniewicz *et al.*, 2007). Up to date, no other PID phosphorylation targets are known besides PIN proteins. Here we identify a synergistic effect between the *bdl* and *pid* mutants, and show that BODENLOS (BDL/IAA12) is a phosphorylation target of PID in *in vitro* assays. Mapping of the phosphorylation site identified the PRXS motif in between the TPL interacting domain I and the TIR1/ABF-interacting domain II of BDL/IAA12 as target for phospho-modification. Our results indicate a phosphorylation-dependent control of the stability and activity of the BDL/IAA12 and IAA13 repressor proteins, implying that the role of PID in plant development, besides regulating auxin transport, extends to the regulation of auxin-responsive gene expression.

MATERIAL AND METHODS

DNA cloning and constructs

For the auxin-responsive GUS assays, a *DR5::GUS* reporter construct with 7 copies of the *DR5* sequence cloned in the plasmid *GusXX-47* (Pasquali *et al.*, 1994) was used. A plasmid carrying the *Renilla reniformis* luciferase (*LUC*)

gene under the control of the *CaMV 35S* promoter was co-transfected as a control for transformation efficiency (De Sutter *et al.*, 2005). All effector plasmids used for protoplast transfections are based on *pART7* carrying the *CaMV 35S* promoter and the *OCS* transcription terminator (Gleave, 1992). GATEWAY® (www.invitrogen.com) destination cassettes derived from *pEarleyGate 201* and *202* (Earley et al., 2006) were transferred into *pART7* to generate plasmids *pART7::HA* and *pART7::FLAG* for the expression of respectively N-terminal HA- or FLAG-tagged proteins in plant cells.

A cDNA encoding *SHY2/IAA3* with an N-terminal HA tag was cloned from *pACT2::SHY2* using *Xho*I/*Xba*I sites into *pART7*, generating *35S::HA-SHY2/IAA3*. The *BDL/IAA12* cDNA was excised with *Bam*HI/*Xba*I from *pETH16-BDL* (Weijers *et al.*, 2006), introduced into *pENTR 3C*, and introduced into *pART7-HA* via LR recombination to create *35S::HA-BDL*. The mutations resulting in the Ser_{67}-Ser_{68} (SS) to Lys_{67}-Ala_{68} (KA), the Ser_{67}-Ser_{68} (SS) to Asp_{67}-Asp_{68} (DD) and the *bodenlos* (P75S) (Hamann *et al.*, 2002) substitutions were introduced in this plasmid using the Quickchange Site-directed Mutagenesis kit (Stratagene) with primer pairs BDL SS>KA F2 5'-GCCATCCTACCACTTGAGCTTTACGAGGAGGAGAAGCTCCTTGGT-3' and BDL SS>KA R2 5'-ACCAAGGAGCTTCTCCTCCTCGTAAAGCTCAAGTGGTAGGATGGC-3' for the SS>KA mutation, bodenlosmtF 5'-GTCAAGTGGTAGGATGGTCACCAATTGGGTTAC-3' and bodenlosmtR 5'-GTAACCCAATTGGTGACCATCCTACCACTTGAC-3' for the *bdl* mutation, IAA12SS>DDF 5'-GGAGCTTCTCCTCCTCGTGATGATCAAGTGGTAGGATGGCC-3' and IAA12SS>DDR 5'-GGCCATCCTACCACTTGATCATCACGAGGAGGAGAAGCTCC-3' for the SS>DD mutation in *35S::HA-BDL*, bdlSS>DDF 5'-GGAGCTTCTCCTCCTCGTGATGATCAAGTGGTAGGATGG-3' and bdlSS>DDR 5'- CCATCCTACCACTTGATCATCACGAGGAGGAGAAGCTCC-3' for the SS>DD mutation in*35S::HA-BDL*, resulting in respectively *35S::HA-BDL KA*, *35S::HA*-bdl, 35S*::HA-bdl KA*, *35S::HA-BDL DD*, and *35S::HA-bdl*

DD. The *35S::PID-FLAG* construct was described previously (Michniewicz *et al.*, 2007). An entry clone for GFP was made by cloning the GFP cDNA from *pTH2* (Chiu *et al.*, 1996) as a *Bam*HI/*Not*I fragment into pENTR 3C (Invitrogen). This clone was used for generating *pART7::FLAG-GFP* and *pART7::HA-GFP* via LR recombination.

BDL N-terminal YFP- fusion was created into pEarleyGate 104 via LR recombination with *pENTR 3C::BDL*. The *YFP-BDL* cassette was PCR amplified with primers BDLYFP ClaI F 5'-CCATCGATATGGGCAAGGGCGAGGAGCTGT-3' and BDLYFP XbaI R 5'-GCTCTAGAAATAGGGTTGTTTCTTTGTC-3', the resulting fragment was cut with *Cla*I and *Xba*I and ligated into *Cla*I/*Xba*I sites of *pART7*. The mutations leading to the *bodenlos* (P75S) and the SS>KA substitutions were introduced into the resulting plasmid *35S::YFP-BDL* as described above, resulting in *35S::YFP-bdl* , *35S::YFP-bdl KA* and *35S::YFP-BDL KA*.

The construct *BDL::3xGFP-BDL* (Weijers *et al.*, 2006) in *pGreen0229* was used to create *BDL::3xGFP-BDL KA* and *BDL::3xGFP-bdl KA* with the primers BDLSS>KA F 5'-ATCTTCCTCTCACCAAGGAGCTTCTCCTCCTCGTTCAAGGTTCGTCCTTTTTCTTA-3' and BDLSS>KA R 5'-AGAAAAAGGACGAACGCTTTACGAGGAGGAGAAGC-3' as described above.

Constructs for production of recombinant protein in His-BDL (Weijers *et al.*, 2006) His-PBP1, GST-PID (Benjamins *et al.*, 2003) in *E.Coli* were previously described.

Plant lines, plant growth and transformation and molecular analysis

The *pid-En197* and *pid-14* (SALK_049736) alleles and the *bdl* mutant have been described before (Hamann *et al.*, 1999; Christensen *et al.*, 2000; Benjamins *et al.*, 2001). Seeds were surface sterilized with 1% commercial chlorine solution, and germinated on MA medium at 21°C and a 16 hours photoperiod. Plantlets were transferred to soil and grown at 20°C, 70% relative humidity and 16 hours photoperiod. The *BDL::3xGFP-BDL* and *BDL::3xGFP-bdl* lines were previously described (Weijers *et al.*, 2006) and kindly donated by

Dolf Weijers (Wageningen University). For generation of the *BDL::3xGFP-BDL KA* and *BDL::3xGFP-bdl KA* lines, *Arabidopsis thaliana* ecotype Columbia (Col) was transformed by the floral dip method (Clough and Bent, 1998). Primary transformants were selected on medium supplemented with 30 mg/L phosphinotricin (PPT), with 50 mg/L nystatin and 100 mg/L timentin to inhibit growth of *Agrobacterium*. For further analysis, single locus insertion lines were selected by germination on 20 mg/L PPT. Resistant seedlings were checked for transgene expression by epifluorescence microscopy, by western blot- or by RT-PCR analysis.

For western blot analysis, around ten 5 day-old plants were frozen in liquid nitrogen, ground and extracted in 0.1 mL cold extraction buffer (Phosphate Buffered Saline, PBS; 1x Roche Complete Protease Inhibitor Cocktail, 1 mM PMSF, 1% Triton X-100). The lysate was cleared by centrifugation at 20.000 g for 10 min. Total protein was quantified by Bradford assay (Bio-Rad) and 80 µL of the extract was mixed with 20 µL 5X Laemmli protein sample buffer and boiled for 5 minuntes. A volume corresponding to 20 µg of total protein was separated on 8 % SDS-PAGE minigels. PAGE-separated proteins were semi-dry blotted onto PVDF membranes, which were subsequently blocked with nonfat dry milk and incubated overnight with 5000-fold diluted anti-GFP rabbit antibody (Invitrogen, A-11122) at 4^{o}C. Membranes were washed and incubated for 1h at 4^{o}C with 10.000-fold diluted goat anti-rabbit IgG antibodies conjugated to HRP (Promega, W4011). Detection of the HRP-conjugated antibody was performed with the LumiGLO Detection Kit (Cell Signalling). Loading was monitored by staining the membrane with Sypro Ruby (BioRad).

For RT-PCR analysis, total RNA was extracted from one-week old seedlings with the Invisorb Spin Plant RNA kit (Invitek). RT-PCRs were performed as described in (Weijers *et al.*, 2001) using 2 µg of total RNA for the RT reaction and transgene-specific primers GFPBDLRT 5'-AGCTGTACAAGAGATCCATGCGTGG-3' and BDLRTR 5'-AACAGGGTTGTTTCTTTGTCTATCC-3' for detection of the 3xGFP-BDL mRNA, or *ROC* (At4g38740) specific primers 3.3F 5'-

CCACAGGCTTCGTCGGCTTTC-3' and 5.2R 5'-GAACGGAACAGGCGGTGAGTC-3' as an internal control.

Root length measurements

Sterile seeds were spread with 0.1 % agarose onto MA solid medium (1.5% agar) containing 0, 10^{-8} and 10^{-7} M of IAA. Seeds were vernalized in the dark for 2 to 4 days at 4°C and transferred to 21°C, 16h light with plates placed vertically to allow root elongation over the medium surface. Plates were scanned after 13 days and root lengths were measured using ImageJ (http://rsb.info.nih.gov/ij/). Average lengths of 15 primary roots were scored from three individual plates. Average groups were compared by One-Way ANOVA followed by Student's T test ($p<0.05$) using SPSS 15.0.

***In vitro* pull down experiments**

GST-tagged PID or the GST-tag alone were used in pull down assays with histidine (His)-tagged BDL and PBP1 (H-proteins). *E. coli* strain BL21(DE3)pLysS containing one of the constructs was grown in 50 ml LC cultures supplemented with antibiotics at 37ºC to OD_{600} 0.8. The cultures were then induced for 4 hours with 1 mM IPTG at 30ºC, after which cells were harvested by centrifugation (10 min. at 2.000 g in tabletop centrifuge) and frozen overnight at -20ºC. 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 Phenylmethanesulfonyl Fluoride (PMSF), 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 $CaCl_2$, supplemented with 0.1 mM PMSF, 0.1 mM, Leupeptin and 0.1 mM Aprotinin) for the His-tagged proteins and sonicated for 2 min on ice. From this point on, all steps were performed at 4ºC. Eppendorf tubes containing the sonicated cells were centrifugated 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 proteins bound to beads, and the mixtures were incubated with gentle agitation for 1 hour. After incubation, the mixtures were centrifugated at 500 g for 3 min, the supernatants were discarded and the beads were subsequently washed 3 times with 20 volumes EB. Elution was performed by mixing 100 µL 2X Laemmli protein loading buffer to the beads, followed by denaturation by 5 min incubation at 95ºC. Proteins were subsequently separated on a 12% polyacrylamide gel prior to transfer to an Immobilon^{TM}-P Polyvinylidene Fluoride PVDF (Sigma) membrane. Western blots were hybridized with 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

All proteins used in *in vitro* phosphorylation assays were His-tagged for purification from several (usually five) aliquots of 50 ml cultures of *E. coli*. strain BL21, which were grown, induced, pelleted and frozen as described above for the *in vitro* pull down experiments. Each aliquot of frozen cell pellet was resuspended in 2 ml Lysis Buffer (25 mM Tris-HCl pH 8.0; 500 mM NaCl; 20 mM Imidazole; 0.1% Tween-20; supplemented with 0.1 mM of the protease inhibitors PMSF, Leupeptin and Aprotinin) and subsequently sonicated for 2 min on ice. From this point on, all steps were performed at 4ºC. Sonicated cells were pelleted in an Eppendorf centrifuge at full speed (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). Mixtures were incubated with gentle agitation for 1 hour. After incubation, mixtures were 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 volumes of Elution Buffer (25 mM Tris.HCl pH 8.0; 500 mM NaCl; 500 mM Imidazole) were added to the resin and the suspension was 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 -80ºC.

Approximately 1 µg of each purified His-tagged protein (PID and substrates) and 1 µg MBP (Sigma #M1891) in maximal volumes of 10 µl were added to 20 µl kinase reaction mix, containing 1x kinase buffer (25 mM Tris-HCl pH 7.5; 1 mM DTT; 5 mM $MgCl_2$) and 1 x ATP solution (100 µM $MgCl_2$/ATP; 1 µCi γ-^{32}P-ATP). Reactions were incubated at 30ºC for 30 min and stopped by the addition of 5 µl of 5 x protein loading buffer (310 mM Tris-HCl pH 6.8; 10 % SDS; 50% Glycerol; 750 mM β-Mercaptoethanol; 0.125% Bromophenol Blue) and 5 min boiling. Reactions were subsequently separated on 12.5% acrylamide gels, which were subsequently washed 3 times for 30 min with kinase gel wash buffer (5% TCA – Trichoroacetic Acid; 1% $Na_2H_2P_2O_7$), coomassie stained, destained, dried and exposed to X-ray films for 24 to 48 hours at -80ºC using intensifier screens.

For the peptide assays, 1 µg of purified PID was incubated with 4 nmol of 9^{mer} biotinylated peptides (Pepscan) in a phosphorylation reaction as described above. Reaction processing, spotting and washing of the SAM^2 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 hours at -80°C using intensifier screens. The phosphorylation intensities of each peptide were determined by densitometry analysis of the autoradiographs using the ImageQuant software (Molecular Dynamics).

Protoplast isolation and transfection

Protoplasts were isolated from *Arabidopsis thaliana* 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) In the *DR5::GUS* transactivation assays 10^6 protoplasts were transfected with 10 µg of the *DR5::GUS* reporter construct and 2 µg of *35S:Rluc* (De Sutter *et al.*, 2005) for experimental normalization. The DNA amounts of the effector constructs varied per experiment and are indicated in the figure legends. All transformations contained 10 µg of *35S::FLAG-GFP* as a control for transformation efficiency, and were split in 2 portions containing 5 x 10^5 protoplasts in a total volume of 2.5 mL of protoplast medium. After 16 h the samples were treated for 4 h either with 1 µM IAA or the same volume of the solvent DMSO. Treated cells were collected by centrifugation at 80 g for 1 minute and the pellets were frozen in liquid nitrogen for GUS (van der Fits and Memelink, 1997) and LUC measurements (Dyer *et al.*, 2000). Triplicate transfections were assayed and mean GUS/LUC relative activities were analyzed by One-way ANOVA using SPSS 15.0 software.

For the Aux/IAA degradation assays, 10^6 protoplasts were transfected with 20 µg *35S::HA-Aux/IAA* construct and 10 µg of *35S::FLAG-GFP*. Treated protoplasts were resuspended by vortexing in cold Extraction Buffer (PBS, 1x Roche Complete Protease Inhibitor Cocktail containing 1% Triton X-100). The lysate was cleared by centrifugation at 20.000 g at 4°C for 10 min. Total protein was quantified by Bradford assay (Bio-Rad) and 20 µg was mixed with protein sample buffer and separated on 10% SDS-PAGE minigels. PAGE-separated proteins were blotted onto nitrocellulose 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 LumiGLO Detection Kit (Cell Signalling).

Microscopy

For imaging of transfected protoplasts and intracellular localization of 3xGFP-BDL in roots a Leica DM IRBE confocal laser scanning microscope equipped with Argon laser line of 488 nm (excitation) and a band pass emission filter of 500-550 nm was used with a 63X water objective, digital zoom and 51% laser intensity. Expression of the 3xGFP-BDL fusions in roots was imaged using a Leica MZ16FA stereomicroscope equipped with a GFP filter set and a DFC 420C camera. Image processing was performed with ImageJ (http://rsb.info.nih.gov/ij/).

RESULTS

Genetic interaction between PID and BDL

Previously, the possibility has been entertained that PID may be involved in regulating the stability of Aux/IAA proteins (Reed, 2001). Since PID is expressed in the embryo and is essential for proper embryonic patterning (Christensen *et al.*, 2000; Benjamins *et al.*, 2001), we decided to test whether PID could affect the stability of the embryonic Aux/IAA protein BODENLOS (BDL)/IAA12. F2 seedlings from a cross between the *pid-14* or *pid-En197* loss-of-function alleles and the *bdl* gain-of-function mutant, displayed a range of phenotypes, varying from wild type and typical *pid* and *bdl* seedlings to seedlings that lack cotyledons (no-cot), or no-cot seedlings that even lack a primary root (Figure 1A to 1D). As the latter seedlings phenocopied the previously identified *gurke* mutants (Chamovitz *et al.*, 1996), their phenotype was referred to as *gurke*-like. The frequency of no-cot or *gurke*-like seedlings matched the expected numbers for respectively *BDL/bdl pid/pid* and *bdl/bdl pid/pid* progeny (Table 1). Few seedlings of the no-cot and *gurke*-like class

were able to develop past this early seedling stage, but the resulting plantlets formed a rosette of twisted leaves with a disorganized phyllotaxis, and developed early pin-like inflorescences (Figure 1E). The no-cot phenotype was also observed in *pid-pin1* double mutants (Furutani *et al.*, 2004), and since we know now that PID regulates PIN polar targeting (Friml *et al.*, 2004) by phosphorylating the PIN hydrophilic loop, our data suggested that a similar functional interaction may exist between PID and BDL.

Table 1. Segregation analysis of phenotypes observed in a *pid* x *bdl* F2 population

	Total	kan$^{s\ \xi}$	tricot†,*	*bdl**	no-cot.*	*gurke*-l*
Observed number of seedlings (%)	198 (100)	50 (25)	6 (3)	17 (8.5)	13 (6)	4 (2)
Expected number of seedlings (%)	198 (100)	50 (25)	6 (3)	25 (12.5)	12 (6)	6 (3)

ξ Number of kanamycin sensitive seedlings. Seeds were germinated on MA medium containing 25 µg/ml of kanamycin, to select for the T-DNA insertion causing the *pid* loss-of-function mutation.
† The three cotyledon phenotype of this *pid* mutant allele shows a penetrance of 50%, indicating that it is a complete loss-of-function allele (Bennett *et al.,* 1995; Christensen *et al.*, 2000).
*The expected number of kanamycin resistant three cotyledon, *bdl*, no-cotyledon and "*gurke*-like" seedlings, based on 1:16 (*BDL/BDL pid/pid*), 1:8 (*bdl/bdl PID/pid*), 1:8 (*BDL/bdl pid/pid*) and 1:16 (*bdl/bdl pid/pid*) segregation ratios, respectively, and a 50% penetrance of the phenotypic changes induced by the homozygous *pid* mutation. The numbers between brackets indicate percentages. The observed numbers did not significantly differ from the expected ones in the X^2 test (X^2=3.69, p<0.05).

Figure 1: The *bdl* gain-of-function mutation enhances the cotyledon defects caused by the *pid* loss-of-function mutation. (A-E) The phenotypes of the *pid* (A) and *bdl* (B) parental lines and the synergistic lack of cotyledons (no-cot) (C) and *gurke*-like (D) phenotypes observed in the *pid* x *bdl* F2 population. No-cot and *gurke*-like seedlings that grow beyond the seedling stage develop a

rosette with curled leaves in a disorganized phyllotactic pattern and form an early pin-like inflorescence (E). White bars represent 2 mm.

PID phosphorylates BDL at a PRSS motif, but does not interact with BDL *in vitro*

To find more evidence for the putative functional interaction between PID and BDL, we tested whether PID could phosphorylate BDL or could bind to it. An *in vitro* protein pull-down assay showing that His-tagged BDL (Figure 2A, lanes 1 to 3) is not pulled down with GST-PID (lane 1) nor with GST alone (lane 2), whereas His-tagged PBP1 (lanes 4 to 6) is specifically pulled down with GST-tagged PID (lane 4) and not with GST alone (lane 5) Although we did not observe a clear interaction between the two proteins in *in vitro* pull down assays, we found that PID was able to phosphorylate BDL in an *in vitro* phosphorylation reaction (Figure 2B). By using the NetPhos software, putative phosphorylation sites were mapped in the BDL protein (Figure 2C). Biotinylated nine amino acid peptides corresponding to these sites were synthesized, and subsequently used in *in vitro* phosphorylation reactions. The peptides with the amino acid sequences MRGVSELEV (Peptide 1), PPRSSQVVG (Peptide 5) and LKDVSMKVN (Peptide 6) in BDL were strongly phosphorylated by PID (Figure 2D), and phosphorylation of peptide 9 was rather variable. Closer inspection of the amino acid sequences of the consistently phosphorylated peptides revealed that peptide 5 comprises the PRXS motif that is also present in the three major PID target sites identified in PIN1 (Huang, F., Zago, M.K. and Offringa, R., in preparation). An alignment of the 27 family members of the Arabidopsis Aux/IAA family shows that the serine pair in the PRXS motif is only found in BDL/IAA12 and IAA13 (Figure 2E). The functional redundancy between these two proteins (Weijers *et al*., 2005) suggests that they might be regulated similarly. In order to determine the significance of the PRSS motif in the phosphorylation of BDL/IAA12, we mutated the coding region so that the two serine residues were substituted by a lysine and an alanine (KA), the sequence that is common to 9 members of the Aux/IAA family, in order to destroy the putative PID recognition site. This mutation abolished the *in vitro*

phosphorylation of BDL/IAA12 by PID (Figure 2B). These observations indicate that, even without showing a detectable physical interaction, BDL is phosphorylated by PID *in vitro* at the serines of the PRSS motif.

The PRSS motif in BDL/IAA12 affects its transcriptional repressor activity and stability

In order to evaluate the *in vivo* significance of the BDL/IAA12 phosphorylation by PID, we tested the transcriptional repression activity of the Aux/IAA protein on the synthetic auxin-responsive *DR5* promoter in Arabidopsis cell suspension protoplasts. In this system, expression of the *DR5::GUS* reporter was highly induced after four hours treatment with 1 µM IAA (Figure 3A). Co-transformation of the reporter with the *35S::HA-BDL/IAA12* construct resulted in a 50% reduction in the IAA-induced reporter gene activity, while co-transfection with *35S::HA-bdl*, encoding the dominant mutant *bodenlos* (P75S), completely abolished this auxin response. The *35S::HA-SHY2/IAA3* construct only had a limited repressive effect on auxin-induced *DR5::GUS* expression. Co-transfection of *35S::PID-FLAG* reduced the overall response of the *DR5* promoter regardless of the co-transfected construct, which probably is a result of the positive effect of PID on auxin efflux (Benjamins *et al.*, 2001; Lee and Cho, 2006), and which makes this experiment less informative. The KA mutation in BDL resulted in a small but statistically significant reduction in its repressive activity (Figure 3B). Additionally, substitution of the two serines by aspartic acid (DD) to mimic phosphorylation resulted in a slightly stronger repression of the *DR5::GUS* reporter (Figure 3B), although this was not statistically significant.

The fact that the identified phosphorylation site is close to the domain II consensus QVVGWPP, makes it tempting to speculate that phosphorylation at this site affects the interaction of the protein with the TIR1/AFB auxin receptors and hence, its stability. To address the effect of these mutations on the stability of the Aux/IAA proteins we transfected Arabidopsis protoplasts with the same HA-tagged constructs and analyzed the protein abundance after auxin

treatment (Figure 3C). The relative abundance of the HA-tagged proteins expressed in protoplasts indicated that

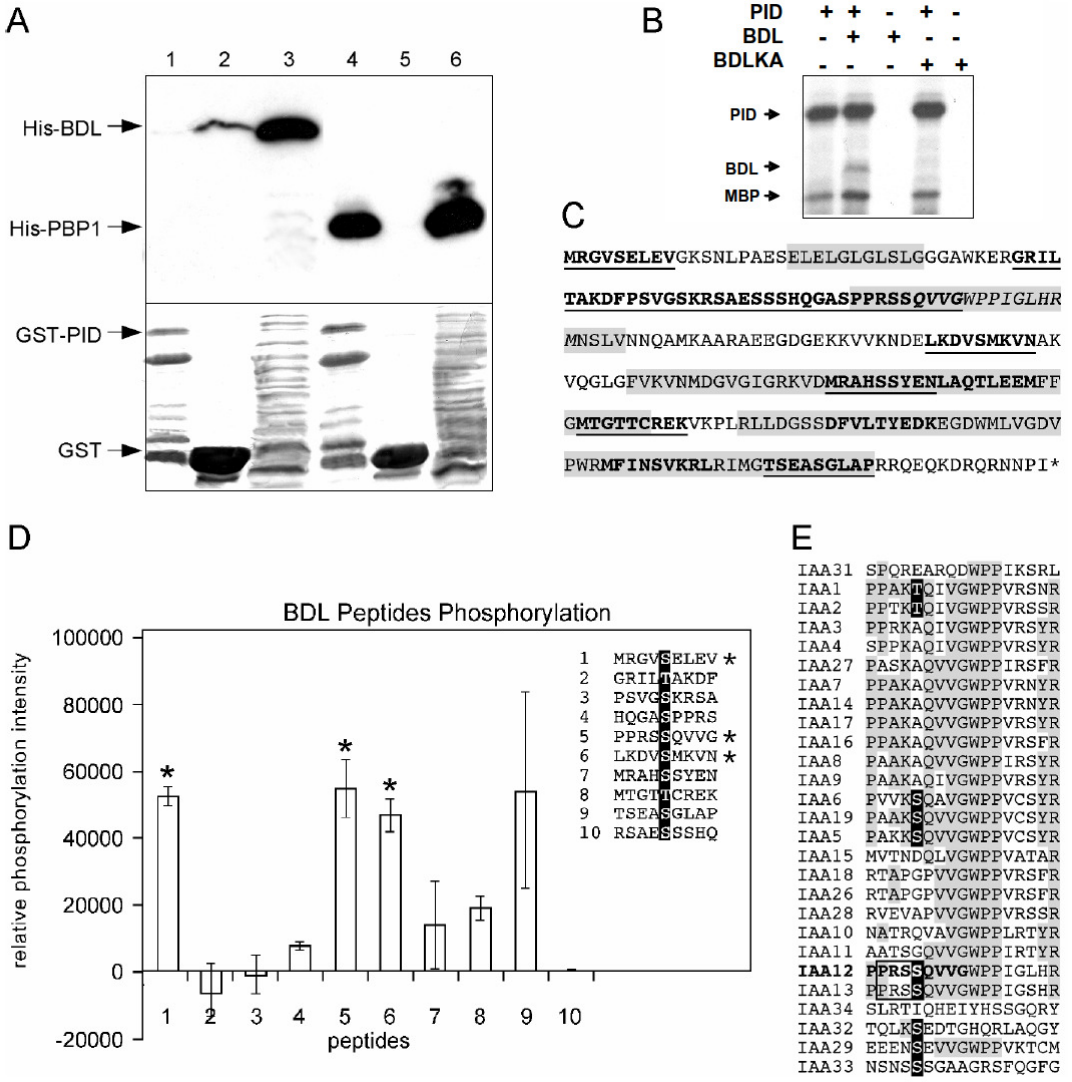

Figure 2: PID phosphorylates BDL in vitro without tightly binding to it.

(A) An *in vitro* protein pull-down assay showing that His-tagged BDL (lanes 1 to 3) pulled down with GST-PID (lane 1) or with GST alone (lane 2), as a positive control His-tagged PBP1 (lanes 4 to 6) is specifically pulled down with GST-tagged PID (lane 4) and not with GST alone (lane 5). Total protein extracts (1% of input) of *E. coli* cells expressing His-BDL (lane 3) or His-PBP1 (lane 6) are loaded as controls. The top panel shows immunodetection of His-tagged proteins, and the coomassie-stained gel is shown in the bottom panel. (B) Autoradiograph of an *in vitro* phosphorylation reaction with His-tagged PID, BDL and BDL KA. MBP is present in all samples as a positive control. Arrows indicate the position of the indicated protein on gel. (C) BDL protein sequence with conserved domains I, II, III and IV shaded, all putative phosphorylation residues identified by NetPhos within nine amino acids peptides indicated in bold, and the peptides used in *in vitro* phosphorylation assays underlined. The highly conserved portion of domain II is in italics. (D)

Relative radioactive labeling intensities of ten BDL-derived peptides by PID in *in vitro* phosphorylation reactions. The BDL-derived peptides that show a reproducible high phosphorylation by PID are indicated with a star. (E) Alignment of the conserved part of domain II of the 27 Arabidopsis Aux/IAAs. Gray shading shows conserved residues. Putative phosphorylation sites at position 5 are shaded in black and the PRSS motif that is unique for BDL/IAA12 and IAA13 is boxed.

their repressive activity was mostly related to the stability of the proteins. The presence of the SS→KA mutation in BDL/IAA12 resulted in a less stable protein, which explains why it works as a milder repressor. Interestingly, the overexpression of PID did not affect the stability of BDL nor of the KA variant suggesting that the *in vivo* phosphorylation had no effect on the stability of the wild type BDL protein. The DD mutant showed slightly stronger repression of the DR5::GUS reporter (Figure 3B) and an enhancement of protein stability, indicating that phosphorylation of the two serines in the wild type protein might fine tune the abundance of the protein via the auxin/TIR1 degradation pathway. These observations indicate that lack of phosphorylation at the PRSS motif on BDL reduces its stability and, hence, its transcriptional repression activity in protoplasts.

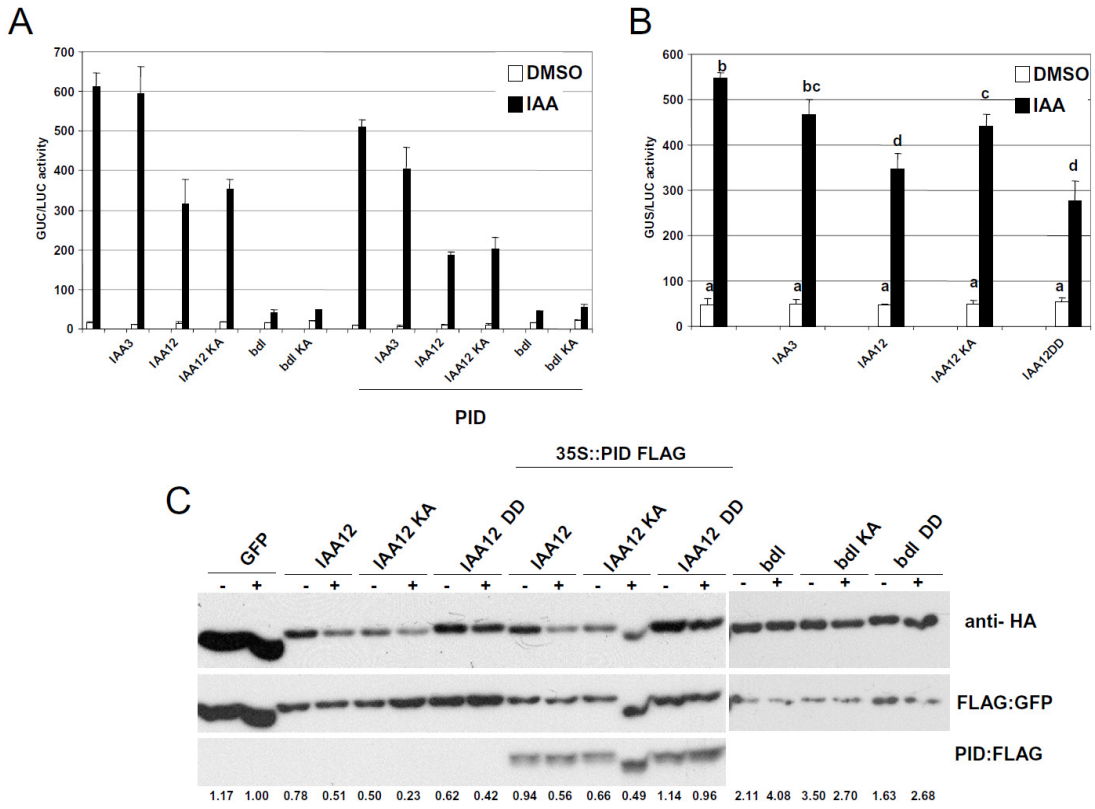

Figure 3: The PRSS motif in BDL/IAA12 affects its transcriptional repressor activity and stability.

(A) Repression of the *DR5::GUS* reporter gene. Values are expressed in arbitrary units of relative GUS/LUC activity with standard deviation of three transfomations. Effector plasmids encoding different HA-tagged Aux/IAA proteins were co-transfected with the auxin-responsive *DR5::GUS* reporter gene into Arabidopsis cell suspension protoplasts, and cells were incubated for 4 hours with (IAA) or without (DMSO) 1 µM auxin. Samples marked with PID were additionally co-transfected with 10 µg of *35S::PID-FLAG.* (B) As in (A) but comparing the effects of the BDL KA and BDL DD mutant versions. Bars indicate means and standard deviation from three repetitions while different letters above bars represent statistically significantly different groups after One-Way ANOVA followed by Student's T test ($p<0.05$). (C) Western blot of total extracts from protoplasts transfected with HA-tagged versions of BDL. Cells were treated for 1 hour with (+) or without (-) 1 µM NAA before harvesting. *35S::HA-GFP* (GFP) is used as a control and *35S::FLAG-GFP* is present in all samples as a transfection efficiency reference. Samples co-transfected with 10 µg of *35S::PID-FLAG* are indicated. The top panel shows detection with anti-HA antibodies and the two lower panels with anti-FLAG antibodies. Numbers at the bottom indicate the HA/FLAG-GFP signal ratio relative to the second lane HA-GFP+.

BDL/IAA12 phosphorylation does not affect its sub-cellular localization or tissue-specific expression

Is well established that Aux/IAA proteins are transcriptional co-repressors that act on auxin-responsive gene expression by dimerization with the ARF transcription factors in the nucleus (Guilfoyle *et al.*, 1998; Tiwari *et al.*, 2001; Tiwari *et al.*, 2004) and one of the mechanisms by which phosphorylation could affect their activity is by regulating their sub-cellular localization (Parry *et al.*, 2006). First we tested the effect of the SS→KA substitution on the sub-cellular localization of YFP-BDL and YFP-bdl fusions in protoplasts. All four variants were nuclear localized, and as previously observed for a GFP-BDL fusion they all accumulated in specific nuclear structures (Figure 4A) (Hamann *et al.*, 2002), which are believed to be sites of proteasomal degradation (Tao *et al.*, 2005). These results suggest that phosphorylation of BDL/IAA12 does not play a role in regulating its sub-cellular localization.

To confirm these results *in planta*, we generated transgenic lines with the *BDL::3xGFP-BDL KA* construct, comprising a fusion between the *BDL KA* genomic clone and a triple *GFP* reporter gene. Two *BDL::3xGFP-BDL KA* lines were selected and compared with the previously generated *BDL::3xGFP-BDL* line (Weijers *et al.*, 2006). For both the *BDL* and the *BDL KA* lines we found expression in the central cylinder close to the meristem in the primary and lateral roots, where the protein localized to nucleus of the expressing cells (Figure 4B). Due to the weaker signal of lines BDL and BDL KA#4 we were not able to get a clear image of the nucleus with these plants. The GFP signal reflected the amount of protein detected by western blot analysis (Figure 4B and C). As for protoplasts, the same signal distribution pattern was observed in roots for both wild-type and mutant BDLKA line #3 (Figure 4B), confirming that the SS→KA substitution did not affect the tissue and sub-cellular localization of BDL.

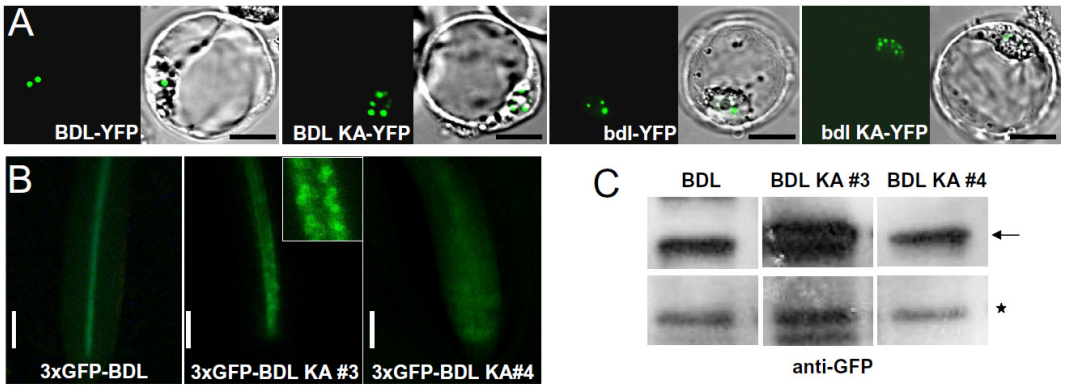

Figure 4: Mutation of the BDL PRSS motif does not affect its sub-cellular localization or tissue-specific expression. (A) Confocal laser scanning microscope images (YFP fluorescence and transmitted light) of Arabidopsis protoplasts transfected with *35S::YFP-BDL*, *35S:: YFP-BDL KA*, *35S::YFP-bdl* or *35S::YFP-bdl KA*. Scale bars represent 10 µm. (B) GFP epifluorescence images of one week old primary roots of the *BDL::3xGFP-BDL* line (Weijers *et al.*, 2006) and two independent *BDL::3xGFP-BDL KA* homozygous transgenic lines. The inset shows the nuclear localization of the GFP signal. Scale bars represent 40 µm (C) Western blot with anti-GFP antibodies of total protein extracts from 10-day-old seedlings of the *BDL::3xGFP-BDL* line (BDL) and two independent *BDL::3xGFP-BDL KA* lines (BDL KA #3 and #4). The arrow indicates the 3XGFP-BDL band and the star a background band crossreacting with the anti-GFP antibodies used as a loading control.

Phosphorylation controls both abundance and activity of the BDL/IAA12 repressor

Seeds of Arabidopsis wild type (Col), or of *BDL::3xGFP-BDL* or *BDL::3xGFP-BDL KA* homozygous T3 lines were germinated on vertical MA plates to which either nothing or 10^{-8} or 10^{-7} M IAA was added (Figure 5A), and the root length of 13 days-old seedlings was measured. Seedlings of the *BDL::3xGFP-BDL* line developed longer roots only on the control plates, indicating that the additional GFP-BDL proteins reduce the limiting effect of endogenous auxin on root elongation, presumably by repressing auxin-responsive gene expression. Interestingly, the roots of the *BDL::3xGFP-BDL KA* seedlings were longer in all treatments, and this increase in root length clearly correlated with the amount of the 3xGFP-BDL KA protein (Figure 5A). The expression level of BDL KA line #4 was comparable to that of BDL line and the reduced sensitivity of line #4 to the

10^{-8} M auxin treatment suggests that the KA mutation enhances the activity of the BDL protein resulting in roots less sensitive to auxin treatment.

In order to further assess the importance of phosphorylation of the PRSS motif on the repressor activity of the BDL protein we also introduced the SS→KA substitution in the stabilized *bdl*-1 mutant protein (Hamann *et al.*, 2002), which shows no or only residual interaction with the auxin receptor TIR1 due to a P → S substitution in domain II (Dharmasiri *et al.*, 2005b). We used a *BDL::3XGFP-bdl* line known to mimic the original *bodenlos* phenotype as a control (Weijers *et al.*, 2006), and used the corresponding construct to generate the *BDL::3xGFP-bdl KA* variant, which was subsequently transformed to Arabidopsis wild type. Transgenic lines homozygous for a single locus *BDL::3xGFP-bdl KA* T-DNA insertion did not show the rootless phenotype characteristic for the expression of the bdl protein, and seedlings and plants showed normal development (Figure 5B). The expression of the proteins was confirmed via observation of the GFP signal which was restricted to the central cylinder of the root vascular tissue (Figure 5B). As observed in the protoplast transfections both the bdl and bdl KA proteins are localized in the nuclei of the cells (Figure 4A and 5B), but curiously the tissue-specific expression in the root tip differed. Expression of the 3xGFP-bdl KA variant was restricted to the central cylinder, whereas the 3xGFP-bdl protein showed strong expression in the root tip including the columella and root quiescent center cells (Figure 5B). When the protein levels in total extracts from these seedlings were analyzed (Figure 5C), 3XGFPbdl KA was expressed much less abundantly in all three lines than 3xGFPbdl. In contrast, based on semi-quantative RT-PCR analysis the *3XGFPbdl KA* mRNA levels were much higher in all three independent lines than the level of *3XGFPbdl* mRNA in the corresponding line (Figure 5C), indicating that the reduced protein level is not due to lower transcription levels, but is caused by a reduced stability of the 3xGFP-bdl KA protein. Our data indicate that phosphorylation is essential for the regulation of the activity of BDL, controlling both its stability and repressor activity.

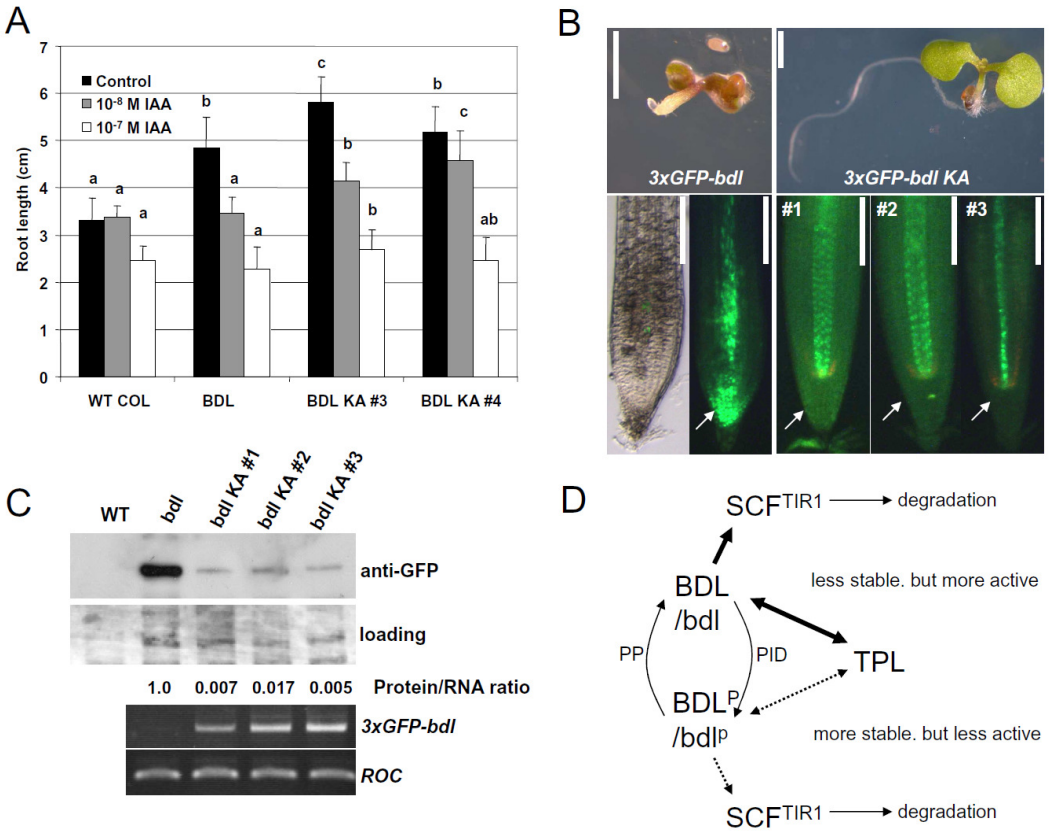

Figure 5: The PRSS motif controls the stability and activity of the BDL/IAA12 repressor.
(A) Average primary root length of 13 day-old seedlings of WT Columbia (WT), 3xGFP-BDL (WT BDL), 3xGFP-BDLKA lines #3 and #4 grown on MA medium (0) containing 10^{-8} and 10^{-7} M of IAA. Bars represent means and standard deviations from three repetitions. Different letters represent statistically significantly different groups after One-Way ANOVA followed by Student's T test ($p<0.05$). (B) One week old transgenic *BDL::3xGFP-bdl* and *BDL::3xGFP-bdl KA* homozygous plants grown on MA medium. The lower panel shows GFP epifluorescence images of one week old primary roots of *BDL::3xGFP-bdl* and three independent *BDL::3xGFP-bdl KA* homozygous lines. Scale bars represent 0.5 cm on top panels and 60 µm on bottom (C) Western blot with anti-GFP antibodies of total protein extracts from 5-day-old seedlings of the *35S::3xGFP-bdl* lines and three independent lines (#1, #2 and #3) for *35S::3xGFP-bdl KA*. The middle panel shows part of the SYPRO-Ruby stained blot as a loading control. The lower panel shows ethidium bromide-stained agarose gels from the RT-PCR on RNA extracted from the same plant samples as used for western analysis, detecting the *3xGFP-bdl* and *ROC* mRNAs. Numbers indicate relative density of protein/RNA signals between samples relative to the bdl lane. (D) Model for the role of phosphorylation in BDL stability and activity. Under high auxin concentrations, non-phosphorylated BDL/bdl is actively degraded by SCF^{TIR1} and might have higher repressor activity due to stronger interaction with TPL. Upon phosphorylation by PID, BDL/bdl would have lower affinity for SCF^{TIR1}

becoming more stable but less active due to weaker interaction with TPL. Tissue-specific kinases/phosphatases would be responsible for maintenance of BDL/bdl relative active levels.

DISCUSSION

Protein phosphorylation is one of the most common post-translational modifications regulating protein activity. Phosphorylation cascades involving multiple protein kinases are central to the classical signaling pathways, and many downstream targets are transcription factors through which the signaling pathway controls gene expression. Interestingly, a classical signaling cascade has not yet been identified for the plant hormone auxin (Zago *et al.*, 2008). Instead, the auxin receptors TIR1/AFB are F-box proteins in SCF E3 ubitquitin ligase complexes, and binding of auxin promotes recruitment of Aux/IAA transcriptional repressors by the TIR1/AFBs, which leads to activation of gene transcription through the ubiquitination and subsequent degradation of the repressors by the proteasome (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005a; Dharmasiri *et al.*, 2005b; Dos Santos Maraschin *et al.*, 2009).

Here we present evidence that phosphorylation controls the activity of the transcriptional repressor BODENLOS (BDL/IAA12), and that, surprisingly, this phosphorylation is dependent on PID, a serine-threonine kinase known to regulate trafficking of PIN auxin efflux carriers (Friml et al., 2004; Michniewicz et al., 2007). In *in vitro* reactions we found that PID was able to phosphorylate a PRSS motif located between conserved domain I and II in BDL/IAA12. This motif is also present in the closely related IAA13, but not in other Aux/IAA proteins. BDL/IAA12 and IAA13 have been described as functional paralogs with similar activities and expression patterns (Weijers *et al.*, 2005), which fits well with the concept that they share similar regulatory mechanisms.

Phosphorylation of Aux/IAA proteins has been reported before, and in this case evidence was provided that phosphorylation was dependent on phytochrome activity (Colon-Carmona *et al.*, 2000). Interestingly, in PsIAA4 phosphorylation was also mapped in the domain I and II containing N-terminal part, and although the phytochrome and PID pathway do not phosphorylate exactly the

same site (in view of the absence of the PRSS motif in PsIAA4), the modification might lead to similar changes in Aux/IAA activity. In the previous paper, however, no function was assigned to phytochrome dependent phosphorylation of Aux/IAA proteins.

PRSS phosphorylation generates a stabilized, less active pool of BDL/IAA12

Substitution of the two serines in the BDL PRSS motif to KA, the sequence most commonly found among different Aux/IAA proteins, abolished *in vitro* phosphorylation by PID, indicating that this is the site of phosphate attachment. Although we can not completely rule out that the PRSS motif is essential for the recognition by the kinase, the absence of a tight interaction between BDL and PID supports the hypothesis that BDL is phosphorylated at the PRSS motif.

We found that the PRSS to PRKA substitution does not affect the sub-cellular localization pattern of YFP-BDL or YFP-bdl in protoplasts, or the cell type-specific localization of 3xGFP-BDL or 3xGFP-bdl *in planta*, indicating that phosphorylation does not play a role in the nuclear trafficking of the protein. Instead, the lack of the phosphorylation site slightly reduced the stability of BDL in protoplasts upon auxin treatment, and this is reflected in a mild reduction of transcriptional repression activity using the *DR5::GUS* reporter construct. The close proximity of the phosphorylation site to the degron in the conserved domain II indicates that it might regulate TIR1 recognition based on the Aux/IAA-auxin-TIR1 interaction structure (Tan *et al.*, 2007). This hypothesis is supported by the effect of mimicking phosphorylation by replacement of the two serines by aspartic acid residues, which renders the protein more stable. The function of many of the *Aux/IAA* genes has been characterized via gain-of-function mutations that cause specific substitutions in the conserved GWPPV motif of domain II, resulting in reduced binding to TIR1, and thus leading to stabilized mutant Aux/IAA proteins (Tian *et al.*, 2002; Dharmasiri *et al.*, 2005b; Uehara *et al.*, 2008). Interestingly, semi-dominant alleles of *SHY2/IAA3, AXR3/IAA17* and *CRANE/IAA18* genes result from the substitution of the glycine in this motif by the phospho-mimic glutamate (Tian and Reed, 1999;

Uehara *et al.*, 2008; Ploense *et al.*, 2009), supporting our hypothesis that phosphorylation close to the GWPPV motif leads to reduced TIR1 binding and thus to enhanced stability of Aux/IAA proteins.

In contrast, *in planta* the BDL KA protein behaved as a mildly stronger repressor of the auxin response. *BDL::3xGFP-BDL KA* plants have significantly longer roots than *BDL::3xGFP-BDL* plants, which again have longer roots than wild type plants. More importantly, *BDL::3xGFP-BDL KA* roots show a reduced response to auxin treatment, correlating with the expression level of the 3xGFP-BDL KA protein, and indicating that BDL KA is a stronger repressor than the wild type BDL protein. We introduced the KA substitution in the gain-of-function bdl mutant protein, and observed an interesting effect. When the *BDL::3xGFP-bdl* construct is introduced into wild type plants, the semi-dominant *bdl* mutant phenotypes are reproduced (Weijers *et al.*, 2006). However, plants transformed with the *BDL::3xGFP-bdl KA* construct developed normal roots, despite the fact that the 3x*GFP-bdl KA* mRNA was expressed at high levels. Analysis of the protein levels showed that the stability of the 3xGFP-BDL KA protein was much reduced compared to the 3xGFP-bdl protein, indicating that phosphorylation at the PRSS motif is necessary to sustain protein stability conferred by the P to S substitution in bdl.

Taken together our data lead to the model that phosphorylation of BDL/IAA12 keeps this repressor in a stabilized but less active form, and that in its unphosphorylated state BDL/IAA12 is most active as repressor, but also more easily recruited for degradation by $SCF^{TIR1/AFB}$ E3 ligases (Figure 4D). In Arabidopsis, a similar mechanism has been described for the bZIP transcription factor HY5. CKII-dependent phosphorylation in the COP1-interacting domain of HY5 reduces binding to COP1. Unphosphorylated HY5 is more active and less stable, allowing fast activation of the light responses by a dynamic balance between phosphorylation and proteasomal degradation (Hardtke *et al.*, 2000).

In this way, phosphorylation provides an additional layer of regulation that dampens the effect of sinusoid levels of BDL repressor caused by its alternating auxin-induced degradation and *de novo* synthesis. This regulation seems specific for BDL/IAA12 and IAA13, as only these Aux/IAA proteins have the

PRSS motif. The observation that the bdl KA mutant did not show reduced repressive activity in the *DR5:GUS* assays in protoplasts indicates that the overexpression of bdl KA might overcome the regulatory step involving phosphorylation or that protoplasts might have rate-limiting expression of accessory proteins that recognize the overexpressed bdlKA protein, as described for TIR1 when BDL was overexpressed in protoplasts (Dos Santos Maraschin *et al.*, 2009).

The observed enhanced repressor activity of BDL KA might be explained by the fact that phosphorylation of the PRSS motif possibly interferes with the binding of TOPLESS (TPL) to BDL domain I. TPL is a transcriptional co-repressor involved in the repression of auxin response genes through its physical interaction with the EAR motif present in conserved domain I of Aux/IAA proteins. BDL was shown to function as a bridge between TPL and MP/ARF5 to repress ARF function (Szemenyei *et al.*, 2008). The loss of function *tpl-1* mutant is able to rescue the *bdl-1* rootless phenotype indicating that the interaction with TPL is important for a strong repressive action by BDL.

Is BDL a direct phosphorylation target of PID?

The experimental evidence that BDL is a direct phosphorylation target of PID is based on the synergistic effect of the *pid* and *bdl* mutations on embryo development, on the *in vitro* phosphorylation assays and on the observation that *PID* overexpression overcomes the repressive effect of *bdl* on parthenocarpic fruit development in the *fwf bdl* mutant background (Adam Vivian-Smith, unpublished observations).

In protoplasts, however, co-transfection with 35S*::PID* does not seem to affect BDL stability or activity, suggesting that phosphorylation might not occur in protoplasts, or that PID indirectly promotes the activity of another kinase to phosphorylate BDL, and that this kinase is rate-limiting in protoplasts. In fact, the spatio-temporal expression patterns of *BDL* and *PID in planta* only partially overlap, and PID is mainly plasma membrane associated and BDL nuclear. We have observed that PID can become nuclear upon binding to its interacting scaffold protein BT1 (Chapter 5 and Robert et al., in prep). Another option might

be that one of the other AGC3 kinases is involved in the phosphorylation. WAG1 and AGC3-4 are both membrane-associated and nuclear localized, and indeed AGC3-4 seems to localize to specific nuclear structures (Galvan and Offringa, unpublished).

PID is able to phosphorylate *in vitro* distinct and unrelated targets such as PINs (Michniewicz *et al.*, 2007), Aux/IAAs and COP9 subunit CSN7 (This thesis Chapter 5). PID seems to play a central role in tuning the downstream effects of polar auxin transport on elevated auxin levels. PID, like BDL, is encoded by an auxin-responsive gene and the control of Aux/IAA levels could involve, inactivation via PID phosphorylation to fine tune the pool of active Aux/IAAs in the cell. The limited overlap of *PID* and *BDL* expression patterns in adult plants indicates that, if such a process occurs, it is probably restrained to specific developmental stages such as root meristem initiation and embryo patterning where both genes are active. First identified as a key regulator of polar auxin transport and trafficking of PIN proteins, the new targets identified for the PID kinase give new biochemical insights into a complex developmental regulatory network.

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Chapter 5

COP9 signalosome assocation 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 autocatalytic 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 *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 *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 *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 *et al.*, 2003). . This led to the isolation of a single pACT2-CSN8/COP9 clone containing the complete *CSN8* (AT4g14110) open reading frame. Interaction in the *Saccharomyces cerevisiae* strain PJ69-4A (James *et al.*, 1996) was performed in using the same system with pACT2-CSN8 directly tested at 20^{o}C with bait plasmids pAS-PID or pAS-PBP2. pAS2-PBP2 was obtained by cloning the *PBP2* cDNA as *Pst*I/*Sal*I-blunted fragment from pSDM6014 into pAS2 digested with *Pst*I/*Xma*I-blunted with Klenow DNA polymerase.

The *CSN7* (AT1g02090) cDNA was amplified by PCR using primers 5'-ACGCAAGTCGACAAGATGGATATCGAGCAGAAGCAAGC-3' and 5'-GATAGATCTAACAGAGGATCTTATACAAGTTG-3', and subsequently digested with *Bgl*II to be ligated into the pBluescript II SK+ plasmid cut with *Eco*RV/*Bgl*II. From this plasmid pBS-CSN7 a *Bam*HI/*Sal*I fragment was ligated into pET16B

(Novagen) digested with *Xho*I/*Bam*HI, resulting in *pHis-CSN7*. The *pHis-CSN8* construct was *created by cloning a SalI fragment from pACT-CSN8 into* pET16H *cut with XhoI/SmaI. 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'- GCCTCGGATTTGCACCTAGAAGCTCGCTTCACGAGC-3' for the ST>AT mutation, csn7ST-SAF 5'-GCTCGTGAAGCGAGCTAGGACTTGCAAATCCGAGGC-3' csn7ST-SAR 5'-GCCTCGGATTTGCAAGTCCTAGCTCGCTTCACGAGC-3' for the ST>SA mutation, csn7ST-DDF 5'-GCTCGTGAAGCGAGCTGATGATTGCAAATCCGAGGC-3' csn7 ST-DD R 5'-GCCTCGGATTTGCAATCATCAGCTCGCTTCACGAGC-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'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGTTACAGGATGCCTCCTC-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 sense orientation. The cDNA of *CSN8* was amplified from pHis-CSN8 with primers attB1 CSN8F 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGATCTTTCGCCTGTT-3' and attB2 CSN8R 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCATGTTCAAGGTGGAACAC-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'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGAAGAGATTTCGACG-3' and attB2 COP1R 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCAGCGAGTACCAGAAC-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 His_6-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 *Sma*I. 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, unpublised) 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 37ºC to OD_{600} 0.8. The cultures were then induced for 4 hours with 1 mM IPTG at 30ºC, after which cells were harvested by centrifugation (10 min at 2.000 g in a tabletop centrifuge) and frozen overnight at -20ºC. 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 $CaCl_2$, 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 4ºC. 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 Laemmli protein loading buffer to the resin samples, followed by denaturation by 5 min incubation at 95ºC. Proteins were subsequently separated on a 12% polyacrylamide gel prior to transfer to a Immobilon^{TM}-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 37ºC to OD_{600} 0.8 in 50 ml LC supplemented with antibiotics. The cultures were

then induced for 4 hr with 1 mM IPTG at 30ºC, after which cells were harvested by centrifugation 10 min at 4.000 g and frozen at -20ºC. 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 $CaCl_2$, 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 4ºC. 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 -80ºC.

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-HCl 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-HCl 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-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM $CaCl_2$ 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-^{32}P] 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-HCl pH 6.8, 10% SDS, 50% glycerol, 7.5 M β-mercaptoethenol 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% $Na_2H_2P_4O7$), 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 9^{mer} biotinylated peptides (Pepscan) in a phosphorylation reaction as described above. Reaction processing, spotting and washing of the SAM^2 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'-CACTGACCATTTGCTCTCTCTTGC-3' with *DdeI*. The *cop15-1/fus5-1* mutation in exon II of the *FUS5* gene creates an early stop codon and an extra *DdeI* 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, 10^6 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* or*, 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 Na_2HPO_4/NaH_2PO_4, 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 *in vitro*. Indeed, CSN7 was efficiently phosphorylated by PID and in our *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 *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 *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). Pep-Chip 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.

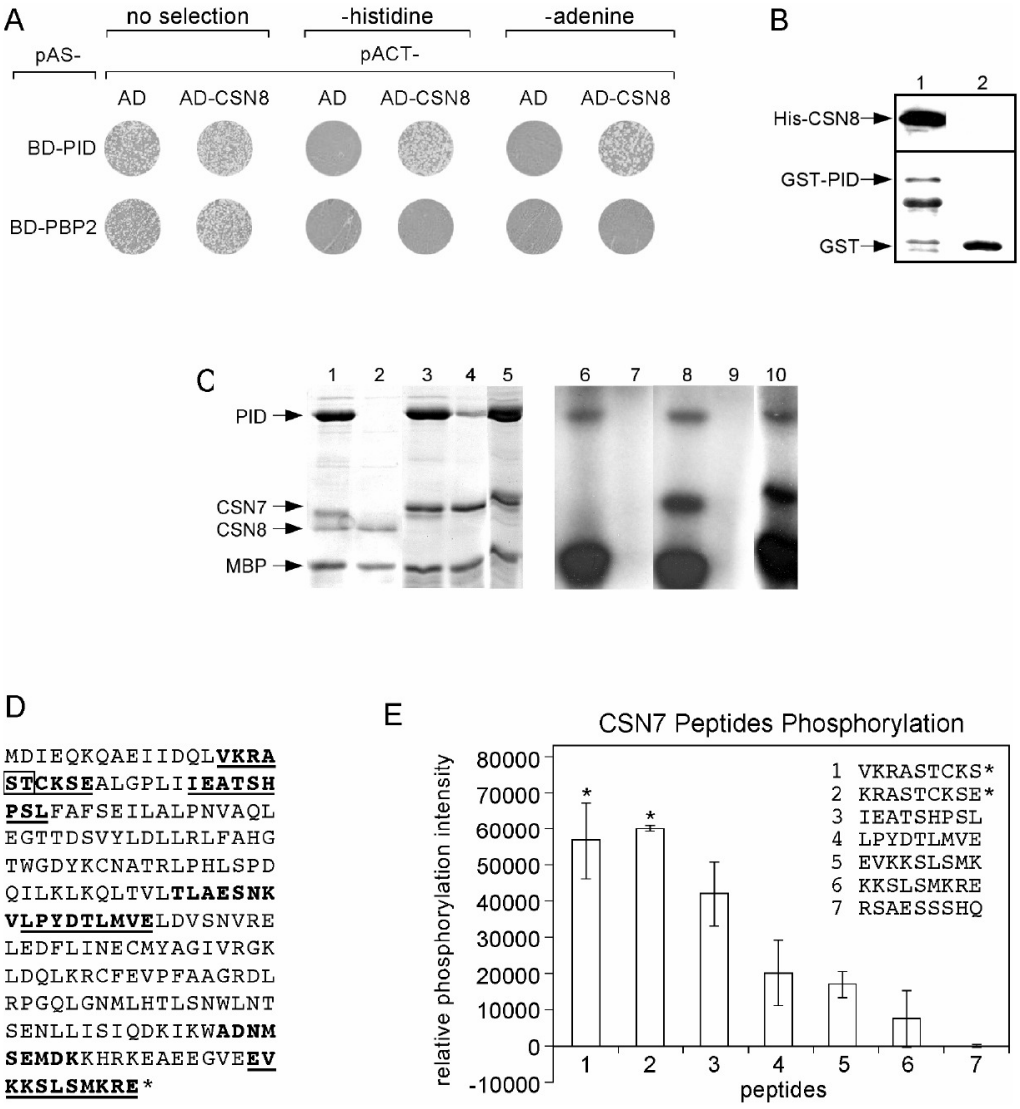

Figure 1. PID interacts with CSN8/COP9 and phosphorylates CSN7/COP15. (A) Yeast two-hybrid assay with PID and PBP2 fused to the GAL4 binding domain (BD; pAS2.1 vector), and CSN8/COP9 fused to the GAL4 activation domain (AD) or the AD alone (pACT2 vector) in non-selective medium or in medium lacking either histidine or adenine. (B) *In vitro* pull-down of His-tagged 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 *pHis-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.

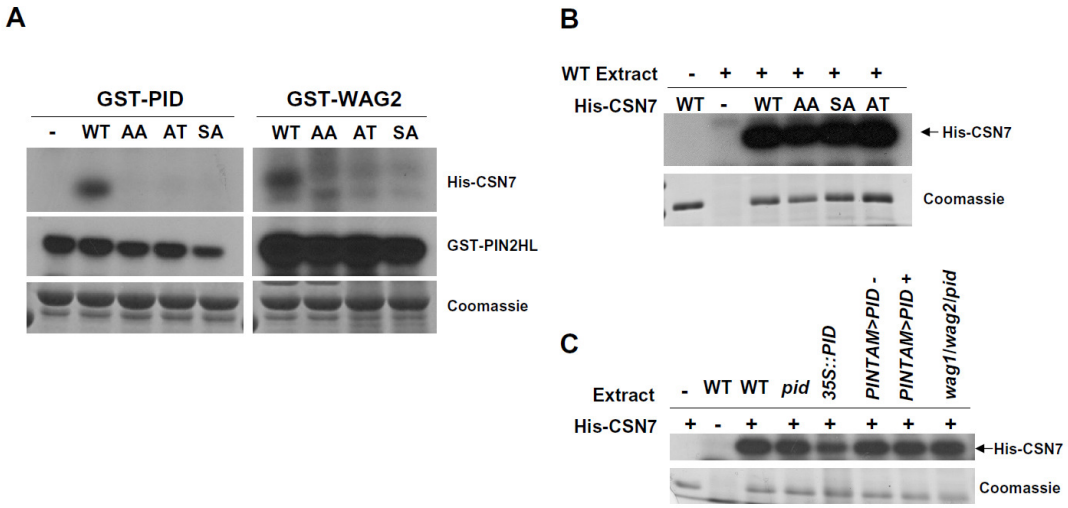

Figure 2: The PID and WAG kinases phosphorylate CSN7 *in vitro*, but do not contribute to the overall CSN7 phosphorylating activity in total protein extracts from Arabidopsis. (A) Autoradiograph (upper two panels) and coomassie-stained gel (PIN2 band, lower panel) of *in vitro* phosphorylation assays containing *E. coli* purified GST-PIN2HL (positive control, all lanes), and His-CSN7-WT, -AA, -AT or -SA incubated with GST-PID or with GST-WAG2. (B) Autoradiograph (upper panel) and coomassie-stained gel (lower panel) of an *in vitro* phosphorylation assay in which His-CSN7-WT, -AA, AT or –SA were incubated with 2 µg of total protein extract from Arabidopsis seedlings. (C) Autoradiograph (upper panel) and coomassie-stained gel (lower panel) of an *in vitro* phosphorylation assay in which 2 µg WT His-CSN7 was incubated with 2 µg of total protein extract of Arabidopsis wild type seedlings (WT), or seedlings from the *pid-14* loss-of function mutant, the *35S::PID* line #21(Benjamins *et al.*, 2001) , the tamoxifen-inducible *PID* line (Friml *et al.*, 2004) treated for 8h with DMSO (*PINTAM>PID-*), or with 2 µM tamoxifen (*PINTAM>PID* +), or the *pid/wag1/wag2* triple loss-of-function mutant (Galvan-Ampudia,C. unpublished).

PINOID does not control the CSN de-rubylation 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-rubylation 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 *pINTAM>PID* showed a wild type CUL1 : CUL1-RUB1 ratio, demonstrating that PID-dependent phosphorylation of CSN7 does not control CSN de-rubylation activity.

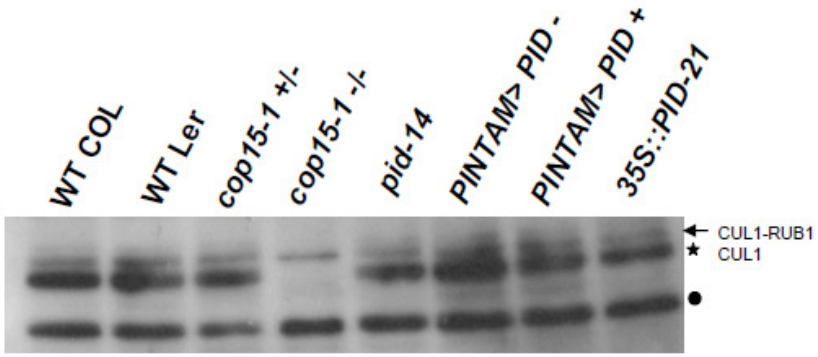

Figure 3: PINOID does not control CSN de-rubylation activity. Twenty µg of total protein extracts from one week old seedlings were analyzed on a western blot probed with anti-AtCUL1 antibodies. Extracts were from wild type Columbia (WT Col) and Landsberg erecta (WT Ler), *cop15-1 (*in Ler background), and in the Columbia background *pid-14, pINTAM>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

	TOTAL	NON-*cop*	*cop*	% *cop*
Cop15-1/cop15-1	493	452	*41*	8.32
CSN7 WT#6	486	485	*1*	0.21*
CSN7 WT#8	559	553	*6*	1.07*
CSN7 AT#1	521	505	*16*	3.07*
CSN7 SA#7	500	500	*0*	0.00*
CSN7 SA#8	499	480	*19*	3.81*
CSN7 DD#1	492	472	*20*	4.07*
CSN7 DD#2	564	561	*3*	0.53*
				*P<0.001

*Significantly different from Cop15-1/cop15-1 based on $\chi 2$ 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

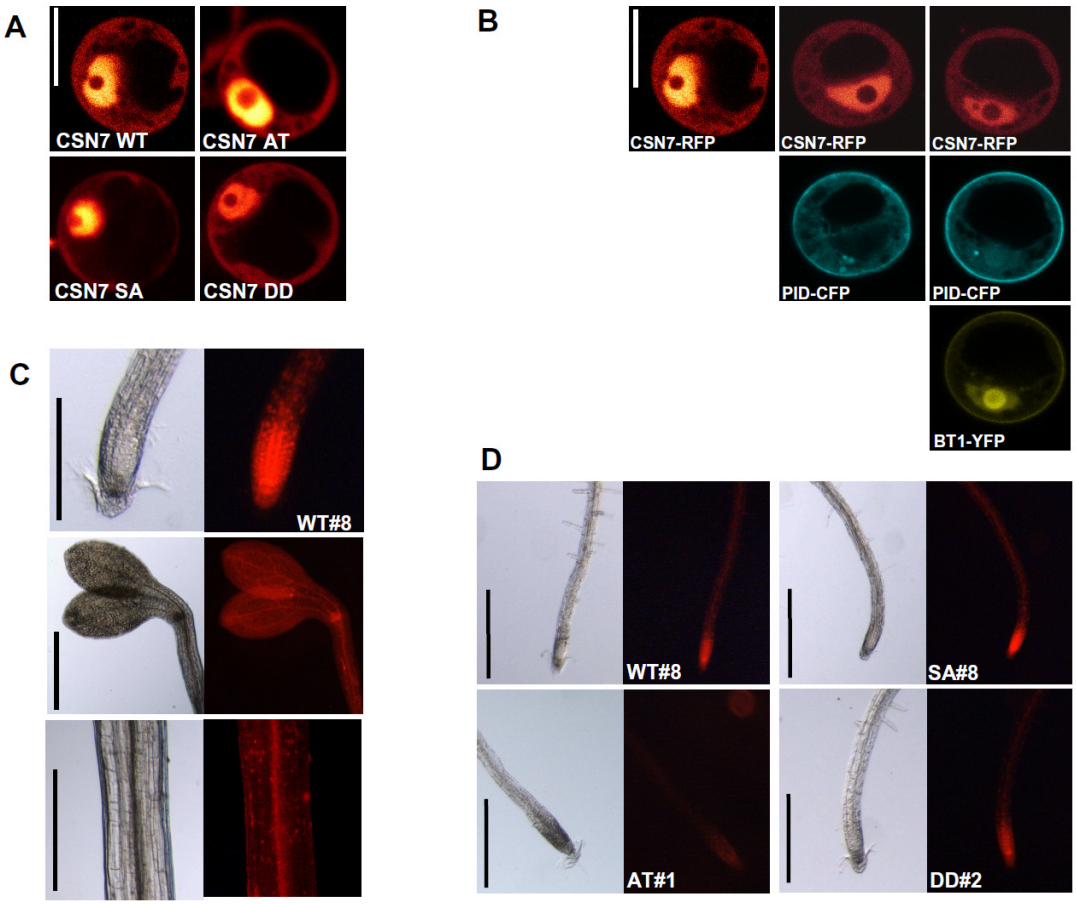

Figure 4: PID-dependent phosphorylation does not affect CSN7 subcellular localization or expression.

(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-

sensitive subcellular distribution. These results provide the first evidence for the interaction between PID and CSN8 *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.

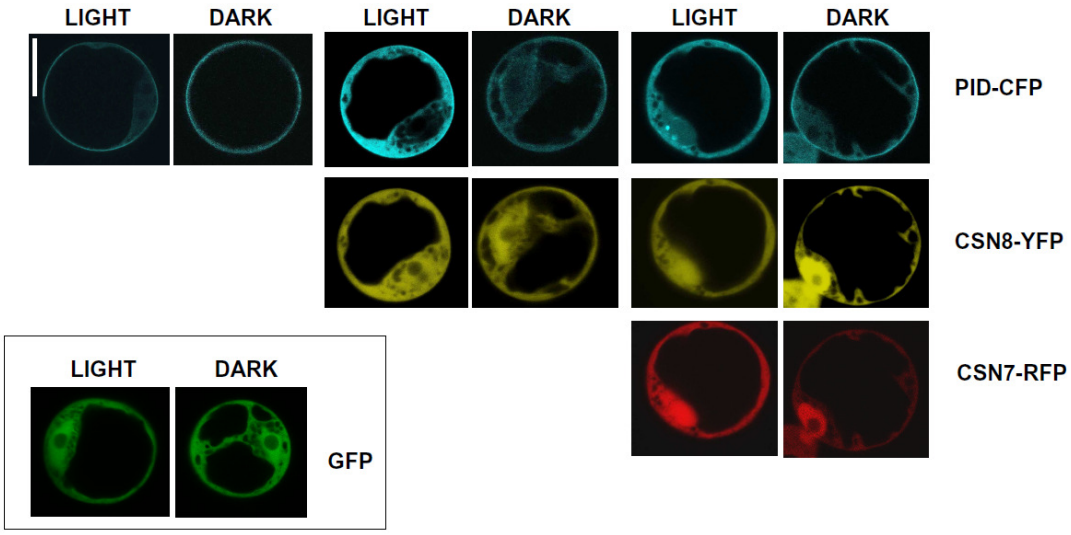

Figure 5: CSN8 sequesters PID from the plasma membrane to the cytoplasm and the nucleus. Confocal laser scanning microscopy images of representative Arabidopsis protoplasts transformed with *35S::PID-CFP* alone (left column) or co-transfected with *35S::CSN8-YFP* (middle column) or *35S::CSN8-YFP* and *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 ubiqutination 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.

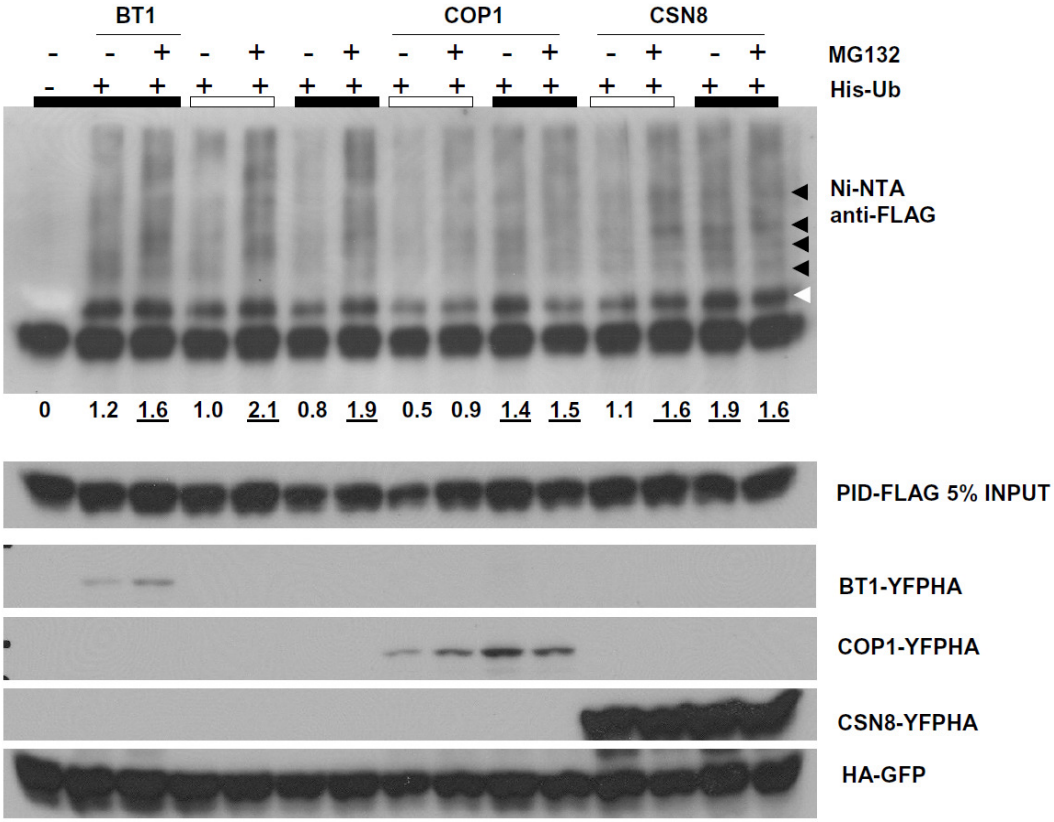

Figure 6: CSN association enhances PID ubiquitination in the dark. Western blot analysis of Ni-NTA purified samples (upper panel) or total extracts (INPUT and lower panels) of Arabidopsis protoplasts transfected with *35S::PID-FLAG* and *35S:HA-GFP*. His-Ub indicates co-transfection with the *35S:5xHis-Ub* 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 turn over 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.

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SUMMARY

Auxin biology is among the oldest fields of experimental plant research, and therefore auxin is one of the most extensively studied plant hormones. The plant hormone auxin (indole-3-acetic acid or IAA) regulates plant development by inducing rapid cellular responses and changes in gene expression. Its regulatory effects on cell division, growth and differentiation are dependent on its transport-driven asymmetric distribution. At the cellular level auxin concentrations are translated into rapid cellular responses and changes in gene expression. Recently, several molecular components involved in auxin-responsive gene expression have been identified. This involves the proteasomal degradation of Aux/IAA transcriptional repressors, thereby allowing auxin response factors (ARFs) to activate the transcription of auxin-responsive genes. Most Aux/IAA proteins are short-lived and degradation of Aux/IAA proteins is essential for auxin signaling. Their half-lives and abundance are dramatically reduced by auxin. Auxin enhances binding of the conserved domain II of Aux/IAA proteins to the receptor TIR1, which is an F-box protein that is part of the E3 ubiquitin ligase complex SCF^{TIR1}. Binding of Aux/IAA proteins to SCF^{TIR1} leads to degradation via the 26S proteasome

Auxin is transported from cell to cell in a polar manner by the asymmetrically distributed PIN auxin efflux carriers. This polar auxin transport (PAT) generates dynamic auxin maxima and gradients. The protein serine/threonine kinase PINOID (PID) is a signaling component in the control of PAT, as it determines the apico-basal polarity of several members of the PIN family of auxin efflux carriers. The PID kinase has been shown to directly phosphorylate the hydrophilic loop of PIN proteins, and to act antagonistically with the PP2A protein phosphatases on the phosphorylation status of PIN proteins. The current model defines that PID regulates polar auxin transport by controlling PIN localization, and thereby determines the direction of auxin flow. PIN proteins are the only functionally characterized PID phosphorylation targets identified to date. In a search for additional targets of PID, yeast-two-hybrid screens identified four interacting partners: the calcium binding proteins TCH3 and PBP1/PBP1H, the BTB/POZ domain protein BT1/PBP2, and subunit 8 of

the COP9 signalosome (CSN8/COP9). None of the PBPs appeared to be phosphorylated by PID, and instead they were found to regulate the activity of this kinase. Moreover, the binding of PID to CSN8 suggested a role for this kinase in regulating protein ubiquitination. The COP9 signalosome (CSN) is a protein complex found in eukaryotic cells that regulates many cellular processes linked to targeted protein degradation. The CSN interacts with, and is essential for the activity of E3 ubiquitin ligases. In Arabidopsis, *cop* mutants display constitutive photomorphogenesis and expression of light-responsive genes in the dark. The interaction network of the CSN is broad and complex, influencing almost every aspect of plant development, among which also auxin response and transport.

The research described in this thesis was directed at unraveling the role of protein ubiquitination in auxin response and transport. As described above, it was well established that the binding of auxin to TIR1 enhances the affinity of this F-box protein for Aux/IAA proteins, and thereby leads to enhanced turn over of these repressor proteins by the 26S proteasome. However, evidence for SCF^{TIR1}-mediated poly-ubiquitination of Aux/IAA proteins was lacking. In **Chapter 2** an *Arabidopsis* cell suspension-based protoplast system was used to find evidence for SCF^{TIR1}-mediated ubiquitination of the Aux/IAA proteins SHY2/IAA3 and BDL/IAA12. Each of these proteins showed a distinct abundance and repressor activity when expressed in this cell system. Moreover, the amount of endogenous TIR1 protein appeared to be rate-limiting for a proper auxin response measured by the co-transfected *DR5::GUS* reporter construct. Co-transfection with *35S::TIR1* led to auxin-dependent degradation, and excess of *35S::TIR1* even led to degradation of Aux/IAAs in the absence of auxin treatment. Expression of the mutant tir1-1 protein or the related F-box protein COI1, which is involved in jasmonate signaling, had no effect on Aux/IAA degradation. The results show that SHY2/IAA3 and BDL/IAA12 are poly-ubiquitinated and degraded in response to increased auxin or TIR1 levels. In conclusion, these data provide experimental support for the model that SCF^{TIR1}-dependent poly-ubiquitination of Aux/IAA proteins marks these proteins for degradation by the 26S proteasome, leading to activation of auxin-

responsive gene expression. It is likely that the differences observed between the two AUX/IAA proteins studied might be linked to differential affinity of these proteins for the TIR1 receptor. For the BDL protein it is hypothesized that affinity might be regulated by phosphorylation (Chapter 4).

The results in Chapter 2 are in line with the paradigm that targeted protein degradation occurs through the specific recognition by the proteasome of proteins that have been marked with ubiquitin chains. In **Chapter 3,** evidence is provided that this paradigm does not hold for all plant proteins. The Arabidopsis calcium binding protein PINOID BINDING PROTEIN 1 (PBP1) was originally identified as an interactor of the PINOID (PID) kinase, which was shown to control PID activity in vitro and its subcellular localization in vivo. Using an Arabidopsis cell suspension protoplast system it was found that PBP1 is a highly unstable, poly-ubiquitinated protein that can be stabilized by inhibition of proteasomal degradation. Co-expression of PID did not affect PBP1 poly-ubiquitination. Surprisingly, substitution of all the lysines (K) in PBP1 for arginines (R) blocked poly-ubiquitination, but did not affect its proteasomal degradation. The mutant (K→R) protein retained all tested wild type functions, including its interaction with PID and its subcellular localization. Translational fusions of the lysine-less PBP1 with YELLOW FLUORESCENT PROTEIN (YFP) were ubiquitinated, which indicates that the PBP1 sequence contains a cis-acting motif that is recognized as an ubiquitination signal but not as a degron, since the YFP fusions were not unstable. PBP1 is the first example of a plant protein that is degraded by the proteasome in an ubiquitination-independent pathway. Although the functions of PBP1 ubiquitination remain to be elucidated, our results show that its proteasomal degradation is not dependent on ubiquitination.

Chapter 4 describes the identification of the labile auxin response repressor BODENLOS (BDL/IAA12) as *in vitro* phosphorylation target of PID. The observation that PID-mediated phosphorylation possibly occurs in the PRSS motif close to the SCF^{TIR1}-interacting domain II of BDL/IAA12 suggests that this event plays a role in the stability of this repressor protein. Blockage of the identified phosphorylation site has minor negative effects on the repressor

activity of the BDL protein in protoplasts and *in planta*, but plants carrying a phosphorylation-insensitive version of the gain-of-function bdl protein fail to reproduce the *bodenlos* phenotype. Additionally, the phosphorylation-insensitive bdl protein is much less stable and has a more restricted tissue distribution in the root tip. This indicates that the control of BDL via phosphorylation might be an important mechanism regulating Arabidopsis root development. Although the mechanisms and roles of PID-mediated regulation of BDL require further elucidation, our data suggest that the PID protein kinase regulates both auxin transport and auxin-responsive gene expression.

Chapter 5 describes a further study on the interaction of PID with the CSN subunit CSN8/COP9. *In vitro* phosphorylation assays showed that not CSN8, but the linked subunit CSN7/COP15 is phosphorylated by PID *in vitro*. PID-dependent phosphorylation of CSN7 appeared not to be essential for CSN functions *in planta,* at least not under the growth conditions tested. In protoplasts CSN8 sequesters PID to the cytoplasm and nucleus and enhances the PID ubiquitination that was already described in Chapter 3. The association of PID with the CSN may be related to the control of PID turn over, however another possibility could be that PID regulates the interaction between its phosphorylation targets BODENLOS/IAA12 and PIN proteins and their corresponding E3 ubiquitin 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 control of ubiquitination.

In conclusion, our results show that poly-ubiquitination of proteins plays a central role in the action of the plant hormone auxin, and that the PID protein kinase provides an unexpected link in the communication between auxin transport and auxin response.

SAMENVATTING

De studie naar het werkingsmechanisme van het plantengroeihormoon auxine heeft een relatief lange geschiedenis, en mede daardoor is auxine één van de best bestudeerde plantenhormonen. Auxine, ofwel indol-3-azijnzuur, reguleert de groei en ontwikkeling van planten via transportgegenereerde dynamische concentratiemaxima en –gradiënten. De auxineconcentraties worden vertaald in snelle cellulaire reponsen en veranderingen in genexpressie, processen die op hun beurt weer celdeling, -strekking en –differentiatie controleren. Recentelijk zijn een aantal moleculaire componenten betrokken bij de signaaltransductie van auxine in kaart gebracht. Auxine-responsieve genexpressie wordt geïnitieerd door de afbraak van Aux/IAA repressoreiwitten, zodat Auxine Respons Factoren (ARFs) de transcriptie van auxine-responsieve genen kunnen activeren. De meeste Aux/IAA eiwitten zijn instabiel, en hun levensduur wordt dramatisch gereduceerd in de aanwezigheid van auxine. Auxine bevordert namelijk de binding van het geconserveerde domein II van Aux/IAA eiwitten aan de auxinereceptor TIR1, een F-box eiwit dat onderdeel uitmaakt van het E3 ubiquitine ligase complex SCF^{TIR1}. Binding van de Aux/IAA eiwitten aan SCF^{TIR1} leidt tot hun afbraak door het 26S proteasoom.

Auxine wordt gerichte van cel naar cel getransporteerd door de asymmetrisch op het celmembraan gelokaliseerde PIN auxine transporters, en dit resulteert in auxine-maxima en -gradiënten. Het proteïne serine/threonine kinase PINOID controleert de richting van het polair auxine transport (PAT) door de apico-basale subcellulaire lokalisatie van PIN familieleden te bepalen. Recentelijk is aangetoond dat PID de centrale hydrofiele lus van PIN eiwitten fosforyleert, en dat het antagonistisch met de PP2A fosfatases de fosforylatiestatus van PIN eiwitten bepaalt. Tot nu toe zijn PIN eiwitten de enige gevalideerde fosforylatietargets van het PID kinase. In een eerdere zoektocht naar nieuwe fosforylatiestargets voor PID via een twee-hybride screen in gist zijn een viertal PID bindende eiwitten (PBPs) geïdentificeerd: de calcium-bindende eiwitten TCH3 en PBP1, het BTB/POZ domein eiwit BT1/PBP2 en subunit 8 van het COP9 signalosoom (CSN8/COP9). Geen van de PBPs bleek door PID gefosforyleerd te worden, echter zij bleken de activiteit van het kinase te

reguleren. De binding van PID met CSN8 suggereerde daarnaast een rol van het kinase in de regulering van eiwitubiquitinering. Het CSN eiwitcomplex reguleert namelijk processen in eukaryote cellen die gepaard gaan met gerichte afbraak van eiwitten. Het CSN interacteert met, en is essentieel voor de activiteit van E3 ubiquitineligases. Het CSN is initieel ontdekt via Arabidopsis *cop* mutanten die constitutieve fotomorfogenese en expressie van licht-gereguleerde genen in het donker vertonen. Het netwerk van interacties van het CSN is echter breed en complex, en het CSN speelt een rol in bijna elk aspect van plantenontwikkeling, waaronder ook auxine respons en transport.

Het in dit proefschrift beschreven onderzoek richtte zich op de ontrafeling van de rol van eiwitubiquitinering in auxine respons en transport. Zoals hierboven beschreven verhoogt binding van auxine aan TIR1 de affiniteit van dit F-box eiwit voor Aux/IAA eiwitten, wat leidt tot hun versnelde afbraak door het 26S proteasoom. Bewijs voor de veronderstelde ubiquitinering van Aux/IAA eiwitten door het SCF^{TIR1} E3 ubiquitineligase ontbrak echter nog. In **Hoofdstuk 2** is gebruik gemaakt van uit Arabidopsis celsuspensies geïsoleerde protoplasten om bewijs te vinden voor SCF^{TIR1}-gemedieerde ubiquitinering van de Aux/IAA eiwitten SHORT HYPOCOTYL 2 (SHY2/IAA3) en BODENLOS (BDL/IAA12). Deze twee eiwitten vertoonde een verschillende abundantie en repressoractiviteit wanneer ze in dit celsysteem tot expressie werden gebracht. De hoeveelheid endogeen TIR1 eiwit bleek limiterend voor een juiste auxine respons, zoals gemeten met het gecotransfecteerde *DR5::GUS* reporterconstruct. Cotransfectie met *35S::TIR1* leidde tot auxine-afhankelijke afbraak van Aux/IAA eiwitten, en een overmaat van het *35S::TIR1* construct zelfs tot Aux/IAA afbraak zonder auxine behandeling. Expressie van het mutante tir1-1 eiwit of het gerelateerde F-box eiwit COI1, dat betrokken is bij jasmonzuursignaaltransductie, had geen effect op de Aux/IAA afbraak. De resultaten laten zien dat SHY2/IAA3 en BDL/IAA12 gepoly-ubiquitineerd en afgebroken worden in respons op verhoogde auxine en TIR1 niveaus. Deze resultaten leveren experimenteel bewijs voor het model dat SCF^{TIR1}-afhankelijke poly-ubiquitinering van Aux/IAA eiwitten deze eiwitten markeert voor afbraak door het 26S proteasoom, leidend tot auxine-responsieve

genexpressie. Het is waarschijnlijk dat de geobserveerde verschillen tussen de twee geteste Aux/IAAs te herleiden zijn tot hun differentiële affiniteit voor de TIR1 receptor. Resultaten beschreven in Hoofdstuk 4 van dit proefschrift suggereren dat de affiniteit van BDL voor TIR1 wordt gereguleerd door fosforylering.

De resultaten in Hoofdstuk 2 passen in het algemeen aanvaarde model dat gerichte eiwitafbraak gepaard gaat met specifieke herkenning door het proteasoom van met poly-ubiquitineketens gelabelde eiwitten. In **Hoofdstuk 3** wordt echter aangetoond dat dit model niet voor alle planteneiwitten opgaat. In de zoektocht naar ubiquitinering van Aux/IAA eiwiten werd het Arabidopsis calcium-bindend eiwit PBP1, eerder geïdentificeerd als PID bindend eiwit, als controle in de protoplasttransfecties meegenomen. Daarbij bleek PBP1 een zeer instabiel gepoly-ubiquitineerd eiwit te zijn, dat in aanwezigheid van de proteasoominhibitor MG132 gestabiliseerd werd. Co-expressie van PID beïnvloedde de poly-ubiquitinering van PBP1 niet, echter dit experiment liet verassend genoeg zien dat PID zelf geubiquitineerd werd. Substitutie van alle lysines (K) in PBP1 voor arginines (R) voorkwam wel de ubiquitinering, maar beïnvloedde niet de proteasomale afbraak van PBP1. Het mutante (K→R) eiwit behield alle geteste wildtype functies, waaronder de interactie met PID, en de subcellulaire lokalisatie. Translationele fusies van zowel het wildtype als het lysinevrije PBP1 met het GEEL FLUORESCERENDE EIWIT (YFP) werden geubiquitineerd, wat aangeeft dat de PBP1 sequentie een *in cis* werkend ubiquitineringsignaal bevat. Het feit dat de YFP fusies relatief stabiel waren suggereert echter dat PBP1 geen degradatiesignaal (degron) bevat. PBP1 is het eerste voorbeeld van een planteneiwit dat onafhankelijk van poly-ubiquitinering door het 26S proteasoom afgebroken wordt. Hoewel de functie van PBP1 ubiquitinering nog onduidelijk is, laten onze resultaten zien dat niet alleen in dieren, maar ook in planten gerichte eiwitafbraak door het proteasoom niet noodzakelijkerwijs afhankelijk is van het labelen van targeteiwitten met poly-ubiquitineketens.

Hoofdstuk 4 beschrijft de identificatie van de labiele auxine respons repressor BDL/IAA12 als *in vitro* fosforylatietarget van PID. De observatie dat fosforylatie

door PID optreedt in het PRSS motief dat zich dicht bij het SCF^{TIR1}-interacterende domein II bevindt, suggereert dat fosforylering mogelijk de stabiliteit van BDL/IAA12 beïnvloedt. Het verwijderen van de fosforyleringsplaats door serine (S) naar alanine (A) substituties had een gering effect op de repressoractiviteit van het BDL eiwit in protoplasten of *in planta*. Echter, planten die een fosforyleringsongevoelige versie van het dominante bdl eiwit tot expressie brachten vertoonden niet de typische bodenlos fenotypes. Het fosforyleringsongevoelige bdl (S→A) eiwit is veel instabieler en laat een sterk beperkte expressie in de wortelpunt zien. Dit suggereert dat de controle op BDL via fosforylering een belangrijk mechanisme is om Arabidopsis wortelontwikkeling te reguleren. Hoewel het mechanisme en de rol van BDL fosforylering door PID verdere analyse behoeven, suggereren onze resultaten dat het PID proteïne kinase zowel auxine transport als auxine-responsieve genexpressie reguleert.

Hoofdstuk 5 beschrijft het verdere onderzoek naar de interactie van PID met CSN8/COP9. *In vitro* fosforyleringsassays lieten zien dat niet CSN8 maar het daaraan gebonden CSN7 door PID kon worden gefosforyleerd. Onder de geteste groeiomstandigheden leek deze fosforylering niet essentieel voor de *in planta* functie van CSN7. Co-expressie van PID en CSN8 in protoplasten leidde tot een cytoplasmatische en nucleaire lokalisatie van PID, en versterkte de reeds in Hoofdstuk 3 beschreven ubiquitinering van PID. De associatie van PID met het CSN kan gerelateerd zijn aan gerichte afbraak van dit kinase, echter een andere rol zou gelegen kunnen zijn in het reguleren van de interactie van de fosforylatietargets BDL en de PIN eiwitten met hun corresponderende E3 ubiquitineligases. De ontdekking van PID als een mogelijk CSN-geassocieerd kinase laat een onverwacht nieuw aspect zien van PID signaaltransductie, en verbindt de actie van dit proteïne kinase met de regulatie van eiwitubiquitinering.

Concluderend laten onze resultaten zien dat poly-ubiquitinering van eiwitten een centraal mechanisme is in de werking van het plantenhormoon auxine, en dat het PID proteïne kinase een onverwachte schakel is in de communicatie tussen auxine transport en auxine respons.

Curriculum vitae

Felipe dos Santos Maraschin was born on the first of December 1980 in Santo Angelo, State of Rio Grande do Sul (RS), Brazil. He attended school in Escola de 1° e 2° Graus da URI in Santo Angelo and went to high school in Colegio Anchieta in Porto Alegre. In 1998 he started the study of Biological Sciences at Universidade Federal do Rio Grande do Sul (UFRGS) in Porto Alegre. From August 1998 he started research training in the Microbiology Department of UFRGS under supervision of Dr. Gertrudes Corção. In September 1999 he joined the Plant Molecular Biology research group of Dr. Giancarlo Pasquali at the Centro de Biotecnologia of UFRGS. In 2002 he obtained his BSc in Biology with specialization in Molecular and Functional Cell Biology. As an MSc student, from April 2002 onwards he was involved in the project "Micropropagation, genetic transformation and analysis of genes involved in alkaloid biosynthesis in *Psychotria brachyceras*" as part of the Graduate Program in Cellular and Molecular Biology at UFRGS under Dr. Pasquali's supervision. In April 2004 he obtained his MSc degree in Cell and Molecular Biology from UFRGS. In January 2004 he was invited to participate in the PhD joint project "Ubiquitous events in auxin and jasmonic acid signal transduction" between the Molecular and Developmental Genetics and the Plant Cell Physiology sections at the Institute of Biology of Leiden University under the supervision of Dr. Remko Offringa and Prof. Dr. Johan Memelink. Working on this project Felipe lived in Leiden, the Netherlands, from May 2004 to December 2008 and now he lives in Porto Alegre, Brazil.