DENEDYLASE1 Deconjugates NEDD8 from Non-Cullin Protein Substrates in Arabidopsis thaliana

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The evolutionarily conserved 8-kD protein NEDD8 (NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED8), also known as RUB (RELATED TO UBQUITIN), is an evolutionarily conserved 8-kD protein closely related to ubiquitin (Rao-Naik et al., 1998; Hochstrasser, 2009). Like ubiquitin, NEDD8 is conjugated to substrates through an enzymatic cascade that includes the E1 NEDD8 activating enzyme (NAE); in Arabidopsis, NAE is a heterodimer of AXR1 (AUXIN RESISTANT1) or AXL (AXR1-LIKE) and ERC1 (E1 C-TERMINAL RELATED1). The NEDD8-conjugating cascade also includes an E2 conjugating enzyme; in Arabidopsis, this is RUB1 CONJUGATING ENZYME1 (RCE1; Pozo et al., 1998; del Pozo and Estelle, 1999; del Pozo et al., 2002; Dharmasiri et al., 2007; Woodward et al., 2007). NEDD8 is ultimately conjugated to its protein substrate with the help of E3 NEDD8 ligases like RBX1 (RING BOX1), a constitutive subunit of cullin-RING E3 ubiquitin ligases (CRLs), and DEFFECTIVE IN CULLIN NEDDYLYATION (DCN; Gray et al., 2002; Duda et al., 2008; Kurz et al., 2008).

The cullin subunits of CRLs are the best-characterized substrates for NEDD8 conjugation (neddylation) (Duda et al., 2008; Huang et al., 2008). Cullin neddylation is promoted by the CRL core subunit RBX1 and required for the assembly of functional CRL complexes that ubiquitylate their cognate substrate proteins to target them for degradation by the 26S proteasome (Gray et al., 2002; Duda et al., 2008). CRL function and protein complex assembly are antagonized by cullin deeneddylation through the COP9 signalosome (CSN) (Schwechheimer et al., 2001; Wei et al., 2008; Schwechheimer and Isono, 2010; Lingaraju et al., 2014). Arabidopsis mutants for all eight CSN subunits have been described, including mutants for the paralogous proteins CSN5A and CSN5B, which are the deeneddylyating subunits of CSN (Gusmaroli et al., 2004, 2007; Dohmann et al., 2005). Whereas csn loss-of-function mutants display the strong characteristic constitutively photomorphogenic (cop) phenotype and accumulate cullins in their NEDD8-modified form, mutants partially impaired in CSN function, such as csn5A and csn5B, have comparatively mild phenotypes and are only partially defective in cullin deeneddylation (Gusmaroli et al., 2004, 2007; Dohmann et al., 2005).

Proper neddylation is essential for plant development. Arabidopsis mutants lacking two of the three RUB genes (Bostick et al., 2004) or mutants defective in both paralogous subunits of the NAE, AXR1 and AXL, have severe developmental defects beginning during embryogenesis (Leyser et al., 1993; Dharmasiri et al., 2007; Hotton et al., 2011). Weaker mutants, such as axr1 single mutants, undergo largely normal embryo differentiation but have substantial growth defects, including a strong insensitivity to the phytohormone auxin when grown on medium containing auxin concentrations that inhibit root growth in the wild type (Lincoln et al., 2001). This auxin insensitivity in the axr1 mutants can be explained by impaired functionality of their cognate E3 ligase SCFTR1 and related CRLs and, consequently, an inability to degrade the auxin-labile AUX/IAA repressor proteins such as AXR2 and AXR3 (Gray et al., 2001). This auxin insensitivity can also be observed when wild-type seedlings are treated with the NAE inhibitor MLN4924, which blocks NEDD8 conjugation in an MLN4924 concentration-dependent manner (Brownell et al., 2010; Hakenjos et al., 2011). Auxin-insensitive root growth is thus an indicator for defects in
neddylation and SCF\textsuperscript{CRL} function. Importantly, weak mutants of CSN such as csn5a and csn5b mutants also display this phenotype, suggesting that an adequate balance of neddylation and de neddylation is required for proper CRL and SCF\textsuperscript{CRL} function (Schwechheimer et al., 2001; Gusmaroli et al., 2004, 2007; Dohmann et al., 2005).

Ubiquitin and ubiquitin-like modifiers such as SMALL UBIQUITIN-LIKE MODIFIER (SUMO) modify hundreds of distinct target proteins and thereby affect protein activity or fate (Miller and Vierstra, 2011; Vierstra, 2012; Kim et al., 2013). Therefore, it is surprising that, to date, only cullins have been recognized as bona fide NEDD8 modification substrates. For NEDD8, a number of non-cullin NEDD8-modified proteins have previously been identified, mainly in animal systems (Xirodimas, 2008; Mergner and Schwechheimer, 2014; Enchev et al., 2015), but recent observations of nonspecific crosstalk between the ubiquitin and the NEDD8 conjugation machineries suggests that at least some of these neddylation substrates may in fact be ubiquitination substrates that have become neddylated rather than ubiquitylated as a consequence of NEDD8 overexpression (Herpe et al., 2012a, 2012b). It has thus been questioned whether other biologically relevant non-cullin neddylation substrates exist in eukaryotes (Enchev et al., 2015). In plants, to date, only one non-cullin substrate, ML3, has been biochemically validated, although the existence of others has been proposed (Hakenjos et al., 2011, 2013; Hotton et al., 2012). However, the biological significance of ML3 neddylation remains to be elucidated since the function of ML3 itself is unknown (Hakenjos et al., 2013).

The Arabidopsis genome encodes three distinct NEDD8-encoding \textit{RUB} genes belonging to two structurally distinct families (Rao-Naik et al., 1998). Whereas Arabidopsis \textit{RUB3} encodes a NEDD8 precursor with a C-terminal extension, \textit{RUB1} and \textit{RUB2} additionally bear an N-terminal extension with full-length ubiquitin (Rao-Naik et al., 1998). Mature NEDD8 is derived from these precursors through the N- and C-terminal processing of these extensions. \textit{DEN1}/\textit{NEDP1}/\textit{SEN8P} (\textit{DENEDDYLASE1}/\textit{NEDD8-SPECIFIC PROTEASE1}/\textit{SENTRIN-SPECIFIC PROTEASE8}; hitherto DEN1) was originally described from \textit{Drosophila melanogaster} and mammals as a NEDD8-specific processing enzyme (Gar-Erdene et al., 2003; Mendoza et al., 2003; Wu et al., 2003; Shen et al., 2005; Chan et al., 2008; Shin et al., 2011). In addition, several ubiquitin C-terminal hydrolases from animals and yeasts were shown to possess a dual specificity for ubiquitin and NEDD8 processing (Wada et al., 1998; Johnston et al., 1999; Linghu et al., 2002; Hemelaar et al., 2004; Fricke et al., 2007; Yu et al., 2007). Although NEDD8 and neddylation are essential in many organisms, none of the mutants described to date from any organism for these NEDD8 processing enzymes are inviable or have an apparent defect in NEDD8 processing. It has therefore been suggested that the different enzymes may act in a functionally redundant manner (Enchev et al., 2015).

Based on sequence similarities, we and others have recently identified four proteins as candidates for NEDD8 processing enzymes from Arabidopsis: a DEN1 homologous protein belonging to the C48 peptidase family (Colby et al., 2006; Mergner and Schwechheimer, 2014) as well as three C12 family peptidases UCH1 (\textit{UBIQUITIN CARBOXYL-TERMINAL HYDROLASE1}), UCH2, and UCH3 (Yang et al., 2007). Here, we examine the DEN1 homologous protein as well as \textit{den1} mutants from Arabidopsis. We show that DEN1 is not essential for NEDD8 processing but required for protein de neddylation since \textit{den1} mutants accumulate a broad range of NEDD8 conjugates. Furthermore, we show that AXR1 is one abundant NEDD8 conjugate in \textit{den1} mutant plants and provide evidence that AXR1 function may be compromised in these mutants. We thus conclude that many non-cullin NEDD8-modified proteins exist in plants and that neddylation and de neddylation may function as regulatory mechanisms of non-cullin proteins in plant development.

**RESULTS**

**Arabidopsis \textit{den1} Mutants Accumulate NEDD8 Conjugates**

Human DEN1 was originally identified as a protein capable of processing the C terminus of NEDD8 propeptides. To understand NEDD8 precursor processing in Arabidopsis, we analyzed ATSG60190, the closest homolog of human and Drosophila \textit{DEN1} from Arabidopsis (Supplemental Figure 1). \textit{DEN1} belongs to the family of cysteine proteases, and Arabidopsis ATSG60190 is a predicted active protease based on the conservation of the residues critical for this biochemical activity (Figure 1A). Due to the overall sequence conservation, the conservation of the active site as well as the biochemical activities of ATSG60190 described in due course, we designated this protein \textit{DEN1}. We further isolated two \textit{den1} mutant alleles, \textit{den1-1} and \textit{den1-2}, from the GABI-Kat (Rosso et al., 2003) and the SAIL (Sessions et al., 2002) T-DNA insertion mutant collections, respectively (Figure 1A). Both mutant alleles carry insertions in close proximity to the catalytic center of the cysteine protease, and the insertions would therefore be predicted to strongly impair protein function.

In Arabidopsis, mature NEDD8 is the product of the proteolytic processing of the precursors derived from \textit{RUB1}, \textit{RUB2}, and \textit{RUB3} (Rao-Naik et al., 1998). Whereas \textit{RUB3} encodes an un fused NEDD8 that only requires C-terminal processing, \textit{RUB1} and \textit{RUB2} encode UB (ubiquitin)-NEDD8 chains that are processed at the UB and NEDD8 C termini, respectively. When we probed total protein extracts of the wild type and \textit{den1} mutants with an anti-NEDD8 antibody that recognizes all three Arabidopsis NEDD8 isoforms (Hakenjos et al., 2011), we observed that NEDD8 processing was not detectably defective in the \textit{den1} mutants since the levels of mature unconjugated NEDD8 and NEDD8-conjugated cullins were indistinguishable between the mutant and the wild type (Figure 1B; Supplemental Figure 2). Surprisingly, however, \textit{den1} mutants accumulated NEDD8-modified proteins of a broad molecular mass range (Figure 1B; Supplemental Figure 2). Particularly prominent were two abundant NEDD8-modified proteins with an apparent molecular mass of 72 and 130 kD, which we designated p72 and p130 (Figure 1B; Supplemental Figure 2). Since the accumulation of the non-cullin as well as the cullin NEDD8 conjugates was reduced in the \textit{den1} mutant following treatment with the NAE inhibitor MLN4924, we concluded that not only the cullins but also the other non-cullin NEDD8-modified proteins were substrates downstream of NAE (Figure 1C). Despite the fact that the \textit{den1} mutants had a prominent molecular phenotype, we did not observe any apparent growth defects when examining the mutants in standard growth conditions or following...
a diverse set of treatments (Figure 1D). In summary, we concluded that the loss of DEN1 as a candidate C-terminal NEDD8 hydrolase was not sufficient to detectably impair NEDD8 processing in planta but resulted in the accumulation of a broad range of NEDD8 conjugates, presumably DEN1 deneddylation targets. Since the two available den1 mutant alleles had identical molecular phenotypes, we performed all subsequent analyses with den1-1.

DEN1 Does Not Deneddylate Cullins in Planta

The CSN subunit CSN5 deneddylates the cullin subunits of cullin-RING ligases, such as CULLIN1 and CULLIN4. At the molecular level, csn5a single as well as csn5a csn5b double mutants accumulate NEDD8-conjugated cullins but not other NEDD8-modified proteins (Figure 2A) (Dohmann et al., 2005). In other systems, DEN1 homologs were reported to also deneddylate cullins (Mendoza et al., 2003). In contrast, we found that the pattern of neddylated protein accumulation in the den1 mutant was distinct from the pattern detected in the csn5a mutant, suggesting that DEN1 and CSN5 may have distinct protein substrate ranges (Figure 2A). Since we occasionally observed slight variations in cullin neddylation between different protein samples when we detected cullin neddylation with the NEDD8 antibody, we also compared the cullin neddylation patterns with anti-CULLIN1 and anti-CULLIN4 antibodies (Figure 2B). However, these analyses also led us to the conclusion that DEN1 does not deneddylate cullins in planta.

While the loss of both CSN5 isoforms in the csn5a csn5b mutant resulted in seedling growth arrest, single mutants of CSN5A or CSN5B are viable and only csn5a mutants have clearly apparent growth phenotypes (Gusmaroli et al., 2004; Dohmann et al., 2005). To examine the possibility of a genetic interaction between DEN1 and CSN5, we introduced the den1 mutation into csn5 single and double mutants. However, the phenotypic analyses indicated that the csn5a den1 double mutants, as well as the csn5a csn5b den1 triple mutants, had the phenotypes of the respective csn5 mutants (Figure 2C). Furthermore, at the molecular level, csn5a den1 double mutants had the combined deneddylation defects of the respective single mutants (Figure 2A). We thus concluded that DEN1 and CSN5 interact in an additive manner in Arabidopsis and may have a differential set of deneddylation substrates in planta.

DEN1 Is a Deneddylating Enzyme

We next tested the ability of recombinant DEN1 to deneddylate NEDD8 conjugates. To this end, we added purified glutathione S-transferase (GST)-tagged wild-type DEN1 and DEN1C166A, a predicted inactive DEN1 variant with an alanine replacement mutation of the catalytically important Cys-166, to total protein extracts from csn5a den1 double mutants. In these experiments,
we observed that non-cullin but also cullin NEDD8 conjugates were hydrolyzed by catalytically active but not by catalytically inactive DEN1 (Figure 3A). We thus concluded that DEN1 has the ability to deconjugate cullin and non-cullin NEDD8 conjugates in vitro. Since our analyses of den7 mutants had suggested that DEN1 cannot deconjugate cullins in planta, we introduced transgenes for the overexpression of untagged and FLAG epitope-tagged DEN1 into the den1 mutant. In line with our previous results, the expression of DEN1 in the den1 background did not affect cullin (de)neddylation, even when DEN1 was strongly overexpressed from the 35S:FLAG:DEN1 transgene, but did result in the disappearance of the non-cullin NEDD8 conjugates (Figure 3B). We thus confirmed our previous observations that DEN1 does not hydrolyze neddylation in planta.

The differences between the in vivo and in vitro deconjugation activity of DEN1 may be explained by the differential protein localization of DEN1 and the predominantly nuclear cullins (Figures 3A and 3B) (del Pozo et al., 2002). We therefore examined the nucleo-cytoplasmic partitioning of DEN1 by differential centrifugation of total protein extracts prepared from the 35S:FLAG:DEN1 transgenic line. Here, we found that DEN1 accumulated in the cytoplasmic and in the nuclear fraction, whereas the vast majority of NEDD8 conjugates accumulated in the nuclear fraction (Figure 3C). This nucleo-cytoplasmic partitioning of the DEN1 protein was also confirmed when we examined the cellular distribution of a YELLOW FLUORESCENT PROTEIN (YFP)-tagged DEN1:YFP:HA using confocal microscopy in a stably transformed line expressing a 35S:DEN1:YFP:HA transgene (Figure 3D). Like the other transgenes used in this study, 35S:DEN1:YFP:HA fully complemented the molecular phenotype of den1 mutants (Supplemental Figure 3). Unfortunately, the sensitivity of the anti-DEN1 antibody only allowed detection of the protein in the overexpression lines, and it can therefore not be stated with absolute certainty that the distribution of the DEN1 protein as observed in the overexpression lines correctly reflects the intracellular distribution of endogenous DEN1 in planta.

Since at least one report has so far described a physical interaction between DEN1 and CSN5 or the CSN complex (Christmann et al., 2013), we also examined a possible interaction between DEN1 and CSN5 in Arabidopsis. However, following immunoprecipitation of FLAG:DEN1 from plants, we did not detect CSN5 after probing DEN1 immunoprecipitates with a CSN5 antibody (Figure 3D). Along the same lines, our analysis of DEN1 and CSN5 distribution by size exclusion chromatography indicated that DEN1 was a predominantly monomeric protein that elutes with a profile that is distinct from that of CSN5, which elutes as a subunit of the CSN protein complex and as a CSN5 monomer (Supplemental Figure 4). From these experiments, we concluded that DEN1 may be present in the cytoplasm and in the nucleus, that it deneddylates nuclear non-cullin NEDD8 conjugates, and that it acts independently from CSN5 in vivo.

DEN1 Can Process RUB1 in Vitro

DEN1 was originally identified as an enzyme required for NEDD8 precursor processing (Gan-Erdene et al., 2003; Mendoza et al., 2003; Wu et al., 2005; Chan et al., 2008; Shin et al., 2011). Since the processing of the Arabidopsis NEDD8 RUB precursors was not detectably affected in the den7 mutant, we tested the enzymatic activity of DEN1 toward an artificial recombinant substrate, UB:NEDD8:His. UB:NEDD8:His encodes a His-tagged variant of the endogenous UB (ubiquitin)-NEDD8 fusion protein as found in Arabidopsis RUB1 and RUB2. Incubation with purified wild-type DEN1 but not with the catalytically inactive DEN1C166A led to the release of a cleaved protein corresponding to the C-terminally processed UB:NEDD8 (Figure 4A). This indicated that Arabidopsis DEN1, just like its mammalian counterparts, was able to process the C terminus of NEDD8 but not the C terminus of ubiquitin in vitro. We then exchanged the UB and NEDD8 moieties to generate NEDD8:UB:His to examine whether DEN1 was able to process NEDD8 also in the context of a longer C-terminal extension and, conversely, whether DEN1...
was able to process ubiquitin with a shorter C-terminal extension. However, DEN1 could neither cleave NEDD8 when present in the NEDD8:UB arrangement nor process the ubiquitin C terminus of NEDD8:UB:His, indicating that it was specific for the C-terminal processing of the NEDD8 moiety of the Arabidopsis RUB propeptides (Figure 4B).

At the same time, we also examined the processing activity of the related cysteine protease UCH3, whose counterpart from the mammalian system has a dual specificity for ubiquitin and NEDD8 processing (Wada et al., 1998; Mergner and Schwechheimer, 2014). In our experiments, UCH3 efficiently cleaved the His C terminus of UB:NEDD8:His as well as the NEDD8:UB:His substrate, indicating that Arabidopsis UCH3 also possessed, at least in vitro, a dual specificity for NEDD8 and ubiquitin C-terminal processing (Figures 4A and 4B). However, just like DEN1, UCH3 was also unable to cleave after NEDD8 or UB when these proteins represented the N-terminal moieties of the fusion proteins. To further examine the DEN1 and UCH3 enzyme activities, we incubated recombinant DEN1 and UCH3 with total protein extracts from plants expressing an N-terminally HA-tagged NEDD8, HA:
DEN1 Is a Ubiquitin-Modified Protein

To gain an understanding of possible molecular associations of DEN1 with other proteins, we also analyzed immunoprecipitates of FLAG:DEN1 by mass spectrometry. In line with our findings that DEN1 was seemingly a monomeric protein (Supplemental Figure 4), we did not detect any stoichiometric protein interactors when analyzing FLAG:DEN1 immunoprecipitates (Figure 5; Supplemental Data Set 1). Interestingly, however, we obtained an indication that DEN1 was modified by either ubiquitin or NEDD8 (Supplemental Figures 5A, 5B, and 6). Since, following trypsin digestion, both modifiers, ubiquitin or NEDD8, leave an identical mass footprint corresponding to two glycines on their substrate proteins, it is not possible to distinguish between these two modifications solely based on these mass spectrometry results. We therefore probed the FLAG:DEN1 immunoprecipitates with antibodies directed against NEDD8 and ubiquitin. Whereas we did not obtain a signal with the NEDD8 antibody, we detected at least two high molecular mass forms of DEN1 with the ubiquitin antibody (Figure 5). Since the mass spectrometric analysis suggested that the ubiquitin modification resided at one of two adjacent lysine residues, Lys-217 and Lys-218, at the DEN1 C terminus (Supplemental Figures 5A and 5B), we also generated transgenic lines for the expression of DEN1 mutant variants where both lysines were replaced by arginine (Supplemental Figure 5C). Indeed, mutagenesis of these residues abolished the ubiquitylation of DEN1, suggesting that one of these two lysines or both lysine residues are ubiquitylation sites in DEN1 in planta (Supplemental Figure 5C).

We also tested whether the ubiquitin conjugation of DEN1 was required for its biochemical activity as a deneddylation and to this end examined the neddylation patterns of den1 mutants in the absence and presence of wild-type and mutant DEN1 transgenes. However, we found that the activity of DEN1 was not compromised by the mutation of the ubiquitylation site (Supplemental Figure 5D). Finally, since ubiquitin conjugation may target the protein for proteasomal degradation, we also examined the stability of DEN1 following treatment of plants for up to 8 h with the protein biosynthesis inhibitor cycloheximide (Supplemental Figure 5E). Since the protein abundance of DEN1 was unaltered when we compared the wild type and the mutant DEN1 protein, we concluded that DEN1 ubiquitylation affected neither its deneddylation function nor its protein stability.

AXR1 Corresponds to the NEDD8-Modified Protein p72

In order to identify NEDD8 conjugates that accumulated in the den1 mutant, we performed a two-step purification of a His- and StreptI-tagged NEDD8 expressed from a 2-kb RUB1 promoter fragment. Samples were analyzed by mass spectrometry after the first native purification step using the StreptI-tag and after a second denaturing purification step using the His-tag (Supplemental Figure 7 and Supplemental Data Set 2). One of the proteins that attracted our attention was the AXR1 subunit of the NAE. AXR1 was strongly enriched after both purification steps, and AXR1 was thereby distinguishable from its interaction partner ECR1, which was only enriched after the first nondenaturating purification step (Figure 6A). We took this as an indication that AXR1 itself might be a neddylated protein and introduced the axr1 mutant and a previously published AXR1p10myc:AXR1 (myc: AXR1) transgene into the den1 mutant background (Hotton et al., 2011). When we analyzed NEDD8 conjugate formation in the den1 axr1 background, we detected a decrease in the intensity of the p72 band specific for den1 in line with the hypothesis that p72 corresponded to a NEDD8-modified form of AXR1 (Figure 6C). Although the p72 band was not completely abolished in the den1 axr1 mutant, we reasoned that AXL, the functional ortholog of AXR1, might be responsible for the residual staining at the molecular mass corresponding of p72. In further support of AXR1 being a NEDD8-modified protein in den1 mutants, we detected a novel high molecular mass form of AXR1 that was present in the den1 mutant but not in the wild-type background when probing
the wild type and the concentrations of the synthetic auxin 2,4-D when comparing the detectable in the absence of the were treated with the neddylation inhibitor MLN4924 (Figures 7A). Since AXR1 was neddylated in Auxin insensitive root growth is a hallmark phenotype of den1 mutants (Lincoln et al., 1990). Since AXR1 was neddylated in AXR1 (Supplemental Data Set 2).

to deneddylation by recombinant DEN1 and con

to a misregulation of cullin neddylation and, thus, the slight but statistically significant increase in 2,4-D insensitivity as observed in the MLN4924-treated den1 mutant. This finding would thus be in line with a further reduction in E1 activity in addition to the partial impairment of E1 function through AXR1 neddylation in den1. A further indication for an impairment of AXR1 function in the den1 mutant background came from our observation that den1 axr1 double mutants expressing myc:AXR1 were less sensitive to 2,4-D than the axr1 single mutant expressing myc:AXR1 (Figures 7C and 7D). Since it had previously been noted that this particular myc:AXR1 transgene cannot fully complement the axr1 mutant defect (Hotton et al., 2011), expression of myc:AXR1 may be seen as a sensitized mutant background for such analyses. In summary, our data provide evidence for a partial impairment of E1 function in the den1 mutants due to the accumulation of neddylated AXR1.

**DISCUSSION**

We genetically and biochemically analyzed DEN1 from Arabidopsis. The founding members of the DEN1 protein family were originally described as enzymes specific for the processing of NEDD8 precursors in mammalian systems, and subsequent studies had implicated DEN1 from animals, yeasts, and fungi also in the deneddylation of, e.g., cullins as bona fide NEDD8-conjugated proteins (Gan-Erdene et al., 2003; Wu et al., 2003; Reverter et al., 2005; Christmann et al., 2013). Our analysis of Arabidopsis DEN1 revealed that DEN1, although capable of processing the Arabidopsis NEDD8 precursors in vitro, is not solely responsible for precursor processing in planta since den1 loss-of-function mutants do not have any detectable defects in processing the Arabidopsis RUB precursors. This is in agreement with observations from other non-plant systems where the loss of any hitherto described NEDD8 processing enzyme including the NEDD8-specific enzyme DEN1 but also hydrolases with dual specificity for NEDD8 and ubiquitin
does not lead to biochemically detectable processing defects or developmental defects indicative for defects in NEDD8 precursor processing (Mergner and Schwechheimer, 2014; Enchev et al., 2015). Besides DEN1, the Arabidopsis genome encodes three further candidate NEDD8 processing enzymes that have also an additional predicted or tested specificity for ubiquitin processing, UCH1, UCH2, and UCH3 (Mergner and Schwechheimer, 2014). Previous work had shown that the defects in the related UCH1 and UCH2 genes would not lead to biochemically discernable ubiquitin-processing defects, again arguing for a functional redundancy among the different processing enzymes (Yang et al., 2007). Here, we show that UCH3 has the predicted dual ubiquitin- and NEDD8-processing activity when tested in vitro and we thus propose that DEN1, UCH3, and possibly also UCH1 and UCH2, which we and others have been unable to recover as biochemically active recombinant proteins when purified from bacteria (Yang et al., 2007), act in a functionally redundant manner in NEDD8 precursor processing.

Our data show that DEN1 is essential for the deconjugation of NEDD8 from a broad range of neddylated proteins that accumulate in the Arabidopsis den1 mutants. Previous studies on DEN1 had indicated that this protein may also deneddylate cullins (Wu et al., 2003; Christmann et al., 2013). In contrast, our den1 mutant and DEN1 protein analysis reveal that DEN1, although possessing cullin deneddylating activity in vitro, is not required for cullin deneddylase in planta, also not in the absence of the cullin deneddylating CSN subunit CSN5. The differential substrate range of the deneddylating subunit CSN5 of CSN and DEN1 had previously been attributed to the inaccessibility of the cullin NEDD8 modification in the CRL E3 ligases (Reverter et al., 2005; Duda et al., 2008). A similar argument may also be used to explain the differences between the in vitro and in vivo activities of Arabidopsis DEN1, under
the premise that CRLs become instable and consequently neddylated cullins accessible to DEN1 in the protein extracts prepared for the respective deneddylation experiments.

Regardless of the functional interplay between DEN1 and CSN5, which may be different between Arabidopsis, where we did not detect an interaction, and Aspergillus nidulans or mammalian cells, where such an interaction had been reported (Christmann et al., 2013), our data clearly indicate the presence of NEDD8-modified proteins other than cullins in planta. Although previous reports about a nonspecificity of the NEDD8 and ubiquitin conjugation machineries in certain experimental conditions had put into question the existence of non-cullin NEDD8 conjugates, our observation of an accumulation of such non-cullin NEDD8 conjugates in the den1 mutant clearly proves their existence. In that regard, our observation is comparable to similar observations with den1 mutants from A. nidulans and Drosophila where NEDD8 conjugate accumulations had also been reported but have remained biochemically unexplored (Chan et al., 2008; Christmann et al., 2013). Taken together, these observations clearly demonstrate that protein neddylation is a common posttranslational modification of many substrate proteins in the respective organisms.

Furthermore, our results identify AXR1 as a novel NEDD8-modified protein that can be detected in this posttranslationally modified form in a genotype comparable to the wild type (an axr1 mutant complemented with an myc:AXR1 transgene under control of the AXR1 promoter), and this form accumulates in the den1 mutant. Our observation that AXR1 in the absence of the deneddylation activity of DEN1 seems partially impaired in protein function suggests that AXR1 neddylation serves to negatively control protein activity and thus indicates that also other as yet unidentified neddylated proteins may be regulated by NEDD8 modification in plants.

Figure 7. AXR1 Neddylation Impairs 2,4-D-Responsive Growth.

(A) and (B) Quantitative analysis of root growth of 5-d-old wild-type seedlings and den1-1 mutants transferred from unsupplemented GM to GM containing MLN4924 and 2,4-D for an additional 5 d. (A) The relative root elongation was measured using ImageJ and normalized to the growth of the untreated roots. Average and SE are shown (n ≥ 12), and asterisks indicate a significant difference (Student’s t test). (B) Representative photographs of 10-d-old wild-type and den1-1 seedlings.

(C) and (D) Root growth assay with wild-type, den1-1, and different axr1 mutant lines. Five-day-old GM-grown seedlings were transferred to GM containing 2,4-D (25, 50, 100, 250, and 500 nM) for 5 d. (C) Root length was measured using ImageJ and is shown as relative to the growth of the untreated roots. Average and SE values are shown (n ≥ 10). (D) Representative photographs of 10-d-old seedlings transferred to media containing 50, 100, and 250 nM, respectively.
The uncertainty of the biological validity of previously reported protein neddylation events as a consequence of the above mentioned risk of them being an artifact of specific biochemical conditions has recently resulted in the compilation of a set of criteria that must be fulfilled for a given protein to be considered a bona fide NEDD8-modified protein (Enchev et al., 2015). According to these criteria, the neddylation target should be conjugated via a C-terminal glycine residue to its protein target. The DEN1 sensitivity of the NEDD8 modification of AXR1 and the stability of the modification under reducing conditions indirectly demonstrate that AXR1 is modified in this manner. Second, genuine NEDD8-modified proteins should be detectable as such under homeostatic conditions in the presence of normal NEDD8 and substrate protein availability. Since we can detect AXR1 in its neddylated form from axr1-30 myc:AXR1 where myc:AXR1 is expressed from the AXR1 promoter, we also see this criterion as being fulfilled. Third, neddylation should be sensitive to treatments with the NAE inhibitor MLN4924, and in agreement with this, we found AXR1 neddylation to be reduced after MLN4924 treatment. Additionally, we suggest that AXR1 neddylation impairs NAE function as concluded from genetic and physiological analyses, and thereby we fulfill the further criterion that protein neddylation has a consequence for the function of the neddylated protein. In conclusion, we judge that AXR1 is a bona fide NEDD8-modified protein from Arabidopsis. Future research will have to elucidate the identity and control of further NEDD8 conjugates, on the one side, and the mechanisms and signals that control neddylation and its substrate specificity, on the other.

METHODS

Biological Material

All experiments were performed in the Arabidopsis thaliana ecotype Columbia, den1-1 (GK381A12) and den1-2 (SAIL_588_H02) were obtained from the Nottingham Arabidopsis Stock Centre and selected for homozygosity by PCR-based genotyping. Other previously reported mutant lines were (SALK_063436), csn5a-2 (SALK_027705), csn5b-1 (SALK_077134), and axr1-30 (SAIL_904_E06), den1-1 was crossed into the csn5a-2, csn5a-1 csn5b double mutant, and myc:AXR1 axr1-30 mutant background, and homozygous double and triple mutants were identified by PCR-based genotyping. Primer sequences for genotyping are listed in Supplemental Table 1. The transgenic AXR1pro:myc:AXR1 (myc:AXR1) line was generously provided by Judy Callis (University of California, Davis, CA). The transgenic line for the dexamethasone-inducible expression of HA:STREP:NEDD8 was previously described (Hakenjos et al., 2011).

Cloning Procedures

To generate the DEN1 overexpression constructs 35S:FLAG:DEN1, 35S: DEN1, and 35S:DEN1:YFP:HA, the DEN1 coding region was PCR amplified from cDNA with primers 14 and 15 or 14 and 16 and cloned using Gateway technology (Invitrogen) into pEarleyGate202 and pEarleyGate101, respectively. Mutagenesis for DEN1K271218R was performed using a nested PCR reaction with the primers 14 and 17 as well as 14 and 15 and subsequent cloning of the fragment into pEarleyGate202. These transgenes were introduced into the den1-1 mutant by the Agrobacterium tumefaciens-mediated floral dip method (Clough and Bent, 1998).

To generate GST-tagged versions of DEN1 and UCH3, the respective open reading frames were PCR amplified from cDNA using primer pairs with additional BamHI (DEN1) or EcoRI (UCH3) restriction sites before and NotI restriction sites after the translational start and stop codons, respectively. After digestion, the PCR fragments were ligated into the pGEX6p-1 vector (GE Healthcare). Mutagenesis of GST:DEN1 to obtain GST:DEN1C166A was performed using DpnI digestion-based site-directed mutagenesis with the primers 22 and 23. UB:NEDD8::His was obtained by generating a PCR fragment of AIRUB1 from Arabidopsis cDNA with primers 24 and 25 and ligating the PCR fragment into pET21a (Novagen). The NEDD8::UB:His construct was obtained by overlap extension PCR with the primers 26 to 29, and the fusion product was cloned into pET21a. To generate a NEDD8 construct with a N-terminal His:StrepII tag expressed under control of the AIRUB1 promoter, RUB1pro::His:StrepII::NEDD8, we first used primers 30 and 31 to substitute the HA-tag by a His-tag in the previously described HSN construct (Hakenjos et al., 2011). The construct was subcloned into pTA7002 (Aoyama and Chua, 1997). To obtain the RUB1 promoter sequence, we used the primers 32 and 33 to amplify a 2-kb region upstream of the start codon of Arabidopsis RUB1 using genomic DNA as a template. The fragments were digested with restriction enzymes and cloned into pGreen0179 (Hellens et al., 2000). Primer sequences are listed in Supplemental Table 1.

Chemical Treatments

To examine the effect of MLN4924 (Millennium Pharmaceuticals) on protein neddylation, wild-type and mutant seeds were grown for 4 d on standard growth medium (GM) and then transferred to GM containing 10 µM MLN4924 for additional 4 d. For the induction of HSN expression, HSN transgenic seedlings were grown on GM for 7 d and then incubated in liquid GM supplemented with 30 mM dexamethasone for 16 h. For 2,4-D response assays, seedlings were grown 5 d on GM and subsequently transferred to MLN4924- and 2,4-D-containing media for 5 d.

Immunobiological Analyses and Size Exclusion Chromatography

For immunoblots and gel filtration analysis, total protein extracts were prepared from 7-d-old Arabidopsis seedlings in protein extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 5 µM N-ethylmaleimidene, and plant protease inhibitor cocktail [Sigma-Aldrich]) or buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 5 µM N-ethylmaleimidene, and protease inhibitor cocktail). Subcellular fractionation was performed as previously published (Folta and Kaufman, 2006). Immunoblots were performed according to standard protocols. The following antibodies were used for protein detection: anti-NEDD8 (1:1000; Hakenjos et al., 2011), anti-CULLIN1 (1:1000; Schwechheimer et al., 2002), anti-CULLIN4 (1:1000; Dohrmann et al., 2005), anti-CSN5 (1:1000; Kwok et al., 1998), anti-CDC2 (1:5000; Santa Cruz Biotechnology), anti-HA- peroxidase (1:1000; Roche), anti-flag (1:2000; Sigma-Aldrich), anti-c-Myc (1:3000; Sigma-Aldrich), anti-RGA (1:1000; Willige et al., 2007), anti-UGFase (1:2500; Agrisera), and anti-Ubiquitin P4D1 (1:2000; Santa Cruz Biotechnology). The anti-DEN1 (1:1000) antibody was raised in rabbits against purified recombinant full-length DEN1 at Eurogentec. Anti-flag M2 affinity gel (Sigma-Aldrich) and anti-c-Myc agarose (Sigma-Aldrich) were used for immunoprecipitations using protein extracts prepared from 0.5 mg (fresh weight) 7-d-old seedlings. Size exclusion chromatography of 1 mg of total plant protein extract was performed using a Superose 6 column (GE Healthcare) as described previously (Schwechheimer et al., 2002).

Mass Spectrometry

For mass spectrometry, proteins were purified from 10 g of 7-d-old RUB1pro::His:StrepII::NEDD8 seedlings using a StreptTactin affinity column (Sigma-Aldrich) as described previously (Hakenjos et al., 2011). Part of the sample (sample I) was supplemented with Laemmli buffer and boiled for 5 min. The other part (sample II) was subjected to a second purification step under denaturing conditions using the Ni-NTA system (Thermo Scientific) as described previously (Miller et al., 2010). For trypptic digestions,
samples I and II were reduced and alkylated by 50 mM DTT and 10 mg/mL chloroacetamide, respectively. Tryptic in-gel digestion was performed according to standard procedures. Nanoflow liquid chromatography-tandem mass spectrometry was performed by coupling an Eksigent nanoLC-Ultra 1D+ (Eksigent) to a LTQ-Orbitrap XL ETD (Thermo Scientific). Peptides were delivered to a trap column (100 μm × 2 cm), packed in house with Reprosil-Pur C 18 -AQ 5 μm resin; Dr. Maisch) at a flow rate of 5 μL/min in 100% solvent A (0.1% formic acid in HPLC-grade water). After 10 min of loading and washing, peptides were transferred to an analytical column (75 μm × 40 cm, packed in house with Reprosil-Pur C 18 -GOLD, 3 μm resin; Dr. Maisch) and separated using a 225-min gradient from 4 to 32% of solvent B (0.1% formic acid and 5% DMSO in acetonitrile; solvent A: 0.1% formic acid and 5% DMSO in water] at 300 nL/min flow rate. The LTQ Orbitrap XL was operated in data-dependent mode, automatically switching between MS and MS2. Full-scan mass spectra were acquired in the Orbitrap at 60,000 (m/Δm 400) resolution after accumulation to a target value of 1,000,000. Tandem mass spectra were generated for up to eight peptide precursors in the linear ion trap using collision-induced dissociation at a normalized collision energy of 35% after accumulation to a target value of 5000 for max 100 ms. Intensity-based label-free quantification was performed using Progenesis (version 4.2; Nonlinear Dynamics). The generated peak list was then searched using Mascot (version 2.4.1) against the NCBI protein sequence databases (download October 26, 2011, 15.8 Mio sequences) and SwissProt (version 57, 0.5 Mio sequences) for protein identification. The variable modification of K (GlyGly) was considered in the database search in order to identify NEDD8- or ubiquitin-modified peptides.

Enzyme Activity Assay

Processing of UB:NEDD8:His and NEDD8:UB:His was performed with DEN1, DEN1C166A, and UCH3 proteins that were cleaved from the purified GST fusion proteins using PreScission Protease (GE Healthcare). Glutathione-Sepharose 4B (GE Healthcare) and Talon Metal Affinity resin (Clontech) were used for protein purification from Escherichia coli [proseta (DE3)PlyS]. For processing reactions, 0.2 μg UB:NEDD8:His or NEDD8:UB:His was incubated with 0.2 μg purified DEN1, DEN1C166A, or UCH3 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM DTT at 20°C for 60 min. The deconjugation assay was performed with protein extract from 7-d-old HSN seedlings containing 15 μg total protein incubated with 0.5 μg purified DEN1 or UCH3 in 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8, 5% glycerol, and 10 mM DTT at 20°C for 30 min. For the in vitro deneddylation assays, crude protein extracts from 7-d-old seedlings containing 350 μg (cullin deneddylation) and 500 μg (myc:AXR1 deneddylation) total protein were incubated with 0.2 or 2 μg purified DEN1 or DEN1C166A in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Triton X-100 at 20°C for 25 min.

Phylogenetic Analysis

Protein sequences for DEN1 orthologous proteins from human (NEDP1, ENSG00000166192) and Drosophila melanogaster (Den1; FBgn0033716) were retrieved from the ensemble genome browser database (http://www.ensembl.org). Alignments were generated using the ClustalW software (BLOSUM, gap open penalty 10 and gap extension penalty 0.1).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: RUB1 (AT1G31340), AXR1 (AT1G05180), CSNSA (AT1G22920), CSN5B (AT1G71230), CUL11 (AT4G02570), CUL14 (AT5G48210), UCH3 (AT5G17510), and DEN1 (AT5G46210). GenBank accession numbers of human and fruitfly genes mentioned in this work are NEDD8 (NP_0006147), NEDP1 (AAG21828), and Den1 (NM_136919).

Supplemental Data

Supplemental Figure 1. DEN1 alignment.

Supplemental Figure 2. den1-1 and den1-2 have indistinguishable molecular phenotypes.

Supplemental Figure 3. 35S:DEN1:YFP:HA complements the den1-1 phenotype.

Supplemental Figure 4. FLAG:DEN1 elutes largely as a monomer after gel filtration.

Supplemental Figure 5. DEN1 is a ubiquitin-modified but stable protein.

Supplemental Figure 6. Result of the mass spectrometric analysis of DEN1.

Supplemental Figure 7. Two-step purification of neddylated proteins.

Supplemental Table 1. List of primers used in this study.

Supplemental Data Set 1. Results of the mass spectrometric analysis following DEN1 immunoprecipitation.

Supplemental Data Set 2. Results of the mass spectrometric analysis following two-step purification of neddylated proteins.

ACKNOWLEDGMENTS

We thank Pascal Genschik (Strasbourg, France) and Judy Callis (University of California, Davis, CA) for providing the anti-CUL4 antibody and the myc:AXR1 transgenic line, respectively. We also thank Johannes Weigl and Christine Plenge for generating GST:DEN1C166A and GST:UCH3 as part of their Bachelor’s theses. This work is supported by a grant from the Deutsche Forschungsgemeinschaft to Claus Schwechheimer (SCHW751/11-1) as part of the Schwerpunktprogramm SPP1365 “Ubiquitin family proteins.”

AUTHOR CONTRIBUTIONS

J.M., B.K., and C.S. designed the experiments. J.M. performed all experiments apart from mass spectrometry. S.H. performed mass spectrometry. S.H., J.M., and B.K. analyzed the mass spectrometric data. J.M. and C.S. wrote the article.

Received December 31, 2014; revised February 5, 2015; accepted February 26, 2015; published March 17, 2015.

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