Loss of the Acetyltransferase NAA50 Induces Endoplasmic Reticulum Stress and Immune Responses and Suppresses Growth

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Stress signaling in plants is carefully regulated to ensure proper development and reproductive fitness. Overactive defense signaling can result in dwarfism as well as developmental defects. In addition to requiring a substantial amount of energy, plant stress responses place a burden upon the cellular machinery, which can result in the accumulation of misfolded proteins and endoplasmic reticulum (ER) stress. Negative regulators of stress signaling, such as ENHANCED DISEASE RESISTANCE 1 (EDR1), ensure that stress responses are properly suspended when they are not needed, thereby conserving energy for growth and development. Here, we describe the role of an uncharacterized N-terminal acetyltransferase, NAA50, in the regulation of plant development and stress responses in Arabidopsis (Arabidopsis thaliana). Our results demonstrate that NAA50, an interactor of EDR1, plays an important role in regulating the tradeoff between plant growth and defense. Plants lacking NAA50 display severe developmental defects as well as induced stress responses. Reduction of NAA50 expression results in arrested stem and root growth as well as senescence. Furthermore, our results demonstrate that the loss of NAA50 results in constitutive ER stress signaling, indicating that NAA50 may be required for the suppression of ER stress. This work establishes NAA50 as essential for plant development and the suppression of stress responses, potentially through the regulation of ER stress.

As sessile organisms, plants frequently encounter and respond to stress conditions such as drought, salinity, heat, and microbial infection. Various adaptations enable plants to defend themselves against these stresses; however, they often come at a significant cost (Cipollini et al., 2014). Plant defense responses require sacrifices by infected cells and tissues, which can negatively impact plant growth. The hypersensitive response, a form of programmed cell death, is a primary mode of defense for infected plant cells (Greenberg and Yao, 2004). Thus, plants must carefully tailor their defense responses to conserve energy for growth and reproduction (Huot et al., 2014). This trade-off is exhibited by enhanced resistance mutants such as suppressor of npr1 constitutive1-1 (snc1-1) and constitutive pr1 (cpr1), which have constitutively active defense responses and are dwarfed (Bowling et al., 1994; Li et al., 2001).

Stress responses place strain upon the cellular machinery, which can result in endoplasmic reticulum (ER) stress (Bao and Howell, 2017). ER stress can occur during biotic or abiotic stress, as well as during normal developmental processes that place increased demands on the protein translation and protein secretion machinery (Vitale and Boston, 2008). Response to ER stress is mediated by the unfolded protein response (UPR), which occurs in two phases. The first phase aims to alleviate ER stress through increased expression of chaperones, removal and degradation of misfolded proteins from the ER, and reduction of protein translation (Liu and Howell, 2010; Williams et al., 2014). If these attempts are unsuccessful, the UPR transitions into a proapoptotic phase (Walter and Ron, 2011; Woehlbier and Hetz, 2011; Srivastava et al., 2018). Recent studies have demonstrated that UPR genes are required for plant growth and development (Kim et al., 2018; Bao et al., 2019). On the other hand, mutations that constitutively activate the UPR cause dwarfism (Iwata et al., 2018). Just as stress responses to external stimuli must be regulated to ensure proper growth and development, so must responses to internal stress and the UPR.

We have previously identified and characterized the ENHANCED DISEASE RESISTANCE1 (EDR1) gene and demonstrated its role in negatively regulating plant
Interestingly, function impacts a diversity of plant stress responses. Mutant edr1 plants display enhanced sensitivity to a variety of stimuli, including drought, pathogen infection, abscisic acid, and ethylene (Frye and Innes, 1998; Frye et al., 2001; Tang et al., 2005). The variety of edr1-related phenotypes implies that EDR1 function impacts a diversity of plant stress responses. Interestingly, edr1 plants appear phenotypically wild type in the absence of external stresses. This transitory requirement of EDR1 indicates that it may be functionally active only after a stress response has been induced.

There remain many unanswered questions regarding EDR1 function. EDR1 encodes a protein kinase bearing similarity to Raf-like MEK kinases (Frye et al., 2001), yet no in vivo substrates of EDR1 have been identified. EDR1 is believed to negatively regulate KEEP ON GOING, an E3 ubiquitin ligase required for postembryonic development and endomembrane trafficking (Wawrzynska et al., 2008; Gu and Innes, 2011; Gu and Innes, 2012). However, it is unclear whether EDR1 itself is a regulator of development or endomembrane trafficking. Interestingly, EDR1 primarily localizes to the ER, yet no ER-associated function of EDR1 has been demonstrated (Christiansen et al., 2011).

To gain a greater understanding of EDR1 function, we performed a yeast two-hybrid screen to identify potential substrates of EDR1. These screens yielded a particularly interesting hit, At5g11340, a predicted N-terminal acetyltransferase (NAT) that bears similarity to the human Naa50 protein (Fig. 1).

Most NATs function as the catalytic component of larger complexes, designated as NatA-F in humans, to modify substrate proteins in a cotranslational manner (reviewed in Polevoda et al., 2009; Aksnes et al., 2016). However, human Naa80 (also known as NatH) appears to function by itself to modify the N terminus of actin subunits using a posttranslational mechanism (Drazic et al., 2018). Similarly, the Arabidopsis (Arabidopsis thaliana) protein AtNAA70 (NatG), which localizes to plastids, does not appear to require additional plant proteins for function, because overexpression of this protein inside Escherichia coli induces N-terminal acetylation (NTA) of over 120 different E. coli proteins (Dinh et al., 2015). Human Naa50 serves as the catalytic component of the NatE complex, which also includes the Naa10 and Naa15 subunits (Arnesen et al., 2006). Naa10, Naa15, and Naa50 are also found in the NatA complex, for which Naa10 provides catalytic function. NAT complexes mediate NTA, a widespread cotranslational protein modification believed to affect the majority of eukaryotic proteins (Brown and Roberts, 1976; Polevoda and Sherman, 2003; Arnesen et al., 2009). These targets unique N-terminal sequences. NAT specificity is largely influenced by the two most N-terminal residues of a given peptide (Polevoda et al., 2009). Human Naa50 preferentially targets N-termini that have retained their initiator Met and have a hydrophobic residue in the second position (Evjenth et al., 2009; Van Damme et al., 2011).

Based on work in yeast (Saccharomyces cerevisiae) and humans (Homo sapiens), there is a solid biochemical understanding of how NATs function; however, the purpose of NTA is not well understood. Emerging evidence suggests that NTA serves various functions. In humans, the Golgi-localized Naa60 specifically targets transmembrane proteins and is required for the maintenance of Golgi integrity (Aksnes et al., 2015). In yeast, NTA mediated by NatC is required for the recruitment of the Arl3p GTPase to the Golgi (Behnia et al., 2004; Setty et al., 2004). Recent work in plants has implicated NTA in the regulation of stress responses and development. Both NAA10 and NAA15 are essential for plant embryonic development, and knockdown of either results in morphological defects and drought resistance (Feng et al., 2016; Linster et al., 2015). Differential NTA of the SUPPRESSOR OF npr1 CONSTITUTIVE1 (SNCl1) protein was found to affect its accumulation, demonstrating a role for NTA in the regulation of defense signaling (Xu et al., 2015). In addition, NatB-mediated NTA of the transcriptional coregulator SIGMA FACTOR-BINDING PROTEIN1 stabilizes this protein, leading to enhanced expression of a subset of defense-related genes, and repression of photosynthesis-associated genes (Li et al., 2020). Plant NATs bear strong similarity to their nonplant orthologs; however, the discovery of the plant-specific, plastid-localized NatG indicates that NTA in plants may serve unique purposes (Dinh et al., 2015). This early work demonstrates that NTA plays an important role in plant physiology and stress responses. However, many aspects of plant NATs have yet to be investigated.

Here, we demonstrate a role for the uncharacterized Arabidopsis NAA50 gene in regulating plant growth and stress responses. Using knockout and transgenic knockdown lines, we show that NAA50 is indispensable for normal plant growth and development. Loss of NAA50 triggers defense response pathways in Arabidopsis, implicating NAA50 in the negative regulation of defense signaling. Loss of NAA50 also induces constitutive ER stress, whereas loss of EDR1 leads to enhanced sensitivity to ER stress. Thus, EDR1 and NAA50 may be involved in the negative regulation of ER stress. This work demonstrates the importance of NAA50 in plant stress responses and development, as well as a potential link between NAA50 and ER stress.

RESULTS

NAA50 Interacts with EDR1

To verify the initial yeast two-hybrid screen that identified NAA50 as a potential interactor of EDR1, we performed additional assays to detect protein-protein interaction. To test for physical interactions between EDR1 and potential substrates, we used a “substrate-trap”
Figure 1. NAA50 physically interacts with EDR1. A, Naa50 is conserved in Arabidopsis. Amino acid alignment depicting Arabidopsis NAA50 and human Naa50. This alignment was generated using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and visualized in Jalview (Waterhouse et al., 2009). Individual residues are colored based upon the Clustal color scheme, which assigns color to residues where amino acid category is conserved. B, EDR1ST interacts with NAA50 in yeast two-hybrid. AD, GAL4 activation domain fusion; BD, GAL4 DNA binding domain fusion; LAM, lamin; T, SV40 large T antigen. C, Immunoblot analysis of yeast strains from B. EDR1-BD accumulated poorly in yeast, and a significant accumulation of degraded EDR1-BD (*) was visible. D, NAA50 coimmunoprecipitates with EDR1. The indicated constructs were transiently expressed in N. benthamiana and then immunoprecipitated using GFP-Trap beads. sYFP-tagged MYC was used as a negative control. E, NAA50 colocalizes with the ER marker SDF2. mCherry-tagged NAA50 and GFP-tagged SDF2 were transiently coexpressed in N. benthamiana. Bars = 50 μm. These experiments were repeated three times with similar results.
mutant of EDR1, EDR1ST (Gu and Innes, 2011). EDR1ST contains a D810A substitution in the phosphotransfer domain, which is necessary for substrate phosphorylation, thus stabilizing the potential interaction between EDR1 and potential substrates (Gibbs and Zoller, 1991). Our initial yeast two-hybrid screen was carried out using EDR1ST as bait. In the yeast two-hybrid assay, NAA50 was found to physically interact with EDR1ST, but not with wild-type EDR1 (Fig. 1B). This result indicates that NAA50 may be a substrate of EDR1. However, immunoblotting demonstrated that wild-type EDR1 accumulation is substantially lower than that of EDR1ST in yeast, potentially explaining the absence of an interaction (Fig. 1C). Coimmunoprecipitation using proteins expressed transiently in Nicotiana benthamiana demonstrated that NAA50 physically associates with both EDR1 and EDR1ST in vivo, contrasting with our yeast two-hybrid results (Fig. 1D). We did not observe NAA50 coimmunoprecipitating with the super Yellow Fluorescent Protein2 (sYFP)-tagged MYC negative control, demonstrating that this interaction is specific (Fig. 1D). EDR1 has been previously demonstrated to localize to the ER (Christiansen et al., 2011). We similarly observed an ER localization of NAA50 tagged with mCherry when transiently expressed in N. benthamiana (Fig. 1E). NAA50 was found to colocalize with the GFP-tagged ER marker STROMAL-DERIVED FACTOR-2 (SDF2; Nekrasov et al., 2009), indicating that NAA50 at least partially localizes to the ER. These experiments indicate that EDR1 and NAA50 physically interact and that both proteins partially localize to the ER.

Arabidopsis NAA50 Is Highly Conserved and Essential for Development

The discovery that NAA50 physically interacts with EDR1 prompted us to investigate its potential functions in Arabidopsis. There is a 51.25% identity match between Arabidopsis and human NAA50 proteins (Fig. 1A). This high degree of sequence similarity indicates that NAA50 function is likely conserved between plants and animals.

To investigate the role of NAA50 in plants, we characterized two T-DNA insertion mutants (SAIL_1210_A02 and SAIL_1186_A03), which we designated naa50-1 and naa50-2, respectively. The naa50-1 mutant contains a T-DNA insertion in intron 2, while naa50-2 contains a T-DNA insertion in intron 1, and neither produces detectable full-length transcript or NAA50 protein (Armbruster et al., 2020). We confirmed the presence of homozygous T-DNA insertions in NAA50 in each line using PCR-based genotyping (see “Materials and Methods’’). Both mutant lines were found to be severely dwarfed compared to wild-type plants (Fig. 2, A and B). Knockout naa50 seedlings displayed abnormal and dwarfed growth (Fig. 2A). As they developed, naa50 plants remained dwarfed and were sterile, although stems and flowers did form (Fig. 2C).

We were able to fully complement the naa50-1 mutant phenotypes by transformation of a transgene carrying NAA50 tagged with sYFP under the control of the native NAA50 promoter, demonstrating that loss of NAA50 is responsible for the dwarf phenotype and sterility (Fig. 2B). These observations establish that NAA50 is essential for normal plant growth and development.

Loss of NAA50 Alters Plant Growth

In addition to being dwarfed, naa50 seedlings displayed a variety of developmental phenotypes. Root morphology was found to be altered in naa50 plants, with root hairs and root cell morphology appearing irregular (Fig. 3A). This led us to hypothesize that loss of NAA50 may result in altered vacuole development. Loss of KEEP ON GOING, another EDR1-interacting protein, has been shown to result in altered vacuolar development (Gu and Innes, 2012). In naa50-1 seedlings expressing the tonoplast marker GAMMA TONO-PLAST INTRINSIC PROTEIN (γTIP; Nelson et al., 2007), altered vacuole shape was observed (Fig. 3B). Many naa50-1 vacuoles appeared fractured and contained many “blebs”, similar to those observed in keg mutant seedlings (Gu and Innes, 2012). Additionally, naa50-1 root cells were larger and irregularly shaped. This could indicate that NAA50 is involved in vacuole maturation.

The severe dwarfing and sterility of naa50-1 homozygous mutant plants compromised our ability to study the role of NAA50 in later stages of plant development. To overcome this limitation, we generated transgenic plants in which NAA50 expression can be reduced based on the expression of an artificial micro-RNA (amiRNA) driven by a dexamethasone-inducible promoter (DEX:NAA50-ami). As a control, we utilized a scrambled amiRNA line (DEX:Scrambled-ami), which contains a dexamethasone-inducible amiRNA with no predicted targets. We generated ten transgenic lines carrying this DEX:NAA50-ami construct, and observed that six of these displayed obvious chlorotic and necrotic leaves in the T2 generation by 7 d post treatment with dexamethasone (Supplemental Fig. S1). We selected one of these lines (no. 7) for further analyses. Quantification of NAA50 mRNA levels in rosette leaves of homozygous T3 plants from this line revealed an approximately 85% reduction in NAA50 transcripts by 16 h post amiRNA induction (Fig. 3C).

Reduced expression of NAA50 in the DEX:NAA50-ami plants caused severe morphological changes. Growth of DEX:NAA50-ami seedlings on Murashige and Skoog (MS) media supplemented with dexamethasone increased the length of root hairs, recapitulating the naa50 root hair phenotype (Fig. 3D). Additionally, dexamethasone treatment caused DEX:NAA50-ami seedlings to grow slower than the control lines, resulting in shorter roots (Fig. 3E). NAA50 knockdown also elicited changes in stem growth. 24 h after dexamethasone...
treatment, the stems of DEX:NAA50-ami plants bent approximately 90° (Fig. 3F). As in the roots, dexamethasone treatment completely halted elongation of the primary stem in DEX:NAA50-ami plants (Fig. 3G). Interestingly, this shoot bending phenotype was suppressed by removal of the shoot apical meristem before dexamethasone treatment (Fig. 3H), suggesting that it is dependent on auxin redistribution. Our observations of knockout and knockdown plants confirm that NAA50 is essential for normal plant growth and development.

Loss of NAA50 Triggers Cell Death

As well as inducing growth changes, reduced expression of NAA50 resulted in early senescence in leaves. Leaves of adult DEX:NAA50-ami plants turned yellow and became necrotic following dexamethasone treatment (Fig. 4A; Supplemental Fig. S1). Senescence also occurred in DEX:NAA50-ami seedlings after transfer to MS plates supplemented with dexamethasone (Fig. 4B). In both adults and seedlings, the senescence phenotype developed about 4 d after the initial dexamethasone treatment, long after the changes in growth rate and stem bending occurred.

The discovery that knockdown of NAA50 induces cell death prompted us to investigate whether loss of NAA50 results in cell death in nna50-1 seedlings. Indeed, roots of nna50-1 and nna50-2 seedlings were readily stained by trypan blue dye, indicating that loss of NAA50 leads to the accumulation of dead cells in roots (Fig. 4C). Trypan blue staining of nna50 roots was spotty and irregular, indicating that only a subset of nna50 root cells died (Fig. 4D). Taken together, these results demonstrate that in addition to being essential for plant development, NAA50 is also required for the repression of cell death and senescence.

Given the interaction between EDR1 and NAA50, we hypothesized that introduction of the edr1-1 allele into DEX:NAA50-ami plants may affect the senescence phenotype. However, we did not observe any major change in the senescence phenotype when edr1-1 was introduced (Fig. 4E). That the edr1-1 mutation did not enhance or suppress the senescence phenotype indicates that NAA50 and EDR1 may regulate senescence through a shared mechanism.

Loss of NAA50 Represses Growth and Induces Stress Signaling

The discovery that reduced expression of NAA50 triggers changes in plant growth and senescence prompted us to investigate the transcriptional changes taking place in these plants. We therefore conducted an RNA sequencing-based analysis of the DEX:NAA50-ami transcriptome. Four-week-old plants were treated with dexamethasone, and RNA was collected 0, 12, and 24 h later. Dexamethasone-treated DEX:Scrambled-ami plants were utilized as a control, with RNA samples collected at the same time points. Transcripts from dexamethasone-treated DEX:NAA50-ami plants were deemed significantly altered only if they differed significantly (adjusted P-value < 0.05) from the dexamethasone-treated Scrambled-ami plants at the same time point. To determine whether these transcripts were significantly up- or down-regulated, we compared expression levels in the 12 and 24 h DEX:NAA50-ami transcriptomes to the time zero DEX:NAA50-ami transcriptome. Transcripts were considered significantly up- or downregulated if they differed from the time zero DEX:NAA50-ami expression level (adjusted P-value < 0.05 and log2 fold-change > 1.5). By comparing the DEX:NAA50-ami and DEX:Scrambled-ami transcriptomes at various time points, we were better able to exclude potential off-target effects of dexamethasone treatment, amiRNA overexpression, or circadian-influenced expression changes.

Our RNA sequencing analysis indicated that reduced expression of NAA50 resulted in altered expression of approximately 2,000 genes by 12 h postdexamethasone application (Supplemental Dataset S1). To determine the biological processes most impacted by loss of NAA50, we analyzed the biological gene ontology (GO) term enrichment in the 12 and 24 h DEX:NAA50-ami datasets. This analysis demonstrated that NAA50 knockdown leads to up-regulation of genes involved in stress hormone signaling (Fig. 5A). Responses to the stress hormones abscisic acid, jasmonic acid, and salicylic acid were induced 12 h after dexamethasone treatment. On the other hand, NAA50 knockdown resulted in the down-regulation of a variety of plant growth and photosynthetic processes (Fig. 5A). In particular, transcripts of genes involved in photosynthesis, light responses, and

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**Figure 2.** NAA50 is required for plant development. A, Loss of NAA50 results in dwarfed seedlings. Representative 7-d-old, MS-grown seedlings are depicted. B, NAA50-YFP complements nna50-mediated dwarfism. Four-week-old adult plants are shown. NP, Native NAA50 promoter (including the 297 nucleotides upstream of the NAA50 start site). C, nna50 plants can develop stems and flowers. A 5-week-old nna50-2 plant is shown. WT, wild type.
Figure 3. Loss of NAA50 results in developmental changes. A, naa50 seedlings have altered root morphology. The seedling roots depicted are from 1-week-old seedlings. B, Vacuole and cell morphology are altered in naa50 seedling roots. Shown are fluorescence micrographs taken of 7-d-old wild type and naa50-1 seedlings expressing mCherry-tagged γTIP. Scale bars = 50 μm. C, Dexamethasone (DEX) treatment induces knockdown of NAA50 in DEX:NAAS0-ami plants. qRT PCR was performed on cDNA generated from multiple adult DEX:NAAS0-ami plants following dexamethasone treatment. Displayed are the averages of three replicates with error bars indicating SD. Asterisk denotes P < 0.05 (by Student’s t test). Expression values were normalized to ACTIN2. This experiment was repeated three independent times with similar results. D, NAA50 knockdown induces changes to root cell morphology. Five-day-old seedlings were transferred from MS plates to MS plates supplemented with DEX. Images were taken 3 d after dexamethasone exposure. E, NAA50 knockdown slows root elongation. Seven-day-old seedlings were transferred to MS plates supplemented with ethanol or dexamethasone. Images were taken 3 d after transfer to ethanol- or DEX-supplemented media. F, NAA50 knockdown induces stem bending. Images were taken 24 h after dexamethasone treatment. Numbers indicate proportion of all stems which displayed the given morphology. G, NAA50 knockdown stalls stem growth. Stem measurements were taken on DEX:Scrambled-ami (n = 8) and DEX:NAAS0-ami (n = 10) immediately before and 6 d after dexamethasone treatment. No stem growth was detected in DEX:NAAS0-ami plants subsequent to dexamethasone application. Error bars indicate
growth hormone responses were negatively impacted. These changes in expression correlate with the altered development and induced senescence phenotypes observed during NAA50 knockdown. For instance, we found that auxin signaling was significantly reduced following NAA50 knockdown, potentially explaining the meristem-dependent stem-bending that occurs following NAA50 knockdown (Fig. 3, F and H). The increase in the expression of defense genes and salicylic acid signaling correlates with the cell death and senescence that results from NAA50 knockdown (Fig. 4).

To further analyze our transcriptome data, we searched for studies that had identified similar transcriptional changes using the Genevestigator Signature tool (Hruz et al., 2008). We selected the 330 most significantly altered transcripts (greatest log_{2} fold-change relative to DEX:NAA50-ami at 0 h) from the 12 h DEX:NAA50-ami dataset, and searched for studies that displayed similar expression profiles. We found that the most similar expression profiles were those of studies investigating plant-pathogen interactions, or light stress (Fig. 5B). This overlap demonstrates that NAA50 knockdown elicits stress signaling in plants.

**NAA50 and EDR1 Repress ER Stress**

The observation that edr1-1 mutants have enhanced sensitivity to diverse abiotic and biotic stresses (Frye et al., 2001; Tang et al., 2005) led us to question whether this mutant might also have enhanced sensitivity to agents that induce ER stress. We therefore tested edr1-1 plants for sensitivity to tunicamycin (TM), an inhibitor of protein glycosylation that induces ER stress. Injection of rosette leaves induced chlorosis and necrosis of the injected region more rapidly in edr1-1 plants than in wild-type plants (Fig. 6A). This observation suggests that EDR1 is required for proper execution of the unfolded protein response, or that loss of EDR1 results in enhanced cell death signaling during ER stress signaling.

Given the potential role of EDR1 in regulating NAA50 function, and the observation that NTA can alter protein stability, localization, and transport (Arnesen, 2011), we hypothesized that loss of NAA50 function might induce the ER stress response. Indeed, many of the observed naa50-mediated developmental phenotypes, such as stunted growth and cell death, can be caused by ER stress. Treatment with TM or dithiothreitol (DTT), which reduces disulfide bonds and induces ER stress, resulted in shorter roots, increased root hair length, and altered cell morphology in wild-type seedlings (Fig. 6, B and C). Additionally, TM and DTT treatments resulted in root cell death like that observed in naa50 seedlings (Fig. 6D). These results demonstrate that ER stress treatment and loss of NAA50 produce similar physiological changes.

To test whether naa50 seedlings display constitutive ER stress responses, we measured the transcription of ER stress marker genes by reverse transcription quantitative PCR (RT-qPCR). naa50-1 seedlings were found to have significantly higher levels of BINDING PROTEIN3 (BIP3) and SECRETORY31A (SEC31A) expression in the absence of any treatment (Fig. 6E). However, BIP3 and SEC31A expression did not significantly differ between wild-type and naa50 seedlings that had been treated with TM. During ER stress, the transcription factor BASIC REGION/LEU ZIPPER MOTIF60 (bZIP60) undergoes splicing, leading to its activation (Deng et al., 2011). Thus, detection of the spliced form of bZIP60 indicates an active ER stress response. Untreated naa50-1 seedlings were found to contain significantly higher levels of spliced bZIP60 relative to the wild type (Fig. 6F). However, similar levels of bZIP60 splicing occurred in TM-treated naa50-1 and wild-type seedlings. These results demonstrate that loss of NAA50 leads to constitutive ER stress, but not an increase in maximum ER stress response signaling. Thus, EDR1 and NAA50 both appear to play important roles in regulating ER stress in plants.

**NAA50 Enzymatic Activity Is Required for Development**

Given the high sequence conservation between Arabidopsis and human NAA50 proteins (Fig. 1A), we hypothesized that the enzymatic activity of NAA50 would be conserved. In addition to functioning as a NAT, human Naa50 has been shown to be capable of auto-acetylation (Evjenth et al., 2009). We therefore tested NAA50 for auto-acetylation activity using recombinant NAA50 protein. In vitro auto-acetylation assays using recombinant NAA50 protein demonstrated that Arabidopsis NAA50 is indeed capable of auto-acetylation (Fig. 7A; Supplemental Fig. S2).

Human Naa50 has previously been shown to associate with the NatA complex, which includes the Naa10 subunit (Arnesen et al., 2006). Transient expression of sYFP-tagged AtNAA50 with mCherry-tagged AtNAA10 indeed demonstrated that these proteins colocalize in plants (Fig. 7B).

Based on the sequence conservation between Arabidopsis NAA50 and human Naa50, as well as the colocalization of AtNAA50 with AtNAA10, we hypothesized that AtNAA50 likely functions as a NAT. To determine whether NAA50 is active in NTA, we tested whether various loss-of-function NAA50 mutants could complement naa50-2 mutant phenotypes. naa50-2

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**Figure 3. (Continued.)**

SD. H. Removal of the apical meristem inhibits NAA50 knockdown-mediated stem bending. Adult DEX:NAA50-ami plants were sprayed with dexamethasone, and images were taken 24 h later. The shoot apical meristem was removed immediately before dexamethasone treatment.
plants were transformed with NAA50\textsuperscript{I145A}-hemagglutinin (HA) and NAA50\textsuperscript{Y34A}-HA. It has been demonstrated that the comparable Y31 and I142 residues in human Naa50 mediate N-terminal peptide binding, and substitution with Ala in these positions reduces NTA activity to below 10\% and 42.2\% of wild-type levels, respectively (Liszczak et al., 2011). Thus, if NAA50-mediated NTA is indeed required for plant development, these mutations may prevent full rescue of the \textit{naa50-2} allele.

We identified numerous transgenic lines expressing both the Y34A and I145A proteins (Fig. 7C). Following transformation with the NAA50\textsuperscript{I145A-HA} transgene, we observed that the \textit{naa50} root phenotype was not fully complemented in the transgenic lines, as roots retained their dwarf phenotype and altered cell morphology (Fig. 7, D and E). Despite retaining the \textit{naa50} root phenotypes, some NAA50\textsuperscript{I145A} lines had wild-type-sized rosettes (Fig. 7F). I145A line 2 plants displayed the greatest expression of the I145A transgene and had a more wild-type-like rosette size than I145A line 3 plants (Fig. 7, C and F). However, although line 2 NAA50\textsuperscript{I145A} transgenic plants had wild-type-sized rosettes, normal siliques did not develop and viable seed was not produced (Fig. 7G).
The Y34A mutation, which has been shown to have a more significant impact on human Naa50 NTA activity than I145A (Liszczak et al., 2011), also prevented full rescue of nna50-2 plants. NAA50Y34A transgenic plants did not have normal roots or rosettes and were infertile (Fig. 7, D–G). As in the I145A transgenic lines, we observed a correlation between NAA50Y34A protein accumulation and rosette size (Fig. 7, C and F). However, unlike NAA50I145A, NAA50Y34A was unable to fully complement the rosette dwarfism phenotype (Fig. 7F). Even in Y34A line 3 plants, which displayed a similar level of NAA50 accumulation to I145A line 2 plants, rosette size was not fully restored.

### Figure 5. Reduction in NAA50 expression induces changes to growth and defense signaling.

**A.** Reducing NAA50 expression results in a down-regulation of growth signaling, and an up-regulation of defense signaling. Significantly altered transcripts were identified by comparing expression levels in dexamethasone-treated DEX:NAA50-amiRNA plants at the given time point to Scrambled-amiRNA plants at the same time point. Furthermore, expression levels in DEX:NAA50-amiRNA plants at 12 and 24 h were compared with NAA50-amiRNA plants at 0 h after DEX treatment. To eliminate circadian effects, all samples were harvested at the same time (12 and 24 h samples were sprayed with dexamethasone 12 and 24 h before harvest). GO term enrichment analysis was performed using the BiNGO application to determine whether the DEX:NAA50-amiRNA transcriptome was enriched for specific biological processes. NS, not statistically significant.

**B.** The DEX:NAA50-amiRNA transcriptome bears similarity to biotic and abiotic stress studies. The 330 most significantly altered transcripts were compared with previous studies using the Genevestigator Signature tool. The five most related transcriptomes based on the calculated Relative Similarity scores are shown. A heatmap was generated using Heatmapper (http://www2.heatmapper.ca/expression/) to display the relative log2 fold-change for each of the 330 transcripts for each study. These 330 transcripts were selected based on log2 fold-change when comparing expression levels in the DEX:NAA50-amiRNA 12-h dataset to the DEX:NAA50-amiRNA time 0 dataset; all 330 were also significantly different when comparing the DEX:NAA50-amiRNA 12-h dataset to the Scrambled-amiRNA 12-h dataset.
The inability of NAA50I145A and NAA50Y34A transgenes to fully rescue naa50-2 plants demonstrates the importance of NAA50-mediated NTA in plant growth and development. That the NAA50I145A mutant was able to complement the rosette dwarfi sm, but not the root phenotypes or sterility demonstrates that NAA50-mediated NTA may be especially required for the growth and development of roots as well as fertility. Furthermore, the inability of the NAA50Y34A transgene to rescue the rosette dwarfism phenotype indicates that this mutation is indeed more deleterious than the I145A mutation. Notably, an in vitro Lys acetyl transferase assay using recombinant NAA50Y34A and NAA50I145A confirmed that these mutations do not block this activity (Supplemental Fig. S3), which further suggests that it is the NTA activity of NAA50 that is required for normal development.

DISCUSSION

NAA50 Is Required for Growth and the Suppression of Stress Responses

The investigation of NTA in regulating cell signaling in eukaryotes is still in its infancy, and identification...
and characterization of all plant NATs is incomplete. Our understanding of how NATs function comes primarily from work in human cell culture and yeast. However, recent work in plants has demonstrated a role for NTAs in regulating diverse processes (Linster et al., 2015; Xu et al., 2015; Huber et al., 2020). Post-translational modification of proteins has long been appreciated as a mechanism by which cell signaling and crosstalk is regulated (Hunter, 2007). NTA may provide a mechanism by which plants regulate responses to external and internal stress signals at the translational level. 

With this work, we have begun to characterize the role of Arabidopsis NAA50 in regulating plant growth and development. Complete loss of NAA50 results in severely dwarfed and sterile plants, as well as altered root morphology. By using hormone-inducible amiRNA transgenic plants, we demonstrated that NAA50 knockdown results in reduced expression of developmental processes and inhibits growth. Taken together, these results indicate that NAA50 is required for plant growth and development.

We initially identified NAA50 as an interactor of EDRI in a yeast two-hybrid screen. Our results indicate that NAA50 and EDRI do indeed interact (Fig. 1). However, it is unclear what the basis for this interaction is. It is possible that NAA50 is a substrate of EDRI, however, we were unable to detect EDRI-mediated phosphorylation of NAA50 during coexpression in N. benthamiana. If NAA50 is indeed a substrate of EDRI,
then phosphorylation by EDR1 may require an unidentified cofactor or signal.

Our work adds to growing evidence that NATs are required for plant development. NAA10 and NAA15 have previously been demonstrated to be essential for development. Loss-of-function mutations in NAA10 or NAA15 are embryonic lethal (Linster et al., 2015; Feng et al., 2016), while partial loss of NAA15 results in dwarfism and enhanced defense signaling (Xu et al., 2015). Reduced expression of NAA10 and NAA15 alters root morphology, enhances the growth of the primary root and inhibits the growth of lateral roots (Linster et al., 2015). NatB also appears to be required for development, as partial loss of NatB components NAA20 and NAA25 result in dwarfism (Ferrández-Ayela et al., 2013; Xu et al., 2015; Huber et al., 2020). Loss of NAA30, the catalytic component of NatC, results in minor dwarfism as well as defects in photosystem II efficiency (Pesaresi et al., 2003). The range of developmental phenotypes resulting from mutations in NATs demonstrate that NATs differ in their involvement in plant development.

Our results demonstrate that, in addition to altering plant development, loss of NAA50 results in the activation of plant stress signaling. Reduced expression of NAA50 elicits senescence in adults as well as seedlings, while the roots of naa50 seedlings contain an abundance of dead cells. Gene expression analysis confirmed that knockdown of NAA50 results in an up-regulation of defense signaling. These results are consistent with the observation that loss of NAA50 results in an accumulation of stress response proteins (Armbruster et al., 2020).

NATs appear to play unique roles in the regulation of plant stress responses. Loss of NatA has been shown to increase drought tolerance (Linster et al., 2015). The NatA and NatB complexes have been previously implicated in the regulation of the nucleotide binding Leu rich-repeat protein SNC1 (Xu et al., 2015). Partial loss of NAA15 results in increased stability and accumulation of SNC1, as well as enhanced defense signaling and resistance. Interestingly, loss of NatB leads to decreased accumulation of SNC1, and suppression of snc1-induced dwarfism (Xu et al., 2015). Partial loss of NAA20 and NAA25 results in enhanced sensitivity to osmotic stress and altered expression of stress-related transcripts (Huber et al., 2020). Our observations add to the growing list of evidence that NATs play an important role in plant stress signaling.

Our results suggest that NAA50-mediated NTA may be required for plant development. Our complementation experiments demonstrate that the NAA50I145A and NAA50Y34A mutations, previously shown to inhibit human Naa50 NTA activity, prevent the NAA50 transgene from fully complementing the dwarfism and infertility of naa50 plants (Fig. 7, D–G). These results are consistent with the recent observation that human Naa50, but not the enzymatically inactive yeast Naa50, can rescue naa50-mediated dwarfism (Armbruster et al., 2020). Taken together, these observations suggest that NAA50 enzymatic activity is essential for plant development and fertility.

The NTA activity of human Naa50 has been demonstrated previously (Liszczak et al., 2011; Van Damme et al., 2011; Reddi et al., 2016; Evjenth et al., 2009). In the companion paper from Armbruster et al. (2020), Arabidopsis NAA50 is shown to have NTA activity in vitro on synthetic peptides, and in vivo when expressed in E. coli. It is thus likely that enzymatic function is conserved between Arabidopsis and humans. In addition, we were able to detect auto-acetylation on Lys residues of recombinant Arabidopsis NAA50 in vitro, confirming that NAA50 is indeed a functional acetyltransferase (Fig. 7A). Because NAA50 possesses both NTA activity and Lys acetyltransferase activity, it is a formal possibility that both activities contribute to normal development. However, our observation that a Y34A substitution retains Lys acetyltransferase activity (Supplemental Fig. S3), but blocks the ability of NAA50 to complement a naa50 knockout mutation (Fig. 7), indicates that it is the NTA activity of NAA50 that is essential. Consistent with this conclusion, Arabidopsis NAA50 localizes at least partially to the ER, where NTA of nascent proteins is likely to occur (Figs. 1 and 7B).

Loss of NAA50 Induces ER Stress

In addition to its role in negatively regulating defense signaling, our results implicate NAA50 in the repression of ER stress. The developmental defects observed in naa50 plants can be recapitulated by TM and DTT treatment, indicating that they may result from constitutive activation of ER stress responses. In support of this hypothesis, we observed increased expression of ER stress genes and bZIP60 splicing in untreated naa50 seedlings (Fig. 6). Following TM treatment, naa50-1 seedlings displayed wild-type levels of ER stress signaling. Therefore, loss of NAA50 induces ER stress signaling, but does not lead to greater induction during TM treatment. Additionally, the expression of BIP3 and SEC31A in naa50-1 seedlings was significantly lower in the absence of TM than during TM treatment. This indicates that the level of constitutive ER stress that occurs in naa50-1 plants is significantly lower than that elicited by chemical treatment. A recent proteome analysis of naa50-2 plants demonstrated an increase in the accumulation of proteins involved in ER stress responses (Armbruster et al., 2020). Based on these results, we hypothesize that NAA50 is required for the prevention of protein misfolding and aggregation, which contribute to ER stress. Plant NATs have not previously been demonstrated to play a role in the regulation of ER stress. Although NatE seems to be required for the repression of ER stress, it is possible that other NAT complexes are required as well.

There is evidence from human and yeast systems for the involvement of NATs in responding to ER stress and protein aggregates. NATs known to contribute to protein stability, trafficking, and translocation to the ER.
The NatA complex has been implicated in the regulation of protein aggregation (Arnesen et al., 2010). HUNTINGTIN YEAST PARTNER K HYPK, a NatA component, has chaperone activity, and has been shown to inhibit the formation of protein aggregates (Raychaudhuri et al., 2008). We have demonstrated that loss of NAA50 in plants results in constitutive ER stress, providing additional evidence that NATs may be involved in the repression of protein aggregation and ER stress.

There is a well-established link between ER stress, the UPR, and defense signaling in plants. Plants carrying loss-of-function mutations in the stearoyl-ACP desaturase suppressor of sa insensitive2 exhibit dwarfism, enhanced accumulation of the ER stress marker BiP3, and higher expression of pathogenesis-related gene1 (Kachroo et al., 2001; Iwata et al., 2018). This mirrors the increased biotic and ER stress signaling observed in naa50 plants. Mutants lacking UPR regulators inositol-requiring1 and bZIP60 display enhanced susceptibility to bacterial pathogens, demonstrating a link between the UPR and salicylic acid-based defense signaling (Moreno et al., 2012). If ER stress cannot be properly maintained, the UPR shifts into a cell death phase (Walter and Ron, 2011; Woehlbieler and Hetz, 2011). A recent investigation of the transcriptional changes that occur during a prolonged UPR demonstrated that transcripts associated with biotic stress responses are elicited during the UPR (Srivastava et al., 2018). Biotic stress signaling is also impacted by the ER quality control (ERQC) pathway. The membrane-bound receptors upon which plant defense signaling relies undergo maturation through the ERQC pathway. Impairment of ERQC machinery can result in enhanced susceptibility, as the receptors required for pathogen recognition are unable to function (Tintor and Saijo, 2014). Thus, compromised ER integrity can hinder plant pathogen responses. Unsurprisingly, plant pathogens have been found to attack the host ER stress pathway for their own benefit. The mutualistic fungus Piriformospora indica induces cell death using an ER stress-dependent mechanism, enabling its colonization of the Arabidopsis root (Qiang et al., 2012). The high degree of overlap between ER stress and biotic stress responses opens the possibility that the observed increase in stress signaling in NAA50 knockout and knockdown plants results from changes to ER stress, rather than direct regulation of stress responses by NAA50.

A link between NTA and osmotic stress in plants has been recently proposed (Linster et al., 2015; Aksnes et al., 2016). NatA knockdown plants display enhanced drought tolerance, and levels of NatA-mediated NTA were shown to fluctuate in response to abscisic acid treatment (Linster et al., 2015). Loss of NatB was also shown to affect plant osmotic stress responses. Mutations affecting NAA20 and NAA25 result in reduced germination in the presence of osmotic stress (Huber et al., 2020). Here, we have demonstrated that loss of a plant NAT induces ER stress. The observed osmotic stress phenotypes of NatA and NatB mutant plants may be an indirect result of induced ER stress. Consistent with this hypothesis, overexpression of the chaperone BiP in tobacco (Nicotiana tabacum) and soybean (Glycine max) results in enhanced drought tolerance (Valente et al., 2009). BiP expression in soybean was found to inhibit both ER- and osmotic stress-induced cell death (Reis et al., 2011). In wheat (Triticum aestivum), treatment with tauroursodeoxycholic acid alleviates osmotic stress-induced cell death and ER stress signaling (Zhang et al., 2017), and strong osmotic stress alters root architecture and induces cell death through an ER stress-dependent mechanism (Duan et al., 2010).

### NAA50 Tissue-Specificity

Most work on NTA has been performed in unicellular organisms, making it impossible to determine whether NATs display tissue-specific functions. There are likely to be differences in the expression patterns of NATs in different tissues. According to the Bio-Analytic Resource for Plant Biology (BAR) ePlant browser (http://bar.utoronto.ca/epplant), root expression of NAA50 and NAA10 is predicted to be the highest in the meristematic region. It is likely that some NAT complexes are specifically active at certain developmental periods, or in specific tissues. If other plant NAT complexes are indeed able to fulfill some functions of NAA50, it is also possible that they are only able to do so in certain tissues or at certain points in development, based upon their own expression profiles. The study of plant NATs has the potential to expose tissue- and development-specific NAT activity.

Loss of NAA50 especially affected certain cell types and tissues. In NAA50 knockdown plants, loss of NAA50 led to reduced growth of both roots and stems. Furthermore, stem bending and altered root morphology were observed. The use of an inducible knockdown line enabled us to compare the effects of reducing NAA50 knockdown in new and old cells. Interestingly, phenotypes resulting from NAA50 knockdown were mainly exhibited in newly developed cells. The sterility of naa50 plants demonstrates that NAA50 is required for reproductive as well as vegetative development. The inability of NAA50H45A and NAA50Y34A transgenes to fully rescue naa50-induced root defects and sterility highlight the necessity of NAA50 for the development of these tissues. These observations demonstrate that NAA50 activity may be especially required by developing cells, or cells undergoing rapid growth and division.

If NAA50 is indeed required for the regulation of ER stress, it follows that roots, shoots, and anthers would be especially impacted by its loss. There is evidence that plant vegetative and reproductive development require an intact UPR to manage ER stress. Roots have been shown to be particularly sensitive to ER stress (Cho and Kanehara, 2017). Substantial changes in root and shoot development may require an intact UPR to manage ER stress. Roots have been shown to be particularly sensitive to ER stress (Cho and Kanehara, 2017). Substantial changes in root and shoot development may require an intact UPR to manage ER stress.
development result from mutations in UPR genes, indicating that a functional UPR is essential for vegetative development (Deng et al., 2013; Kim et al., 2018; Bao et al., 2019). Mutations in UPR genes also have a strong impact on plant reproductive development (Deng et al., 2013; Deng et al., 2016). In fact, the UPR is constitutively active in anthers (Iwata et al., 2008). The requirement for UPR signaling in unstressed plants implies that ER stress occurs during normal development and must be managed by the UPR, or that UPR genes are involved in the direct regulation of developmental genes (Kim et al., 2018). A requirement for the UPR in development has long been demonstrated in animals. The rapid production of immunoglobulins by B cells is preceded by an up-regulation of the UPR, which manages potential ER stress (van Anken et al., 2003). Roots, shoots, and anthers may rely upon UPR signaling due to the high level of protein translation which occurs in these tissues during development. That these tissues were indeed particularly affected by the loss of NAA50 demonstrates that NAA50 may be required for the management of ER stress which occurs during development.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Arabidopsis (Arabidopsis thaliana) accession Col-0 and Col-0 mutants rdr1-1 (Frye and Innes 1996), naa50-1 (SAIL_1210_A02), and naa50-2 (SAIL_1186_A03) were used in this study. The presence of homozygous T-DNA insertions in NAA50 in the SAIL lines was confirmed by PCR-based genotyping using NAA50 gene-specific primers and a T-DNA left border primer (Supplemental Table S1) following the primer design criteria and protocol given by the T-DNA Express Primer Design tool (http://signal.salk.edu/tdnaprimer2.html).

For growth on MS plates, seeds were surface sterilized with a solution of hydrogen peroxide and ethanol (1:19) and planted on half-strength MS plates supplemented with 0.8% (w/v) agar. For soil-grown plants, seed was directly sown onto Pro-Mix PGX Biofungicide plug and germination mix plates supplemented with 0.8% (w/v) agar. For soil-grown plants, seed was grown under the same growth room conditions as Arabidopsis. The growth room was set to 23°C and a 12-h light (150 μmol m⁻² s⁻¹) cycle of 2× Laemmli sample buffer, supplemented with 5% (v/v) β-mercaptoethanol, 1% (v/v) Protease Inhibitor Cocktail (Sigma), and 50 μmol 2,2'-dithiodipyridine (Sigma) or, for co-immunoprecipitations, immunoprecipitation buffer (50 mmol Tris-HCl, pH 7.5; 1% (v/v) Nonidet P-40; 0.1% (v/v) Sodium Deoxycholate; 0.1% (v/v) glycine; 1% (v/v) Plant Proteinase Inhibitor Cocktail [Sigma]; and 50 mmol 2,2'-dithiodipyridine [Sigma]). For protein extraction from yeast, yeast grown on solid SD-Tp-Leu medium for 16 h at 30°C. Cultures were resuspended in water to an optical density at 600 nm of 1.0, serially diluted, and plated on appropriate SD media. Plates were grown for up to 4 d at 30°C.

**Plasmid Construction and Generation of Transgenic Arabidopsis Plants**

NAA50 clones were derived by PCR amplification using complementary DNA (cDNA) from Col-0. Site-directed mutagenesis was used to introduce the same growth room conditions as Arabidopsis. The growth room was set to 23°C and a 12-h light (150 μmol m⁻² s⁻¹) cycle. The rapid production of immunoglobulins by B cells is preceded by an up-regulation of the UPR, which manages potential ER stress (van Anken et al., 2003). Roots, shoots, and anthers may rely upon UPR signaling due to the high level of protein translation which occurs in these tissues during development. That these tissues were indeed particularly affected by the loss of NAA50 demonstrates that NAA50 may be required for the management of ER stress which occurs during development.

**Yeast Two-Hybrid Assays**

For yeast two-hybrid assays between EDR1 and NAA50, pGBK7 and pGADT7 clones were transformed into haploid yeast strain AH109 (Clontech) by electrotransformation and selected on SD-Trp-Leu medium. Successful transformants were selected after 48 h of growth at 30°C and then struck onto fresh SD-Trp-Leu medium and allowed to grow for another 48 h. Before carrying out yeast two-hybrid assays, yeast was grown in liquid SD-Trp-Leu medium for 16 h at 30°C. Cultures were resuspended in water to an optical density at 600 nm of 1.0, serially diluted, and plated on appropriate SD media. Plates were grown for up to 4 d at 30°C.

**Immunoprecipitations and Immunoblots**

For total protein extraction, tissue was ground in lysis buffer (50 mmol Tris-HCl [pH 7.5]; 150 mmol NaCl; 0.1% [v/v] Nonidet P-40; 1% [v/v] Plant Proteinase Inhibitor Cocktail [Sigma]; and 50 mmol 2,2'-dithiodipyridine [Sigma]) or, for co-immunoprecipitations, immunoprecipitation buffer (50 mmol Tris [pH 7.5]; 1% [v/v] Nonidet P-40; 0.1% [v/v] sodium deoxycholate; 0.1% [v/v] glycine; 1% [v/v] Plant Proteinase Inhibitor Cocktail [Sigma]; and 50 mmol 2,2'-dithiodipyridine [Sigma]). For protein extraction from yeast, yeast grown on solid SD-Leu-Trp plates were resuspended in lysis buffer (100 mmol NaCl; 50 mmol Tris-CI [pH 7.5]; 50 mmol NaF; 50 mmol Na₂β-glycerophosphate, pH 7.4; 2 mmol EDTA; 2 mmol EDTA; 0.1% [v/v] Triton X-100; and 1 mmol NaVO₃). Glass beads were then added to the suspension, and the solution was vortexed for 1 min three times. After the addition of 1 volume of 2× Laemmli sample buffer supplemented with 5% (v/v) β-mercaptoethanol, samples were boiled for 10 min. Immunoblots were performed using anti-HA-HRP (3F10; Sigma), mouse anti-GFP (ab6556; Abcam), and goat antimonoset-HRP antibodies (A-10668; Invitrogen).

For protein extraction from yeast, yeast grown on solid SD-Leu-Trp plates were resuspended in lysis buffer (100 mmol NaCl; 50 mmol Tris-CI [pH 7.5]; 50 mmol NaF; 50 mmol Na₂β-glycerophosphate, pH 7.4; 2 mmol EDTA; 2 mmol EDTA; 0.1% [v/v] Triton X-100; and 1 mmol NaVO₃). Glass beads were then added to the suspension, and the solution was vortexed for 1 min three times. After the addition of 1 volume of 2× Laemmli sample buffer supplemented with 5% (v/v) β-mercaptoethanol, samples were boiled for 10 min. Immunoblots were performed using anti-HA-HRP (3F10; Sigma), mouse anti-GAL4DBD (RKSC1; Santa Cruz Biotechnology), and goat antimonoset-HRP (A-10668; Invitrogen).
antibodies. Visualization of immunoblots from yeast strains used in the two-hybrid assay were performed using the KwikQuant Imager (Kindle Biosciences).

Fluorescence and Light Microscopy

Confocal laser scanning microscopy was performed on a Leica TCS SP8 confocal microscope (Leica Microsystems) equipped with a 63×, 1.2-numerical aperture water objective lens and a White Light Laser. mYFP fusions were excited at 514 nm and detected using a 522 to 545 nm band-pass emission filter. mCherry fusions were excited at 561 nm and detected using a custom 595 to 620 nm band-pass emission filter.

To capture detailed images of Arabidopsis roots, images were captured using a Stermi 305 compact Greenough stereo microscope (Zeiss). Digital images were captured using Labscope software (Zeiss).

Reverse Transcription Quantitative PCR

For RT-qPCR experiments, RNA was extracted using the Spectrum plant total RNA kit (Sigma-Aldrich) according to the manufacturer’s instructions. cDNA was produced from 1 μg total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific). Relative RNA amounts were determined by quantitative PCR using the Power Up SYBR Green Master Mix (Thermo Fisher Scientific). A comparative Ct method was used to determine relative quantities (Schmittgen and Livak, 2008). ACTIN2 was used for normalization.

NAA50 Knockdown Transcriptome Profiling

For RNA sequencing, plants were first sprayed with a solution containing 50 μM dexamethasone and 0.02% (v/v) Silwet-L-77 (OSI Specialties) 24 h, and immediately before tissue collection. This experimental design avoided changes in gene expression caused by circadian effects because all samples were harvested at the same time. Three biological replicates were performed per genotype per treatment, each consisting of approximately 0.4 g leaf tissue taken from the fourth leaf of four unique plants. RNA was extracted from 4-week-old Arabidopsis leaves using the Spectrum plant total RNA kit (Sigma-Aldrich) according to the manufacturer’s instructions. Total RNA was prepared into equimolar pools for each sample submitted to Indiana University’s Center for Genomics and Bioinformatics for cDNA library construction using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) following the standard manufacturing protocol. Sequencing was performed using an Illumina NextSeq500 platform with 75 cycle sequencing kit generating 84-bp single-end reads. After the sequencing run, demultiplexing was performed with bcftools v2.0.10.422. RNA sequencing data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database and is accessible through the accession number GSE145580.

In Vitro Acetylation Assays

E. coli strain BL21-A1 was transformed with a pDEST17 vector carrying NAA50. 5×His-tagged NAA50 was purified from E. coli using a Nickel-His column (Sigma). A 5 ml culture was incubated at 37°C for 16 h, and then subcultured to a final volume of 100 ml. The culture was grown until the optical density at 600 nm reached 0.5. Expression of NAA50 was induced by adding 1 mM isopropylthio-galactoside and 0.2% (w/v) arabinose to the culture. The culture was then incubated at 30°C for 3 h. Cells were then harvested and resuspended in 8 ml of native purification buffer (50 mM NaH2PO4, 500 mM NaCl) supplemented with 8 mg lysozyme and a protease inhibitor tablet (Roche). The suspension was then incubated on ice for 30 minutes, and then sonicated. After sonication, the cell debris was pelleted by centrifugation (5,000g, 15 min) at 4°C. The Ni-NTA resin was washed twice with native purification buffer and then incubated with the lysate for 1 h at 4°C. The resin was then washed four times with wash buffer (native purification buffer supplemented with 6 mM imidazole). Fractions were eluted with elution buffer (native purification buffer supplemented with 250 mM imidazole).

For in vitro auto-acetylation assays, 4 μg recombinant NAA50 was incubated with 100 μM acetyl-coenzyme A (Roche) in a 2X acetylation buffer (50 mM Tris HCl, pH 7.5; 2 mM EDTA; 200 mM NaCl; 10% [v/v] glycerol) at 30°C. Detection of auto-acetylation activity was performed through immunoblotting using acetylated Lys monoclonal antibody (1C6 Invitrogen).

Trypan Blue Staining

Trypan blue staining of Arabidopsis roots was performed by soaking seedlings in a solution of 10 mg ml⁻¹ trypan blue (Sigma) in water for 20 min. Seedlings were then washed three times with deionized water.

ER Stress Treatments

ER stress treatments of Arabidopsis seedlings were performed by growing seeds directly on MS plates supplemented with TM (Sigma) or DTT (Bio-Rad). For treatment of adult plants, TM was injected directly into one half of an Arabidopsis leaf using a needleless syringe.

Accession numbers

Arabidopsis sequence data are available under the following Arabidopsis Genome Initiative accession numbers: EDR1 (At1g08720), NAA50 (At5g11340), γ-TIP (At2g36830), NAA10 (At5g13780), SD2 (AT2G31110), RNA sequencing data are accessible through the NCBI GEO database (GSE145580).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Transgenic Arabidopsis plants carrying the DEX:NAA50-amirna transgene display chlorosis and necrosis following dexamethasone treatment.

Supplemental Figure S2. NAA50 displays auto-acetylation activity in vitro.

Supplemental Figure S3. NAA50 mutant proteins retain Lys autoacetylation activity.

Supplemental Table S1. Primers used in this study.

Supplemental Dataset S1. RNA-seq data from NAA50 amiRNA transgenic lines.
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LITERATURE CITED

Aksnes H, Drazic A, Marie M, Arnesen T (2016) First things first: Vital protein marks by N-terminal acetyltransferases. Trends Biochem Sci 41: 746–760

Aksnes H, Van Damme P, Goris M, Starheim KK, Marie M, Stove SJ, Hoel C, Kalvik TV, Hole K, Glomnes N, et al (2015) An organellar n-acyltransferase, naa60, acetylates cytosolic N termini of transmembrane proteins and maintains Golgi integrity. Cell Rep 19: 1362–1374

Armbruster L, Linster E, Boyer JB, Brünje A, Eirich J, Stephan I, Bienvenut WV, Friese J, Meineel T, Hell R, et al (2020) NAA50 is an enzymatically active N-acyltransferase that is crucial for development and regulation of stress responses. Plant Physiol 183: 1502–1516

Arnesen T (2011) Towards a functional understanding of protein N-terminal acetylation. PLoS Biol 9: e1001074

Arnesen T, Anderson D, Torsvik J, Halseth HB, Varhaug JE, Lillehaug JR (2006) Cloning and characterization of hNAT5/ISAN: An evolutionarily conserved component of the NatA protein N-acetyltransferase complex. Gene 371: 291–295

Arnesen T, Starheim KK, Van Damme P, Evjenth R, Dinin H, Betts MJ, Rynningen A, Vandekerckhove J, Gevaart K, Anderson D (2010) The chaperone-like protein HYPK acts together with NatA in cotranslational N-terminal acylation and prevention of Huntingtin aggregation. Mol Cell Biol 30: 1898–1909

Arnesen T, Van Damme P, Povelova B, Helsens K, Evjenth R, Coalaert N, Varhaug JE, Vandekerckhove J, Lillehaug JR, Sherman F, Gevaart K (2009) Proteome analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci USA 106: 8157–8162

Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, Wishart DS (2016) Heatmapper: Web-enabled heat mapping for all. Nucleic Acids Res 44(W1): W147–W153

Bao Y, Bassham DC, Howell SH (2019) A functional unfolded protein response is required for normal vegetative development. Plant Physiol 179: 1834–1843

Bao Y, Howell SH (2017) The unfolded protein response supports plant development and defense as well as responses to abiotic stress. Front Plant Sci 8: 344

Bheena R, Panic B, Whyte JRC, Munro S (2004) Targeting of the Arf-like GTPase Arf3p to the Golgi requires N-terminal acetylation and the membrane protein Sys1p. Nat Cell Biol 6: 405–413

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–20

Bowling SA, Guo A, Cao G, Gordon AS, Klessig DF, Dong X (1994) A mutation in Arabidopsis is required to constitutively express of systemic acquired resistance. Plant Cell 6: 1845–1857

Brown JL, Roberts WK (1976) Evidence that approximately eighty percent of the soluble proteins from Ehrlich ascites cells are Nalpha-acetylated. J Biol Chem 251: 1099–1014

Cho Y, Kanehara K (2017) Endoplasmic reticulum stress response in Arabidopsis roots. Front Plant Sci 8: 144

Christiansen KM, Gu Y, Rödibaugh N, Innes RW (2011) Negative regulation of defence signalling pathways by the ERD1 protein kinase. Mol Plant Pathol 12: 746–758

Cipollini D, Walters D, Voelckel C (2010) Costs of resistance in plants: From theory to evidence. In C Voelckel, and G Jander, eds, Annual Plant Reviews: Insect-Plant Interactions. John Wiley & Sons, Hoboken, NJ, pp 263–307

Clough SJ, Bent AF (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743

Deng Y, Humbert S, Liu J-X, Srivastava R, Rothstein SJ, Howell SH (2011) Heat induces the splicing by IRE1 of a mRNA encoding a transcription factor involved in the unfolded protein response in Arabidopsis. Proc Natl Acad Sci USA 108: 7247–7252

Deng Y, Srivastava R, Howell SH (2013) Protein kinase and ribonuclease domains of IRE1 confer stress tolerance, vegetative growth, and reproductive development in Arabidopsis. Proc Natl Acad Sci USA 110: 19633–19638

Deng Y, Srivastava R, Quilichini TD, Dong H, Bao Y, Horner HT, Howell SH (2016) IRE1, a component of the unfolded protein response signaling pathway, protects pollen development in Arabidopsis from heat stress. Plant J 88: 193–204

Dinh TV, Bienvenut WV, Linster E, Feldman-Salit A, Jung VA, Meinell T, Hell R, Giglione C, Witzt M (2015) Molecular identification and functional characterization of the first N-acetyltransferase in plastids by global acylome profiling. Proteomics 15: 2426–2435

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR (2013) STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29: 15–21

Drazic A, Aksnes H, Marie M, Bozckowska M, Varland S, Timmerman E, Foyen H, Glomnes N, Repowskbi N, Geavall I, Thommesen F, et al (2019) NAA80 is an acetyltransferase that is required for N-terminal acetylation of Arabidopsis thaliana. Plant J 98: 161–168

Duan Y, Zhang W, Li B, Wang Y, Li K, Södergren, Han C, Zhang Y, Li X (2010) An endoplasmic reticulum response pathway mediates programed cell death of root tip induced by water stress in Arabidopsis. New Phytol 186: 681–695

Earley KW, Haãn JR, Platanes O, Oppe K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J 45: 616–629

Evjenth R, Hole K, Karlsen OA, Ziegler M, Arnesen T, Lillehaug JR (2009) Human Naa50p (Nats5/San) displays both protein N- and N-ε-acyltransferase activity. J Biol Chem 284: 31122–31129

Feng J, Li R, Yu J, Ma S, Wu C, Li Y, Yao T, Ma L (2016) Protein N-terminal acetylation is required for embryogenesis in Arabidopsis. J Exp Bot 67: 4779–4789

Ferrández-Ayela A, Micol-Ponce R, Sánchez-García AB, Alonso-Peral MM, Micol JL, Ponce MR (2013) Mutation of an Arabidopsis NatB N-alpha-terminal acetylation complex component causes pleiotropic developmental defects. PLoS One 8: e60697

Forte GMA, Pool MR, Stirling CJ (2011) N-terminal acetylation inhibits protein targeting to the endoplasmic reticulum. PLoS Biol 9: e1001073

Frye CA, Innes RW (1998) An Arabidopsis mutant with enhanced resistance to powdery mildew. Plant Cell 10: 947–956

Frye CA, Tang D, Innes RW (2001) Negative regulation of defense responses in plants by a conserved MAPKK kinase. Proc Natl Acad Sci USA 98: 373–378

Gibbs CS, Zoller MJ (1991) Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. J Biol Chem 266: 8923–8931

Greenberg JT, Yao N (2004) The role and regulation of programmed cell death in plant-pathogen interactions. Cell Microbiol 6: 201–211

Gu Y, Innes RW (2011) The KEEP ON GOING protein of Arabidopsis recruits the ENHANCED DISEASE RESISTANCE1 protein to trans-Golgi network/early endosome vesicles. Plant Physiol 155: 1827–1838

Gu Y, Innes RW (2012) The KEEP ON GOING protein of Arabidopsis regulates intracellular protein trafficking and is degraded during fungal infection. Plant Cell 24: 4717–4730

Hrzu T, Laule O, Szabo G, Wessendorf F, Bleuer S, Oertle L, Widmayer P, Gruissem W, Zimmermann P (2008) Genevigator v3: A reference expression database for the meta-analysis of transcriptomes. Adv Bioinformatics 2008: 420747

Huber M, Bienvenut WV, Linster E, Stephan I, Armbruster L, Sticht C, Layer D, Lapouge K, Meinell T, Sinning I, et al (2020) NatB-mediated N-terminal acetylation affects growth and abiotic stress responses. Plant Physiol 182: 792–806

Hunter T (2007) The age of crosstalk: Phosphorylation, ubiquitination, and beyond. Mol Cell 28: 730–734

Huot R, Yao J, Montgomery BL, He SY (2014) Growth-defense tradeoffs in plants: A balancing act to optimize fitness. Mol Plant 7: 1267–1287

Ignatiadis N, Klaus B, Zaugg JB, Hüber W (2016) Data-driven hypothesis weighting increases detection power in genome-scale multiple testing. Nat Methods 13: 577–580
Iwata Y, Fedoroff NV, Koizumi N (2008) Arabidopsis bZIP50 is a proteolysis-activated transcription factor involved in the endoplasmic reticulum stress response. Plant Cell 20: 3107–3121

Iwata Y, Iida T, Matsunami T, Yamada Y, Mishiba K-I, Ogawa T, Kurata T, Koizumi N (2018) Constitutive BiP protein accumulation in Arabidopsis mutants defective in a gene encoding chloroplast-resident stearyol-seryl carrier protein desaturase. Genes Cells 23: 456–465

Kachroo P, Shanklin J, Shah J, Whittle EJ, Klessig DF (2001) A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proc Natl Acad Sci USA 98: 9448–9453

Kim J-S, Yamaguchi-Shinozaki K, Shinozaki K (2008) Arabidopsis bZIP60 is a immunity and abiotic stress responses. PLoS One 21:

Raychaudhuri S, Sinha M, Mukhopadhyay D, Bhattacharyya NP, Qi D, Scholthof K-BG (2009) Proteome-derived peptide libraries allow detailed analysis of the substrate specificity of amino-terminal acetylation: Detailed structural and bio-chemical analysis using tetrapeptide library. J Biol Chem 291: 20530–20538

Reis PA, Rosado GL, Silva LA, Oliveira LC, Oliveira LB, Costa MDL, Alvim FC, Fontes EPB (2011) The binding protein BiP attenuates stress-induced cell death in soybean via modulation of the N-rich protein-mediated signaling pathway. Plant Physiol 157: 1853–1865

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101–1108

Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell 18: 1121–1133

Serrano I, Gu Y, Qi D, Dubiella U, Innes RW (2014) The Arabidopsis EDR1 protein kinase negatively regulates the AT1L E3 ubiquitin ligase to suppress cell death. Plant Cell 26: 4532–4546

Setty SRG, Stochlic TI, Tong AYH, Boone C, Burd CG (2004) Golgi targeting of ARF-like GTPase Arl3p requires its Nalpha-acetylation and the integral membrane protein Sys1p. Nat Cell Biol 6: 414–419

Shao F, Golstein C, Ade J, Strotmeyer M, Dixon JE, Innes RW (2003) Cleavage of Arabidopsis PFS1 by a bacterial type III effector. Science 301: 1230–1233

Srivastava R, Li Z, Russo G, Tang J, Bi R, Muppilara U, Chudalayandi S, Severin A, He M, Vaitkevicius SI, et al (2018) Response to persistent ER stress in plants: A multiphasic process that transitions cells from pro-survival activities to cell death. Plant Cell 30: 1220–1242

Tang D, Christiansen KM, Innes RW (2005) Regulation of plant disease resistance, stress responses, cell death, and ethylene signaling in Arabidopsis by the EDR1 protein kinase. Plant Physiol 138: 1018–1026

Tintor N, Saijo Y (2014) ER-mediated control for abundance, quality, and signaling of transmembrane immune receptors in plants. Front Plant Sci 5: 65

Valente MA, Faria JA, Soares-Ramos JRL, Reis PA, Pinheiro GL, Piovesan ND, Morais AT, Menezes CC, Cano MA, Fietto LG, et al (2009) The ER luminal binding protein (BiP) mediates an increase in drought tolerance in soybean and delays drought-induced leaf senescence in soybean and tobacco. J Exp Bot 60: 533–546

van Anken E, Romijn EP, Maggioni C, Meghznati A, Sittia R, Braakman I, Heck AJR (2003) Sequential waves of functionally related proteins are expressed when B cells prepare for antibody secretion. Immunity 18: 243–253

Van Damme P, Evijenth R, Foyin H, Demeyer K, De Bock P-J, Lillehaug JR, Vandekerckhove J, Arnesen T, Gevaert K, Jenrette J, Greenberg JT (2012) The mucoattractant protein NAA50 interacts with glycosyltransferases of the myxosporidian Pseudomona syringae pv. syringae B728a and its role in survival and disease on host and non-host plants. Microb Cell 3: 26–44

Viale A, Bostrom RS (2008) Endoplasmic reticulum quality control and the unfolded protein response: Insights from plants. Traffic 9: 1581–1588

Walter P, Ron D (2011) The unfolded protein response: From stress pathway to homeostatic regulation. Science 334: 1081–1086

Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ (2009) Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25: 1189–1191

Wawrzynska A, Christiansen KM, Lan Y, Rodibaugh NL, Innes RW, Christiansen KM, Lan Y, Rodibaugh NL, Innes RW (2008) Powdery mildew resistance conferred by loss of the ENHANCED DISEASE RESISTANCE1 protein kinase is suppressed by a missense mutation in KEEP ON GOING, a regulator of abscisic acid signaling. Plant Cell 14: 1516–1531

Williams B, Verchot J, Dickman MB (2014) When supply does not meet demand ER stress and plant programmed cell death. Front Plant Sci 5: 211

Woehlher U, Heiz C (2011) Modulating stress responses by the UP donor: A matter of life and death. Trends Biochem Sci 36: 329–337

Xu F, Huang Y, Li L, Gannon P, Linster E, Huber M, Kapos P, Bienvenut W, Polevoda B, Meinelt T, et al (2015) Two N-terminal acetyltrans- ferases antagonistically regulate the stability of a nod-like receptor in Arabidopsis. Plant Cell 27: 1547–1562

Zhang L, Xin Z, Xu X, Ma C, Liang W, Zhu M, Cheng Q, Li Z, Niu Y, Ren Y, et al (2017) Osmotic stress induced cell death in wheat is alleviated by tauroursodeoxycholic acid and involves endoplasmic reticulum stress-related gene expression. Front Plant Sci 8: 667

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