MLN4924 Is an Efficient Inhibitor of NEDD8 Conjugation in Plants

Jana Pia Hakenjos, René Richter, Esther Mirjam Natascha Dohmann, Anthi Katsiarimpa, Erika Isono, and Claus Schwechheimer*

Plant Systems Biology, Technische Universität München, 85354 Freising, Germany (J.P.H., R.R., A.K., E.I., C.S.); and Developmental Genetics, Center for Plant Molecular Biology, Tübingen University, 72076 Tuebingen, Germany (J.P.H., E.M.N.D., C.S.)

The conjugation of the ubiquitin-like modifier NEURAL PRECURSOR CELL-EXPRESSED DEVELOPMENTALLY DOWN-REGULATED PROTEIN8/RELATED TO UBIQUITIN1 (NEDD8/RUB1; neddylation) is best known as an important post-translational modification of the cullin subunits of cullin-RING-type E3 ubiquitin ligases (CRLs). MLN4924 has recently been described as an inhibitor of NEDD8-ACTIVATING ENZYME1 (NAE1) in human. Here, we show that MLN4924 is also an effective and specific inhibitor of NAE1 enzymes from Arabidopsis (Arabidopsis thaliana) and other plant species. We found that MLN4924-treated wild-type seedlings have phenotypes that are highly similar to phenotypes of mutants with a partial defect in neddylation and that such neddylation-defective mutants are hypersensitive to MLN4924 treatment. We further found that MLN4924 efficiently blocks the neddylation of cullins in Arabidopsis and that MLN4924 thereby interferes with the degradation of CRL substrates and their downstream responses. MLN4924 treatments also induce characteristic phenotypes in tomato (Solanum lycopersicum), Cardamine hirsuta, and Brachypodium distachyon. Interestingly, MLN4924 also blocks the neddylation of a number of other NEDD8-modified proteins. In summary, we show that MLN4924 is a versatile and specific neddylation inhibitor that will be a useful tool to examine the role of NEDD8- and CRL-dependent processes in a wide range of plant species.

The small ubiquitin-like modifier NEURAL PRECURSOR CELL-EXPRESSED DEVELOPMENTALLY DOWN-REGULATED PROTEIN8 (NEDD8) or RELATED TO UBIQUITIN (RUB), hitherto referred to as NEDD8, is the closest sequence paralog of ubiquitin in the family of ubiquitin-like proteins (Hochstrasser, 2009). NEDD8 is conjugated to target proteins via an enzymatic cascade that is composed of an E1 NEDD8-activating enzyme (a heterodimer composed of NEDD8-ACTIVATING ENZYME1 [NAE1] and a UBA3 subunit), an E2 conjugating enzyme (Ubc12p), and an E3 ligase (RING BOX1 [RBX1] or DEFECTIVE IN CULLIN NEDDYLATION1; Gong and Yeh, 1999; Gray et al., 2002; Kurz et al., 2005). Neddylation is best studied as a posttranslational modification of the cullin (CUL) subunits of CUL-RING-type E3 ubiquitin ligases (CRLs; Hori et al., 1999), where it has been shown that neddylation is important for the proper enzymatic function of CRLs (Hotton and Callis, 2008). CRLs are evolutionarily conserved E3 ligase complexes that share the CUL and RBX1 subunits and that, depending on the identity of the CUL subunit, associate with different adaptor and degradation substrate receptor modules, such as S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1) and F-box proteins to form CUL1-containing SCF-type E3 ligases or DNA DAMAGE-BINDING PROTEIN1 (DDB1) and DDB1-CUL4-ASSOCIATED FACTOR (DCAF) subunits to form CUL4-containing DCAF E3s (Hotton and Callis, 2008; Deshaies and Joazeiro, 2009). NEDD8 is hydrolyzed from target proteins by CUL-deneddylating enzymes including subunit 5 of the CONSTITUTIVELY PHOTORMOPHOCENIC SIGNALOSOME (Lyapina et al., 2001; Schwechheimer et al., 2001; Cope et al., 2002) or the deneddylase DEN1 (Gan-Erdene et al., 2003; Wu et al., 2003). Besides CULs, several other NEDD8-modified proteins have been reported from animal species, and in some cases a functional understanding of the role of their neddylation has been obtained (Rabut and Peter, 2008; Xirodimas, 2008). While the neddylation and deneddylation pathways are conserved in plants and the role of NEDD8 conjugation of plant CULs is well docu-
Neddylation was initially identified based on Arabidopsis (Arabidopsis thaliana) auxin resistant1 (axr1) mutants that are impaired in growth responses mediated by the phytohormone auxin (Leyser et al., 1993; del Pozo et al., 1998; Lammer et al., 1998; del Pozo and Estelle, 1999). In Arabidopsis, NEDD8 is known as RUB and is encoded by three different genes (Bostick et al., 2002; Dharmasiri et al., 2004); NAE is composed of the NAE1 subunit AXR1 or its homolog AXR1-LIKE (AXL) and the UBA3 subunit E1 C-TERMINAL RELATED (ECR1; del Pozo et al., 2002; Dharmasiri et al., 2007). axr1 and ecr1 mutants are insensitive to the root growth-inhibiting effect of the phytohormone auxin and have reduced root gravitropism (Leyser et al., 1993; Woodward et al., 2007). axr1 mutants are partially impaired in NEDD8 conjugation, and the effect of the axr1 mutation on NEDD8 conjugation and development is enhanced when the AXR1 homolog AXL is dysfunctional, resulting in embryo lethality (Dharmasiri et al., 2007; Hotton et al., 2011).

The auxin-related phenotypes of axr1 and ecr1 mutants are best explained by a functional impairment of SCF-type E3 complexes that are associated with the F-box protein TRANSPORT INHIBITOR RESISTANT1 (TIR1) and its homologs AUXIN-BINDING F-BOX1 (AFB1), AFB2, and AFB3 (Dharmasiri et al., 2005a, 2005b). SCFTIR1/AFB1-3 promotes the degradation of AUXIN/INDOLE ACETIC ACID (AUX/IAA) repressor proteins in response to auxin, and several AUX/IAA family members have been attributed roles in different auxin-dependent processes, including the initiation of root formation during embryogenesis, primary root growth, and root gravitropism (Reed, 2001; Lokser and Weijers, 2009). In the absence of auxin, AUX/IAAs repress the activity of AUXIN RESPONSE FACTOR (ARF) transcription factors. In the presence of auxin, AUX/IAAs are degraded and ARFs can regulate the expression of their target genes (Lokser and Weijers, 2009). As part of a negative feedback mechanism, the transcription of many AUX/IAAs is regulated by AUX/IAA–ARF regulons. Besides SCFTIR1/AFB1-3, CUL1-type E3s have been implicated in the degradation of DELLA regulators in response to the phytohormone GA [SCFSLEEPYL1; Dill et al., 2004] and the cell cycle inhibitor KIP1-RELATED PROTEIN1 (KRP1; [SCFSKIP2]; Ren et al., 2008). The best-characterized CUL4 CRL from Arabidopsis acts together with DDB1, CONSTITUTIVELY PHOTOMORPHOGENIC1, DEETIOLATED1, and SUPPRESSOR-OF-phyA-1051 to promote the degradation of the transcription factor ELONGATED HYPOCOTYL5 (HY5) that normally represses hypocotyl elongation in light-grown Arabidopsis seedlings (Osterlund et al., 2000; Yanagawa et al., 2004; Chen et al., 2006, 2010).

MLN4924 has recently been characterized as an inhibitor of human NAE (Soucy et al., 2009; Bennett et al., 2010) and as a candidate drug for the treatment of mammalian cancers (Mihollen et al., 2010; Swords et al., 2010). Structural studies revealed the molecular mode of function of MLN4924 as a mechanism-based inhibitor that acts through the formation of a NEDD8-AMP mimic (Brownell et al., 2010). These structural data have allowed us now to predict that MLN4924 may also be functional in plants. Here, we show that MLN4924 inhibits NEDD8 conjugation in Arabidopsis and other plant species and that MLN4924 treatment of wild-type seedlings induces morphological and molecular phenotypes that are reminiscent of those observed in mutants with partially impaired neddylation. Therefore, we propose that MLN4924 is an important tool that will allow studying the role of neddylation in the growth and development of Arabidopsis and other plant species.

RESULTS

Sequence Comparisons Predict an Inhibitory Role for MLN4924 in Arabidopsis

To be able to predict whether MLN4924 may function as a neddylation inhibitor in plants, we aligned the protein sequences of human NAE subunit UBA3 (HsUBA3) with those of their plant ECR1 and yeast UBA3p counterparts (Fig. 1A). The high sequence similarity between ECR1 and HsUBA3 as well as the previously published structure of the NEDD8-MLN4924 adduct bound to NAE allowed us also to model the interaction of Arabidopsis ECR1 with MLN4924 (Fig. 1B; Brownell et al., 2010). Both approaches, sequence alignment and structure prediction, indicated that the amino acid residues required for MLN4924 interaction are conserved between the human and Arabidopsis NAE subunits and that their predicted spatial orientation and positions in ECR1 may allow binding of MLN4924 in the same way as proposed for HsUBA3.

MLN4924 Is an Inhibitor of Neddylation in Arabidopsis

In order to examine the effect of MLN4924 on neddylation in plants, we established transgenic lines that express hemagglutinin (HA)-STREPII-tagged NEDD8 (HSN) under the control of a dexamethasone (Dex)-inducible promoter (Aoyama and Chua, 1997). Following Dex induction, we found that HSN is efficiently translated and conjugated to proteins (Fig. 2A). We then also showed in a western blot with an anti-CUL1 antibody that the most prominent NEDD8-modified protein comigrates with CUL1 and, based on this observation, that this prominent neddylated band corresponds to NEDD8-modified CUL1 (Supplemental Fig. S1). Since the NEDD8 conjugation was efficiently suppressed in an MLN4924 dose-dependent manner (Fig. 2B), we judged that MLN4924 inhibits NEDD8 conjugation, most likely by inhibiting ECR1 function.

Since NEDD8 and ubiquitin as well as NAE1 and ubiquitin-activating enzymes are highly related proteins (Fig. 1), we also wanted to examine whether MLN4924 blocks ubiquitin-activating enzymes and...
ubiquitin conjugation. To this end, we generated transgenic lines for the Dex-inducible expression of HA-STREPII-tagged ubiquitin (HSUB). Consistent with the multitude of known and expected ubiquitylation substrates and the fact that ubiquitin is known to form polyubiquitin chains of different lengths and topologies, we observed the accumulation of HSUB conjugates of varying lengths as well as monomeric HSUB following Dex induction (Fig. 2C). Since we detected only a comparatively moderate reduction of ubiquitylation when we applied elevated concentrations of MLN4924 to the Dex-induced HSUB transgenic seedlings, we concluded that MLN4924 blocks neddylation more efficiently than ubiquitylation (Fig. 2D). Because the inhibition of neddylation impairs CRL E3 ligase activity and since our in vivo assay does not allow us to distinguish between direct and indirect effects of MLN4924, we cannot exclude the possibility that the observed reduction in ubiquitylation may be the result of this reduced E3 ligase activity rather than the result of a direct inhibition of the ubiquitin-activating enzymes. This possibility is also supported by the fact that several amino acids required for MLN4924 binding in NAE1 are not conserved in the animal and plant UBA1 ubiquitin-activating enzymes (Fig. 1) and that in vitro studies had shown that MLN4924 is more specific for human NAE1 than for human UBA1 (Soucy et al., 2009). In combination, our physiological and biochemical data thus support the conclusion that MLN4924 is an inhibitor of NAE in Arabidopsis.

Plants May Have Additional MLN4924-Sensitive NEDD8-Conjugated Proteins

Following Dex induction and detection of HSN using an anti-HA antibody, we noticed that plants expressing HSN accumulate a number of other HSN conjugates besides CULs, which we purified using the STREPII tag of HSN and analyzed by mass spectrometry. This analysis identified NEDD8, subunits of the neddylation machinery (AXR1, AXL, ECR1), as well as all Arabidopsis CULs, providing proof of an overall successful purification of neddylated proteins (Supplemental Table S1). Interestingly, we also identified a range of proteins that had previously not been identified as neddylated proteins, which could be grouped into two categories. First, we identified proteins that may have been copurified with the above-mentioned proteins because they interact or are likely to interact with NEDD8 or NEDD8-modified CULs in CRL complexes (e.g. RBX1, SKP1, F-box proteins, DCAF proteins, or components of the ubiquitin-proteasome pathway;
Supplemental Table S1). Second, we identified proteins that are not functionally connected to the ubiquitin-proteasome system, such as proteins involved in protein folding, protein synthesis, intracellular transport, signal transduction, as well as proteins with metabolic functions (Supplemental Table S1). We thus hypothesize that these proteins are either NEDD8 modified or are associated with NEDD8-modified proteins.

MLN4924 Treatment Induces the Auxin Insensitivity Phenotype of Arabidopsis Neddylation Mutants

Although AXR1 mediates CRL neddylation and activity at a global level, axr1 mutants have a number of phenotypes that are characteristic for mutants impaired in the auxin receptor CRL SCF^{TIR1/AFB2-4}. axr1 mutants are auxin insensitive and have reduced gravitropism in...
root growth; they also fail to efficiently degrade AUX/IAA repressors and are consequently impaired in auxin-induced gene expression (Lincoln et al., 1990; Gray et al., 2001). We reasoned that MLN4924 treatments should lead to a phenocopy of the axr1 phenotype in the wild type. Indeed, when we grew wild-type Arabidopsis seedlings on MLN4924 in the presence of critical concentrations of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), we noted that MLN4924 treatment confers auxin insensitivity to the roots of wild-type seedlings (Fig. 3A; Supplemental Fig. S2). In addition, we also observed a significant reduction of root growth following MLN4924 treatment at elevated concentrations (greater than 5 μM) as well as agravitropic root growth (Fig. 3B and C; Supplemental Fig. S2). We thus concluded that MLN4924 treatment induces the auxin-insensitive and agravitropic root growth of neddylation-deficient axr1 mutants. The strong root growth reduction of MLN4924-treated seedlings may be the consequence of the inhibition of both NAEs, AXR1 and AXL, rather than the inhibition of AXR1 alone. Similar root growth defects, for example, have been reported for an axr1-12/s-12 axl-1/AXL mutant (Dharmasiri et al., 2007).

We next examined whether MLN4924 treatment leads to a stabilization of AUX/IAA repressors, which may be explained by the inhibition of SCFTIR1/AFB1-3 by MLN4924. Indeed, we observed an MLN4924-dependent accumulation of the AUX/IAA protein BODENLOS (BDL)/IAA12 by examining a transgenic line expressing a translational fusion between BDL and the reporter GUS (BDL:BDL:GUS; Fig. 3D; Weijers et al., 2005). We further observed that the transcripts of two representative auxin-induced genes, IAA3 and IAA13, whose expression is normally repressed by AUX/IAAs and induced following AUX/IAA degradation, is compromised in MLN4924-treated wild-type seedlings and even more so in the genetically sensitized axr1-12 mutants (Fig. 3E). In addition, we found that the auxin induction of the established and widely used auxin response reporter DR5:GUS was compromised following MLN4924 treatment (Fig. 3F; Sabatini et al., 1999). We thus conclude that MLN4924 treatment of wild-type seedlings mimics the phenotypes of Arabi-
dopsis neddylation pathway mutants at the morphological, physiological, and molecular levels. MLN4924 may thus act as an inhibitor of neddylation in Arabidopsis and thereby impair SCF<sup>SKP1b/AFB3</sup> and auxin responses.

**MLN4924 Inhibits the Function of Other CRLs**

We next tested whether the predicted inhibition of neddylation by MLN4924 also affects the degradation of other CRL E3 ligases. The deetiolation of seedlings in response to light is a well-characterized process, which requires the activity of a CUL4-containing CRL that promotes the degradation of photomorphogenesis regulators such as HY5 in the dark (Osterlund et al., 2000; Holm et al., 2002). While dark-grown etiolated seedlings are characterized by an elongated hypocotyl, closed cotyledons, and an apical hook, light-grown deetiolated seedlings have a short hypocotyl, open cotyledons, and express a large set of light-induced genes. With MLN4924 treatment, we were able to induce deetiolated seedling growth in dark-grown wild-type seedlings (Fig. 4, A and B). The even stronger decrease in hypocotyl elongation together with the visible increase in anthocyanin accumulation suggested that axr1 as well as ecrl mutants are already sensitized in this pathway (Fig. 4, A and B; Leyser et al., 1993; Woodward et al., 2007). We then also found that HY5 protein but not HY5 transcript level increases in dark-grown MLN4924-treated seedlings and that this correlates with an increase in transcript abundance of light-induced genes in these seedlings (Fig. 4, C–E). These findings suggest that MLN4924 treatment promotes deetiolation in dark-grown seedlings, which may be explained by a dysfunction of CUL4-containing CRLs that normally repress this process in the dark (Chen et al., 2006, 2010).

In order to confirm the effect of MLN4924 on additional CRLs, we also examined the behavior of the SCF<sup>SLY1</sup> substrate and DELLA repressor RGA as well as that of the SCF<sup>SKP2b</sup> substrate and cell cycle regulator KRP1 following MLN4924 treatment (Dill et al., 2004; Ren et al., 2008). Using immunoblots, we found that RGA accumulates in MLN4924-treated but not in untreated wild-type seedlings and that its GA-induced degradation is compromised after MLN4924 treatment (Fig. 5A). In agreement with an impairment of SCF<sup>SKP2b</sup> function, we observed that a translational fusion protein of KRP1 to GUS (KRP1::KRP1:GUS) is stabilized in wild-type seedlings treated with MLN4924 or the proteasome inhibitor MG132 (Fig. 5B). We thus concluded that MLN4924 treatment also impairs other CRL-dependent protein degradation events and growth responses. MLN4924, therefore, may act as a general inhibitor of CRL-dependent processes by inhibiting neddylation.

**MLN4924 Inhibits Neddylation in Other Plant Species**

The availability of MLN4924 as a neddylation inhibitor would allow examining the role of this posttranslational modification also in species where neddylation-defective mutants are not available. Our sequence comparisons had revealed that MLN4924-binding sites are conserved in all plant ECR1 proteins available in the databases (Fig. 1). To test this, we examined the effects of MLN4924 on plant development by treating *Cardamine hirsuta*, tomato (*Solanum lycopersicum*), and purple false brome (*Brachypodium distachyon*) with MLN4924. For each of the tested plant species, we observed in part overlapping and in part distinct growth phenotypes. In all species, we noted a dosage-dependent reduction in primary root growth, lateral root growth, and lateral root initiation, suggesting that these processes require neddylation for proper function (Fig. 6, A–D). We also noted that the sensitivity toward the inhibitor was different for each species and that different critical concentrations were required to induce similar phenotypes in the three species tested. Interestingly, *Cardamine*, similar to Arabidopsis but not tomato or *Brachypodium*, showed a strong induction of root hair formation in response to MLN4924 treatment (Fig. 6, E and F). On the other hand, *Cardamine*, unlike Arabidopsis, tomato, and *Brachypodium*, was at least partially insensitive to MLN4924 with regard to gravitropic growth (Supplemental Fig. S3). In all cases, the observed phenotypes may be attributed to the stabilization of CRL substrates or the dysfunctional NEDD8 conjugation of hitherto uncharacterized NEDD8 conjugation substrates. Since the available NEDD8 antibody from Arabidopsis cross-reacts with NEDD8 from other species, we were able to demonstrate a reduction in *Cardamine* culin neddylation after MLN4924 treatment on immunoblots (Fig. 6E). We thus conclude

![Figure 5](image-url)
that MLN4924 is a functional NEDD8 inhibitor in plants that can now be used for developmental and comparative studies on neddylation in plant species other than Arabidopsis.

**DISCUSSION AND CONCLUSION**

In this paper, we evaluate the role and mode of action of the compound MLN4924, which had previously been shown to inhibit NEDD8 conjugation in mammalian cells. Plant responses to the phytohormone auxin and light are both well-established processes that require the activity of NEDD8-dependent CRL E3 ubiquitin ligases. In a set of physiological and molecular experiments, we demonstrate that the application of MLN4924 to wild-type Arabidopsis seedlings produces phenotypes that strongly support the conclusion that MLN4924 inhibits neddylation and CRL function in this plant species. Furthermore, we find that mutants with a partial deficiency in neddylation are hypersensitive to MLN4924 and that MLN4924 treatment of wild-type seedlings induces growth phenotypes that are strongly reminiscent of those observed in the neddylation mutants. Finally, we show that NEDD8 conjugation to CULs and other proteins is blocked when MLN4924 is applied to Arabidopsis seedlings expressing a tagged version of NEDD8. Since the amino acid residues required for MLN4924 binding are conserved in all plant ECR1 orthologous sequences available to date (Fig. 1), we hypothesize that MLN4924 may be functional in all plant species. Interestingly, we also noted that several MLN4924-interacting amino acids are not conserved in the budding and fission yeast Uba3p counterparts, suggesting that MLN4924 may not bind to yeast Uba3p proteins (Fig. 1A). In summary, our data suggest that NEDD8 is a functional neddylation inhibitor in plants. To date, studies on the role of neddylation for plant growth could only be performed with genetic mutants that are impaired in NEDD8 conjugation. Our results now open the possibility to use MLN4924 to examine the requirement of neddylation in any plant species and at any stage of growth and development, thus facilitating interspecies comparisons of the regulation of developmental processes.

Particularly in plants, the availability of a small molecule inhibitor that interferes with NEDD8 conjugation, and thereby also with the function of CRL-type E3 ubiquitin ligases, is of importance. First, it is appar-

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**Figure 6.** MLN4924 induces characteristic phenotypes in plant species. A to D, Photographs of Arabidopsis (A), Cardamine (B), tomato (C), and Brachypodium (D) seedlings grown on MLN4924. Eight-day-old Arabidopsis seedlings were germinated on MLN4924-containing medium. Cardamine (14 d old) and tomato (10 d old) were grown for 6 d before transfer to MLN4924-containing medium. Brachypodium (11 d old) was grown for 1 d before transfer to MLN4924-containing medium. E, Root segments of 8-d-old Arabidopsis seedlings grown in the absence and presence of MLN4924. Bar = 200 μm. F, Root segments of 14-d-old Cardamine seedlings grown in the absence and presence of MLN4924. Seedlings were germinated without MLN4924 for 6 d and then transferred to MLN4924. Bar = 2 mm. G, Immunoblot detection of NEDD8-conjugated CULs from Cardamine using an anti-NEDD8 antibody. Coomassie Brilliant Blue (CBB) staining served as a loading control. [See online article for color version of this figure.]
ent from genome-wide analyses in Arabidopsis that the different CRL families with CUL1, CUL3, and CUL4 subunits have, in comparison with yeast and mammalian species, greatly expanded in plants; thus, it can be anticipated that CRL-dependent protein degradation plays an important role in plant development and plant physiology (Gagne et al., 2002; Figueroa et al., 2005; Bernhardt et al., 2006; Chen et al., 2006; Jain et al., 2007). This has been analyzed in great detail in Arabidopsis (Bernhardt et al., 2006; Chen et al., 2006; Jain et al., 2007). To date, only a few CRL functions are known in plants, and the roles of only a very small number of these CRLs, such as SCF TIR1/AFB2-4 and SCF SLY1, are understood at the molecular level (Schwechheimer et al., 2009). This may be due to the fact that specific CRL functions, and in this particular example, F-box protein functions, cannot easily be identified with genetic mutants due to the functional redundancy of individual CRLs and F-box proteins. With MLN4924, it will now be possible to test for the involvement of a CRL in a particular growth process or response.

Second, MLN4924 will allow testing for the stability of proteins that are destined for degradation by CRLs and the ubiquitin-proteasome system. The anticipated large number of CRLs suggests that a similarly large number of substrate proteins exist that are targeted for proteasomal degradation by these CRLs. However, to date, only a few proteins have been shown to be regulated by CRLs and the proteasome pathway (Schwechheimer et al., 2009). This can be attributed at least in part to the restricted number of genetic tools but also to the restricted number of chemical tools that are available for this field of plant research. Commonly, 26S proteasome inhibitors, most importantly MG132, are being used in plant studies to demonstrate that selected proteins are turned over by the 26S proteasome (Schwechheimer et al., 2009). The availability of MLN4924 will now allow for specific, and possibly also more efficient, blocking of CRL-dependent protein turnover and thereby allow determining whether protein instability is promoted by a CUL-containing E3 ligase.

Our observation that proteins other than CULs are modified by NEDD8 in plants is intriguing (Fig. 5; Supplemental Table S1). Importantly, several studies conducted in animal cells also report a similar accumulation of NEDD8-modified proteins, suggesting that many NEDD8 targets remain to be identified (Norman and Shiekhattar, 2006; Chan et al., 2008). Few of these proteins have been characterized as yet, and to date, besides CULs, only a very limited number of NEDD8 targets are known from mammalian studies. In plants, no NEDD8 conjugates other than CULs have been identified as yet. This is in sharp contrast to the related modifiers ubiquitin and SMALL UBQUITIN-LIKE MODIFIER, for which a steadily increasing number of target proteins are being identified and for which a diverse range of regulatory functions are continuously being discovered (Ravid and Hochstrasser, 2008; Acconcia et al., 2009; Bergink and Jentsch, 2009). This invites the hypothesis that many NEDD8 targets still remain to be identified. The most prominent studies of the known non-CUL NEDD8 targets are studies that demonstrate the neddylation of the tumor suppressor protein p53 and the ribosomal protein L11 (Xirodimas et al., 2004, 2008). In the case of p53, neddylation is mediated by Mdm2, and neddylated p53 has reduced transcriptional activity (Xirodimas et al., 2004). Neddylation was also reported for ribosomal proteins (Xirodimas et al., 2008), and specifically L11 was shown to be deneddylated in response to nucleolar stress and to then relocalize from the nucleolus to the cytoplasm. In addition, other mammalian proteins such as APP1 (Lee et al., 2008), BCA3 (Gao et al., 2006), EGFR (Oved et al., 2006), and pVHL (Stickle et al., 2004; Russell and Ohh, 2008) have been described to be neddylated, and regulatory roles have been attributed to the NEDD8 modification. Future research will now allow for the confirmation and examination of the role of NEDD8 as a modifier of the set of putative NEDD8-modified proteins presented in this study.

MATERIALS AND METHODS

Biological Material and Physiological Experiments

Arabidopsis (Arabidopsis thaliana) ecotype Columbia as well as previously published mutants as referenced in the text were used for all physiological experiments. DR5:GUS, BDL::BDL:GUS, KRP1:GUS, and KRP1::KRP1:GUS were obtained from Tom Guilfoyle (University of Columbia), Dolf Weijers (Wageningen Agricultural University), and Mark Estelle (University of San Diego), respectively. Brachypodium distachyon, Cardamine hirsuta, and tomato (Solanum lycopersicum ‘Moneymaker’) wild-type seeds were obtained from Monika Kavanova (Technische Universität München), Miltos Tsiantis (Oxford University), and Catarina Brancato (Universität Tübingen), respectively.

Quantitative Real-Time PCR

Quantitative RT-PCR was performed as described previously (Richter et al., 2010). For auxin response assays, RNA was extracted from 8-d-old seedlings that had been grown on standard growth medium (GM + 1% Suc) supplemented with 50 μM MLN4924 and that were treated for 45 min with 50 μM 2,4-D. For the analysis of light-induced gene expression, RNA was extracted from 4-d-old dark- and light-grown seedlings that had, where indicated, been grown on 10 μM MLN4924. Primer sequences are listed in Supplemental Table S2.

Cloning Procedures

The HSN construct was generated by overlap extension PCR with the primers HSN-FW1 and HSN-RV1 using the vector pXCS-HASstep as a template for the STREPII-HA tag (a gift from Tina Romeis) and HSN-FW2 and HSN-RV2 using Arabidopsis cDNA as a template for NEDD8/RUB1 (ATIG31340). The fusion construct was cloned in pTA7002. The HSUB construct was generated in a similar manner with the primers HSUB-FW1 and HSUB-RV1 as well as HSUB-FW2 and HSUB-RV2. Primer sequences are listed in Supplemental Table S2. HSN and HSUB were transformed into Arabidopsis wild-type plants using the floral dip method and selected using standard protocols (Clough and Bent, 1998).

Immunoblot

Immunoblotting was performed according to standard protocols. The following antibodies were used for protein detection: anti-HA-peroxidase.

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spectrometry was performed using an nLC-LTQ-Orbitrap tandem mass buffer, and boiled for 5 min. The solution was subjected to SDS-PAGE, and the gift of MLN4924 from Millennium Pharmaceuticals (Cambridge, MA). for preparing the HSUB construct, and Jeff Boeren (Biqualys, Wageningen, a 15-h induction with 30 m
M Dex in liquid GM/0.01% Tween 20. Ten grams of seedlings were extracted from liquid nitrogen-frozen samples at 4°C with extraction buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10 mM dithiothreitol [DTT], 5 mM N-ethylmaleimide, 100 μg mL
-1 avidin, and plant protease inhibitor cocktail [Sigma-Aldrich]) using a HomGen homogenizer (Schuett). After centrifugation at 50,000 g and 4°C for 30 min, the supernatant was loaded on a 1-mL Stre'pTactin affinity column (IBA). The column was washed five times with ice-cold washing buffer (100 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, pH 8.0, 150 mM NaCl, 2 mM DTT, and 0.05% Triton X-100) and then with ice-cold washing buffer without Triton X-100. Proteins were eluted with elution buffer (100 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.5 mM EDTA, pH 8.0, 150 mM NaCl, 2 mM DTT, and 4%/C176 precipitated with acetone/0.2% DTT at
-20°C overnight. After centrifugation at 10,000 g and 4°C for 15 min, the pellet was dried, dissolved in 2× Laemmli buffer, and boiled for 5 min. The solution was subjected to SDS-PAGE, and the samples were prepared for liquid chromatography–tandem mass spectrometry as described elsewhere (http://biochemistry.wur.nl/Biochem/). Mass spectrometry was performed using an nLC-LTQ-Orbitrap tandem mass spectrometer at Biqualys, and the data were analyzed using the Bioworks software (ThermoFisher). The same procedure was performed with Columbia wild-type seedlings as a negative control.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Immunoblot with an anti-CUL1 antibody of total protein extracts prepared from MLN4924-treated wild-type and axr1 mutant seedlings.

Supplemental Figure S2. MLN4924 treatment induces auxin-insensitive growth in Arabidopsis wild-type seedlings.

Supplemental Figure S3. MLN4924 induces agravitropic root growth in different plant species.

Supplemental Table S1. List of proteins identified by mass spectrometry following purification of HSN.

Supplemental Table S2. List of primers employed in this study.

ACKNOWLEDGMENTS

We thank Julia Mengner (Technische Universität München) for critical comments on the manuscript, Katrin Schachtel (Technische Universität München) for preparing the HSUB construct, and Jeff Boeren (Biqualys, Wageningen, The Netherlands) for mass spectrometric analysis. We greatly appreciate the gift of MLN4924 from Millennium Pharmaceuticals (Cambridge, MA).

Received March 19, 2011; accepted April 28, 2011; published April 28, 2011.

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