Downregulation of N-terminal acetylation triggers ABA-mediated drought responses in Arabidopsis

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N-terminal acetylation (NTA) catalysed by N-terminal acetyltransferases (Nats) is among the most common protein modifications in eukaryotes, but its significance is still enigmatic. Here we characterize the plant NatA complex and reveal evolutionary conservation of NatA biochemical properties in higher eukaryotes and uncover specific and essential functions of NatA for development, biosynthetic pathways and stress responses in plants. We show that NTA decreases significantly after drought stress, and NatA abundance is rapidly downregulated by the phytohormone abscisic acid. Accordingly, transgenic downregulation of NatA induces the drought stress response and results in strikingly drought resistant plants. Thus, we propose that NTA by the NatA complex acts as a cellular surveillance mechanism during stress and that imprinting of the proteome by NatA is an important switch for the control of metabolism, development and cellular stress responses downstream of abscisic acid.
N-terminal acetylation (NTA) is a common modification of eukaryotic proteins occurring on more than 50 and 80% of yeast and human cytosolic proteins, respectively. NTA is catalysed by ribosome-associated N-terminal acetyltransferases (Nats), when 25–50 amino acids of the nascent chain protrude from the ribosome. Three major Nat complexes, NatA, NatB and NatC, accept acetyl-CoA as the donor for the transferred acetate moiety, are present in yeast and humans and are thought to be responsible for the majority of NTA events. NTA may occur on the initiator Met (iMet) or on the first residue after iMet cleavage by methionine aminopeptidases. NatA potentially acetylates Ser-, Ala-, Thr-, Val-, Gly-, and Cys- N termini after iMet-cleavage, thus, NatA is the predominant Nat with respect to the number of substrates in yeast and human. NatB and NatC potentially acetylate Met- N termini when the second residue is either acidic or hydrophobic, respectively. In yeast and humans, NatA is composed of the catalytic subunit NAA10 (Ard1) and NAA15 in the NatA complex can modulate the active site of substrate N termini by the respective Nats or by differential subunits of protein complexes for ubiquitin-dependent proteasomal degradation. In these proteins, the acetylated N terminus acts as a preformed degradation signal, called the Ac/N degron, which must be unshielded before it can be recognized by the ubiquitin ligase, DOA10 (ref. 6). Several endogenous yeast proteins have been shown to bear an Ac/N degron in wild-type yeast, but are degraded in natC mutants in an Arg/N end rule pathway-based manner.

Although deacetylases of protein N termini have not been identified so far, 9% of all experimentally identified N termini are only partially acetylated in human cells. The partial acetylation might be explained by constitutive poor identification of these substrate N termini by the respective Nats or by differential regulation of Nat activity. Indeed, the interaction of NAA10 with NAA15 in the NatA complex can modulate the active site of Naa10 for substrate-specific NTA, but this interaction is mandatory for ribosome attachment. Thus, no regulatory mechanisms have been evidenced for ribosome-associated Nats so far. The only known factor that can limit co-translational NatA activity is supply with cytosolic acetyl-CoA.

Total loss of NatA, NatB or NatC mutants of humans are not known. However, a point mutation in human NAA10 causes lethality in male infants due to NTA deficiency, which demonstrates the essentiality of NatA for humans. Mutations in the gene encoding the presumed orthologue of human NAA10 have been shown to result in embryonal defects in Drosophila melanogaster and Arabidopsis thaliana. In contrast, all studied yeast nat mutants are viable; thus, NatA, NatB and NatC are not essential in this unicellular organism. Yeast mutants of the major Nats display a number of different phenotypes suggesting that these Nats, and thus probably NTA in general is implicated in numerous cellular processes reviewed in ref. 5. The discrepancy between phenotypes of NatA-depleted human cells and yeast nata provides evidence for relevant differences in the biological function of NTA by the NatA complex in yeast and higher metazoans.

In spite of the fact that NTA is a very common modification in Arabidopsis, the significance of NTA in phototrophic eukaryotes is almost unaddressed. Arabidopsis T-DNA insertion mutants for genes encoding putative NatB subunits or the catalytic subunit of NatC (NAA30, Mak3p) were viable but displayed a pleiotropic growth retarded phenotype. However, neither enzymatic activities of putative plant Nat complexes have been demonstrated in vitro, nor an in vivo substrate of a distinct plant Nat complex has been experimentally identified. Thus, the current knowledge on the plant NTA machinery is based on homology to characterized yeast and human Nat complexes.

Drought is the main reason for crop failure in world agriculture and provokes annually up to 50% of yield losses caused by environmental factors. Consequently, drought stress tolerance of crops was a significant trigger for total yield in the last decades and its significance for yield is supposed to even increase in the future as a result of global warming. The drought stress response of plants is mainly controlled by the phytohormone abscisic acid (ABA), which regulates the transcription of drought-stress-related genes, closure of stomata and root branching. Ubiquitin-dependent proteasomal degradation is of particular importance for successful drought stress response in plants, since it regulates turnover of many ABA-controlled transcription factors and removes dehydration-induced malformed proteins. The latter avoids aggregation of the damaged proteins and ensures proteostasis, which is under control of sophisticated cellular surveillance mechanisms in eukaryotes reviewed in ref. 21. These cellular surveillance mechanisms not only control protein synthesis but also translation and correct folding of proteins at the ribosome. Thus, the ribosome has been proposed recently as a central hub for protein quality control in eukaryotes. Here we ask the question if co-translational NTA of proteins by the ribosome-attached NatA complex contributes to cellular surveillance during stresses in higher plants and address the biological significance of NatA activity for regulation of metabolism and development.

We demonstrate that NAA10 and NAA15 are mandatory for NatA activity and target nearly 50% of soluble proteins in leaves. Thus, composition and substrate specificity of the NatA complex is evolutionary conserved within higher eukaryotes. Plant NatA activity is essential for proper embryogenesis and controls drought stress-induced developmental plasticity of the root system. Surprisingly, the turnover of the NatA complex is under the control of the stress-dedicated phytohormone ABA. Decrease of NatA is found to be sufficient for the induction of the canonical drought stress response and downregulates many biosynthetic pathways. Thus, we propose that imprinting of the cytosolic proteome by NatA is a master switch for the control of metabolism, developmental and cellular stress responses and integrates stress signals by perceiving the hormone ABA.

Results

Biochemical characterization of the Arabidopsis NatA complex.

A search for orthologues of the NatA complex in Arabidopsis with the TAIR BLASTP 2.2.17 algorithm (www.arabidopsis.org) revealed two candidates, At5g13780 (AtNAA10; AY091419) and At1g80410 (AtNAA15; AY087018), which show 67 and 41% identity with the human Naa10 and Naa15 proteins, respectively. Transcription of AtNAA10 and AtNAA15 was verified by quantitative reverse transcription–PCR (qRT–PCR) in roots, leaves, stem and flowers of Arabidopsis (Supplementary Fig. 1a). Purified recombinant AtNAA10 protein (MBP-His-AtNAA10) acetylated specifically an oligopeptide with a known NatA-type substrate sequence (STPD (ref. 1)) (Supplementary Fig. 1b). Formation of NatA complex was independently demonstrated by co-immunoprecipitation of full-length AtNAA10 and AtNAA15 in fusion with V5 tag and Xpress tag (Supplementary Fig. 1c), a bimolecular fluorescence complementation (BiFC) assay, which also revealed cytoplasmic localization of both proteins (Supplementary Fig. 1d) and a yeast two-hybrid approach (Supplementary Fig. 1e). Separation of soluble Arabidopsis leaf proteins by differential ultracentrifugation revealed co-fractionation of AtNAA15 with the ribosomal protein S14, strongly suggesting the association of the auxiliary subunit AtNAA15 with ribosomes (Supplementary Fig. 1f).
The NatA complex is essential for Arabidopsis embryogenesis. The in vivo function of NTA by the NatA complex in Arabidopsis was investigated by T-DNA insertion alleles for AtNAA10 (naa10-1) and AtNAA15 (naa15-1, naa15-2). Using differential interference contrast microscopy, we found that on heterozygous naa10-1 plants 20% of analysed embryos (n = 120) arrested at the dermatogen to early globular stage, demonstrating that loss of NAA10 activity causes embryo lethality (Fig. 1a,b). Homozygous naa15-1 (25%, n = 236) and naa15-2 (22%, n = 60) also arrested at the same embryonic stage (Fig. 1c,d). Genetic complementation of homozygous naa10-1 plants with AtNAA10 under control of the CaMV3SS promoter rescued the embryo lethal naa10-1 phenotype and resulted in wild-type-like growth of complemented naa10-1 plants, confirming the lack of NAA10 as single cause of the observed phenotype (Fig. 1e, Supplementary Fig. 2).

Downregulation of NatA complex results in retarded growth. To overcome the embryo lethal phenotype of the NatA loss-of-function mutants, artificial microRNAi sequences directed against NAA10 and NAA15 were expressed under the control of the CaMV3SS promoter in stably transformed wild-type or NAA10 function mutants, artificial microRNAi sequences directed against NAA15 (amiNaa15) were identified and displayed a significant reduction of growth. Transcription of the artificial microRNA (21 bp) against NAA10 was verified by RNA–DNA hybridization and qRT–PCR in selected amiNaa10 plants and demonstrated a strong correlation between steady-state level of the NAA10 microRNA and growth retardation (Supplementary Fig. 3). We selected two independent lines for amiNaa10 and amiNaa15 that have intermediate to strong growth phenotypes (Fig. 2a) for further analyses. Downregulation of the NAA15 transcript in the amiNaa15 lines 8 and 10 and the NAA10 transcript in the amiNaa10 lines 18 and 23 resulted in significant decrease of Naa10 and Naa15 protein level, respectively (Fig. 2b,c). Interestingly, downregulation of the catalytically active or the auxiliary subunit of NatA by the artificial microRNA caused also decreased the abundance of the second subunit of the NatA complex (Fig. 2b,c). Analysis of individual amiNaa15 plants revealed significant correlation between decrease of NAA15 abundance and growth retardation (Supplementary Fig. 3, r² = 0.99). Despite of the growth retardation, all isolated transgenic lines harboursing pBin-amiNAA10 (amiNaa10) and pBin-amiNAA15 (amiNaa15) were identified and displayed a significant reduction of growth. Transcription of the artificial microRNA (21 bp) against NAA10 was verified by RNA–DNA hybridization and qRT–PCR in selected amiNaa10 plants and demonstrated a strong correlation between steady-state level of the NAA10 microRNA and growth retardation (Supplementary Fig. 3). We selected two independent lines for amiNaa10 and amiNaa15 that have intermediate to strong growth phenotypes (Fig. 2a) for further analyses. Downregulation of the NAA15 transcript in the amiNaa15 lines 8 and 10 and the NAA10 transcript in the amiNaa10 lines 18 and 23 resulted in significant decrease of Naa10 and Naa15 protein level, respectively (Fig. 2b,c). Interestingly, downregulation of the catalytically active or the auxiliary subunit of NatA by the artificial microRNA caused also decreased the abundance of the second subunit of the NatA complex (Fig. 2b,c). Analysis of individual amiNaa15 plants revealed significant correlation between decrease of NAA15 abundance and growth retardation (Supplementary Fig. 3, r² = 0.99). Despite of the growth retardation, all isolated transgenic lines were fertile.
Depletion of NAA10 and NAA15 results in the same retarded vegetative growth phenotype. (a) Growth phenotype of amiNaa15 lines 10 and 8 and amiNaa10 lines 18 and 23 grown for 7 weeks on soil under short-day conditions. (Scale bar, 4 cm) (b) Immunological detection of NAA10 or NAA15 with specific antisera in leaves of NatA-depleted plants. Coomassie blue-stained protein served as loading control. Dashed lines indicate rearrangement of immunological signals. The original blot is shown in the Supplementary Fig. 7. (c,d) Quantification of NAA10 (c) and NAA15 (d) signals shown in b. Data are represented as mean±s.d. Asterisks indicate significant differences (P<0.05, N=3, Student’s t-test).

Supplementary Fig. 4b). NatA substrates of D. melanogaster display an intermediate kind of distribution for preference of the second amino-acid residue. Also the overall distribution of the acetylome substrates specificity show higher similarity between higher eukaryotes (D. melanogaster22, Arabidopsis and human23) when compared with lower eukaryote yeast1 (Fig. 3b).

To allow for direct quantification of free N termini in NatA-depleted plants, we established a fast and sensitive assay for the detection of free N\textsuperscript{\alpha}-terminal group of polypeptides with the fluorescent dye, 4-chloro-7-nitrobenzofurazan (NBD-Cl\textsuperscript{23}). The suitability of the optimized assay for N\textsuperscript{\alpha}-terminal groups in complex mixtures of non-denatured soluble proteins was demonstrated by detection of an about twofold increase in free N\textsuperscript{\alpha} termini of native soluble proteins of the yeast NatA loss-of-function mutants Scnaa15 (nat1) and Scnaa10 (ard1; Fig. 3g). This is in agreement with earlier studies demonstrating that NatA approximately acetylates half the yeast proteome\textsuperscript{1}. All analysed transgenic plants with decreased NAA15 or NAA10 subunits displayed a significant increase of free N termini that was in the same order compared with the yeast NatA loss-of-function mutant (Fig. 3h). Furthermore, the increase in detected free N termini correlated with the degree of NatA subunit depletion and the retardation of growth (Fig. 2).

NatA-depleted plants are highly drought tolerant. The hypothesis of NTA-dependent quality control of proteins\textsuperscript{7} prompted us to test if the NatA complex is involved in response to stresses. The NatA-depleted mutants were compared with NatB T-DNA insertion mutant (naa20) and wild type of the same age and the same developmental stage and in a time series for its drought resistance. Noteworthy, the naa20 mutant displays a similar decrease in growth rate as NatA-depleted plants\textsuperscript{15}. NatA-depleted plants were significantly more resistant to drought than both wild-type controls and naa20, demonstrating that the slower growth of NatA-depleted mutants is not the reason for the higher drought resistance compared with wild type (Fig. 4a). The relative water content (RWC) of leaves decreased in the two wild-type controls as result of prolonged drought (day 10 to 20, Fig. 4b). After 20 days of drought, wild-type plants died and could not be recovered by resupply of water (Supplementary Fig. 5a,b). In contrast, all tested amiNaa10 and amiNaa15 lines were viable after 20 days of drought, did not show any visible drought-related phenotype and completed life cycle after resupply of water (Supplementary Fig. 5b). Astonishingly, the NatA-depleted plants retained almost 95% RWC even after 20 days of drought, which explains the drought-tolerant phenotype (Fig. 4b) and the soil water content was higher in pots that contained NatA-depleted plants (Supplementary Fig. 5c).

Depletion of NatA activity causes closure of stomata. The capability of NatA-depleted plants to retain almost all water even after 20 days of drought prompted us to test the transpiration rate of truncated leaves in a short-term experiment. Detached leaves
of amiNaa10 and amiNaa15 lines lost significantly less water over time than detached wild-type leaves (Fig. 4c). Transpiration of leaves is controlled by abundance and aperture of stomata in higher plants. The density of stomata was unaffected in leaves of NatA-depleted plants (Supplementary Fig. 5d), but the stomata aperture in the NatA-depleted mutants was significantly decreased by B2m, which represents the almost fully closed state of stomata (Fig. 4d, Supplementary Fig. 5e). In search for a functional explanation for the closure of stomata in NatA-depleted plants, we quantified hydrogen peroxide (H2O2)
in guard cells of stomata (Fig. 4e). H$_2$O$_2$ is a canonical trigger for stomata closure and its production is known to be regulated in plants by the hormone ABA. Guard cells of NatA-depleted plants display two to three times higher steady-state levels of reactive oxygen species (ROS) than the wild-type guard cells in the absence of exogenous applied ABA (Fig. 4e,f). Application of...
ABA to wild-type leaves increased ROS in guard cells to the same levels as in guard cells of non-treated NatA-depleted plants. The concentration of ROS in guard cells of NatA-depleted plants was not increased further by ABA treatment (Fig. 4f).

NatA-depleted plants display drought-adapted root morphology. Since drought tolerance is often mediated by improved water uptake by the root system, we analysed the root morphology of the wild type, the nna20 mutant and the NatA-depleted mutants. NatA depleted showed a significant increase of total root length to shoot fresh weight ratio when compared with wild type and the nna20 mutant (Fig. 4g). This increase is driven by growth of the primary root in the NatA-depleted plants. The formation of lateral roots is lowered in the NatA-depleted mutants, which results in a significant decrease of lateral root density under non-stressed conditions when compared with wild type (Fig. 4h, Supplementary Fig. 6a). The increase of primary root length and the inhibition of lateral root formation are both known adaptations of the wild type towards drought and are triggered by the phytohormones IAA and ABA18 via the transcription factors MYB77 and ARF7 (ref. 24). Transcript steady-state levels of both transcription factors are significantly downregulated in amiNaa10 and amiNaa15 lines (Supplementary Fig. 6b), suggesting that these transcription factors contribute to the observed constitutive drought stress adaptation of root morphology in NatA-depleted plants under non-stressed conditions.

NatA depletion induces ABA responses. Lateral root formation and closure of stomata are triggered by ABA in the wild type on drought. We therefore tested the expression of the well-established ABA marker gene, LEA7, in leaves of NatA-depleted plants and the wild type. In the amiNaa10 line 23, which shows strongest depletion of NatA, the steady-state transcript level of LEA7 was ~60-times higher than the wild type (Fig. 5a). The increase of LEA7 transcript steady-state level correlated with the degree of NatA depletion and was not significantly changed in amiNaa15 line 10. To test if LEA7 transcription is triggered by higher ABA levels in NatA-depleted plants, we quantified ABA levels in leaves and roots of NatA-depleted plants by LC-MS/MS. Neither in the leaves (Fig. 5b) nor in the roots (Supplementary Fig. 6c) a significant increase of ABA could be detected in NatA-depleted plants when compared with wild type.

In search for NatA-regulated processes, we determined the global transcriptome in leaves of NatA-depleted mutants and compared it with the response of the wild type on drought stress. Transcriptomes of amiNaa10 line 23 and amiNaa15 line 8 displayed a remarkable degree of co-regulation (73%), showing that the downregulation of the catalytic and the auxiliary subunit cause a similar transcriptional response in leaves of both mutants. Downregulated genes (32%) and 19% of upregulated genes in NatA-depleted plants are part of the drought stress-induced response in wild-type plants (Fig. 5c,d). A gene annotation enrichment analysis revealed significant downregulation of flavonoid and phenylpropanoid biosynthesis pathways in NatA-depleted plants (Table 1), which both use phenylalanine as precursor. Interestingly, sulfate assimilation was downregulated in leaves of both mutants, but nitrate assimilation was unaffected. This strongly indicates that the downregulation of sulfate assimilation is specific and not a simple consequence of slower growth of NatA-depleted plants. The only pathway that was significantly upregulated in NatA-depleted plants was ubiquitin-mediated proteolysis (Table 1).

Drought stress and ABA cause increase of free N termini. Depletion of the NatA complex resulted in higher amount of free N termini on the global scale (Fig. 3) and constitutively induced

![Figure 5 | Transcriptional response to NatA depletion.](image)

**Figure 5 | Transcriptional response to NatA depletion.** ABA steady-state levels and transcription of ABA-related genes were determined in leaves of 6-week-old wild type and NatA depleted grown on soil with regular water supply. (a) Transcript steady-state level of the ABA-inducible marker gene, LEA7. (N = 3) (b) Steady-state levels of ABA. (N = 5) (a,b) Data are represented as mean ± s.e. Asterisks indicate significant differences to wild type. (P < 0.05, Student’s t-test). (c,d) Venn diagrams for comparison of downregulated (c) and upregulated genes (d) in wild-type plants by drought stress (10 days) with genes that are regulated in NatA-depleted plants under control conditions when compared with wild type. Global transcriptional response in leaves of well-watered amiNaa10 (green), amiNaa15 (blue) was compared with non-stressed wild-type plants (control) as determined by hybridization of total mRNA to the Gene 1.0 ST Array (Affymetrix, Germany). (N = 4).
part of drought stress response in presence of low ABA levels (Figs 4 and 5). We therefore hypothesized that the generation of free N termini might be part of the drought stress response and act downstream of ABA. Quantification of free N termini in soluble protein extracts from leaves of wild type challenged with drought revealed a significant correlation between decrease of RWC and quantity of free N termini (Fig. 6a). Exogenous application of ABA caused a significant increase of free N termini in the soluble protein fraction within 2 h. The amount of free N termini increased linearly for 6 h and reached a plateau after 8 h (Fig. 6b). ABA treatment resulted in significant downregulation of NAA10 and NAA15 transcript steady level to ~60% of wild-type level after 6 h (Fig. 6c). Immunological detection of NAA10 and NAA15 revealed significant and specific decrease of the catalytic (74%) and the auxiliary subunit (45%) of NatA (Fig. 6d,e), while stable cytosolic (OAS-TL A) and plastidic control proteins (GR2) were not affected by short-term ABA treatment (Fig. 6d). Taken together, these data demonstrate that ABA downregulates NatA abundance and causes an increase of free protein N termini within a few hours’ time range. Consequently, the amount of free N termini increases on drought stress in wild-type plants.

### Table 1 | Gene annotation enrichment analysis of transcripts regulated in NatA-depleted plants.

| Pathway                        | Annotation         | Fold enriched | P value |
|--------------------------------|--------------------|---------------|---------|
| Sulfate assimilation           | PO2778             | Downregulated | 17.45   | 0.01   |
| Indole alkaloid biosynthesis   | ATH00901           | Downregulated | 13.17   | 0.02   |
| Flavonoid biosynthesis         | ATH00941           | Downregulated | 12.94   | 0.00   |
| α-Linolenic acid metabolism    | ATH00592           | Downregulated | 7.52    | 0.00   |
| Phenylpropanoid biosynthesis   | ATH01061           | Downregulated | 2.13    | 0.01   |
| Ubiquitin-mediated proteolysis | ATH04120           | Upregulated   | 3.82    | 0.01   |

Wild-type and NatA-depleted plants (aminNaa10 and aminNaa15) were grown for 6 weeks under short-day condition with regular water supply. Transcripts were quantified with the Gene 1.0 ST Array (Affymetrix, Germany). Significantly regulated genes were defined by P < 0.05 and ≥1.5-fold change when compared with wild type (N = 4). Gene annotation analysis of these genes was performed with the DAVID Bioinformatics Resources tool v.6.7 (http://david.abcc.ncifcrf.gov).

### Discussion

The here-applied biochemical and reverse genetics approaches demonstrate the identity of At5g13780 as the catalytic subunit NAA10 and At1g80410 as the auxiliary subunit NAA15 of the Arabidopsis NatA complex. The first in vivo substrate characterization of a Nat complex from a phototrophic eukaryote demonstrates high similarity of the Arabidopsis NatA substrate specificity to the substrate specificities of the human and yeast NatA complex. Thus, NatA acts co-translationally after the excision of iMet by methionine aminopeptidases in all analysed organisms so far, which is in agreement with complementation of yeast loss-of-NatA mutants by reconstituted human NatA complex. Remarkably, the human catalytic or auxiliary NatA subunits do not complement respective single loss-of-function yeast mutants.

Loss-of-NatA activity in Arabidopsis or NAA10 activity in D. melanogaster and humans is fatal, but causes only slight growth retardation in yeast grown under optimal conditions in full medium. A possible explanation for this discrepancy might be provided by the different distribution of NatA substrates in higher eukaryotes and yeast. While in higher eukaryotes most NatA substrates starts with A > S > T, G, the frequency of these

![Figure 6](https://example.com/figure6.png)

**Figure 6 | Drought stress and ABA cause significant increase of free N termini.** (a) Correlation between free N termini of soluble proteins and the RWC determined in leaves from 6-week-old wild-type plants after application of drought (N = 3–5). (b) Quantification of free N termini in soluble proteins extracted from leaf discs of 6-week-old wild-type plants treated with 50 μM ABA for indicated time (N = 4). (c) NAA10 and NAA15 transcript steady-state level in the wild-type leaf discs after application of 50 μM ABA for 6 h. (d) Immunological detection of NatA subunits and two cytosolic control proteins (OAS-TL A, GR2) in wild-type leaf discs after application of 50 μM ABA for 6 h. Coomassie-stained Rubisco large subunit (LSU) served as loading control. (e) Quantification of NAA10 and NAA15 signal shown in d after normalization to loading control. The amount of NAA10 or NAA15 in wild-type leaf discs treated with water was set to 100% (N = 4). Data are represented as mean ± s.e. Asterisks indicate significant differences to wild type. (P < 0.05, Student’s t-test).
substrates is significantly different in yeast.\textsuperscript{4,26} The experimentally identified NatA-dependent acetylome of Arabidopsis thaliana shares highest similarity to the human NatA-dependent acetylome.

A hallmark of higher eukaryotes is multicellularity, which provides the basis for the specification of cells in tissues. This specification is mainly controlled by defined hormone gradients in higher eukaryotes. In the higher eukaryote Arabidopsis, NatA activity is regulated by the phytohormone ABA (this work) and the developing NatA-deficient plant embryos arrest exactly in the apical–basal axis.\textsuperscript{27} However, abortion of embryogenesis can be caused by specific deregulation of developmental processes or by defects in primary metabolism. For that reason, arrest of embryogenesis in NatA-deficient Drosophila or Arabidopsis is indicative, but does not prove the direct involvement of the NatA complex in the regulation of development.

Downregulation of the NatA complex in plants causes not only growth retardation in the vegetative stage but also significantly affects the root-to-shoot ratio and the root morphology (Fig. 4). Both developmental processes are known to be ABA regulated and comprise significant transcriptional adaptation of key regulators that has been also been evidenced in NatA-depleted roots.\textsuperscript{18,24} In view of the fact that the abiotic factor, drought, impacts the N\textsuperscript{2}-acetylome, we hypothesize a mediating role of the NatA complex in the enormous plasticity of plants towards environmental challenges reviewed in ref. 28.

NTA has been designated as co-translational and irreversible imprinting of the proteome, and not considered subject to regulation.\textsuperscript{29} NTA imprinting was thought to influence the fate of any given protein constitutively. This dogma is also supported by the lack of proven N-terminal deacetylases in any organism. NTA of proteins was suggested to be important for targeting specific proteins for degradation after malfolding and/or release from multi-subunit complexes by unshielding of a preformed degradation signal, or to assist proteins in obtaining their proper subcellular localization.\textsuperscript{30,31} Nonetheless, both suggestions have not taken into account the possible relevance of the partially modified acetylome or even its modulation in response to biotic or abiotic stresses.

Up to now, no reports for active regulation of the NatA acetylome or any other N\textsuperscript{2}-acetylome have been reported. The only identified factor that impacts Nat activity was limitation by acetyl-CoA supply, which will affect nonspecifically all Nat complexes and many other biosynthetic routes in the cytosol.\textsuperscript{10} Here we show that 10% of all NatA substrates are partially acetylated under non-stressed conditions in leaves of Arabidopsis. A similar degree of partially acetylated N termini has been also found in humans. Astonishingly, only 16 NatA substrates (6.6% of all identified NatA substrates) were less acetylated in HeLa cells after the downregulation of hNAA10 to 5% of wild-type level.\textsuperscript{1} In higher plants, the NatA-dependent acetylome is much more sensitive to regulation of NatA abundance. Depletion of NatA to 20% of the wild-type levels resulted in a significant increase of free and partially acetylated N termini (up to 30% of all identified NatA substrates, 148 proteins) and caused an overall decrease in the NatA acetylation frequency. Such a decrease in acetylation frequency was also found in leaves of water-deprived plants and revealed for the first time a physiological adaptation of the N\textsuperscript{2}-acetylome in response to an abiotic stress in higher eukaryotes. In agreement with a regulatory function of protein NTA in plants on stress, a recent study has shown that the e subunit of chloroplast ATP synthase occurs as both acetylated and non-acetylated form and the level of the latter preferentially decreases under drought conditions.\textsuperscript{32} Taken together, these results imply a paradigm shift for the biological function of protein NTA.

The first time-resolved quantification of the global acetylation status in a higher eukaryote demonstrates quick adaption of the leaf N\textsuperscript{2}-acetylome after application of the stress-related hormone ABA (\textless 6 h). The increase in unacetylated protein N termini was at least partially triggered by fast and significant downregulation of NatA abundance on ABA treatment, which provides a solid explanation for the remarkable correlation between unacetylated N termini and the RWC in leaves. Thus, in higher plants, NTA by NatA is an ABA-regulated dynamic process that adopts the N\textsuperscript{2}-acetylome in response to environmental changes. Such a hormone-regulated adaptation of the N\textsuperscript{2}-acetylome on stress has not been reported for any other eukaryote up to now, but is in agreement with the sensitivity of yeast NatA loss-of-function mutants to abiotic stresses like heat shock or nutrient starvation.\textsuperscript{25} Remarkably, also NatB and NatC yeast knockout mutants have been shown to be sensitive to specific sets of abiotic stresses.\textsuperscript{25}

The molecular reason for the stress-resistant phenotype of NatA-depleted plants is the constitutive induction of major components of the drought stress response. This constitutive induction comprises metabolic adaptation of single cells (increase of H\textsubscript{2}O\textsubscript{2}-induced stomata closure), alteration of global transcriptional response and adjustment of developmental processes (inhibition of lateral root hair formation). All these processes are known to be regulated by ABA in higher plants, but ABA levels were unaffected in roots and leaves of NatA-depleted plants. The latter strongly argues against the activation of ABA synthesis or decreased degradation of ABA as molecular trigger for constitutive induction of drought stress response in NatA-depleted plants. The significant overlap between ABA-mediated processes and the response to NatA depletion in combination with the decreasing effect of ABA on NatA activity in the wild type suggests that NatA acts downstream of ABA during the induction of the drought stress response. This could be either achieved by altered biological activity of many NatA substrates in different pathways or by modified activity of a few key regulators of the drought response. Indeed, many drought stress-responsive transcription factors are regulated by ubiquitination-dependent proteasomal degradation.\textsuperscript{20} In humans, NAA10 is a negative regulator of the proteasome\textsuperscript{33}, which is in agreement with the significant upregulation of the ubiquitination-mediated proteolysis pathway in NatA-depleted plants. Furthermore, degradation of proteins by the Ac/N end rule pathway that accepts acetylated N termini starting with A, V, S, T and C\textsuperscript{6} could be perturbed in the plant NatA mutants. The unacetylated protein N termini might earmark these proteins for different degradation routes in plants. A similar scenario has been evidenced for proteins starting with iMet in yeast natC mutants.\textsuperscript{8}

In summary, our findings demonstrate that imprinting of the proteome by the NatA complex is an evolutionary conserved process in higher eukaryotes and is dynamic in higher plants. We propose that the regulation of NatA by ABA triggers the response of plants towards drought stress by regulation of global transcription and development (root morphology). Consequently, NatA-depleted plants are preadapted and highly drought resistant. Thus, we propose that N\textsuperscript{2}-terminal acetylation is a general cellular surveillance mechanism in higher plants that contributes significantly to the response to abiotic stresses. These findings suggest new targets to genetically engineer plants that are more resistant towards water limitation, an important trait for crop yield.

**Methods**

**Plant material and growth conditions.** All work was performed with Arabidopsis thaliana ecotype Col-0. One T-DNA insertion line for AtNAA10, naa10-1 (Wisc DsLOX289_292G3), AtNAA20, naa20-1 (SALK_027687) and two T-DNA

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insertion lines for AtNAA15, naa15-1 (SAIL_812_B10) and naa15-2 (WiscDsLoX481_48420) were obtained from the SAIL, SALK and Wisconsin collections, respectively. Transformation of Agrobacterium tumefaciens vectors and subsequent transformation and selection of Arabidopsis thaliana cv Col-0 was carried out as described in ref. 34. Heterozygous T-DNA lines for NAA10 and NAA15, homozygous T-DNA lines for NAA20, wild-type and Arabidopsis plants expressing amiRNA-Naa10 and amiRNA-Naa15 (F2 or F3-generation) were grown in climate chambers in growth medium containing one half soil and one half substrate 2 (Klasmann-Deilmann, Germany) under controlled conditions: 8.5 h light, 100 μE m⁻² s⁻¹ photon flux density, 24 °C at day, 18 °C at night and 50% humidity. Rosette leaves of 8-week-old complemented nai10-1 plants were pooled before fresh weight determination.

PCR. PCR for identification of T-DNA insertion lines was performed with the Taq-DNA Polymerase from New England Biolabs (M0267L). Genotyping of T-DNA insertion lines for NAA10 and nai10-1, nai15-1, nai15-2 was performed with specific primer combinations for the wild-type allele (NAA10_1_LP, NAA10_1_RP, NAA15_1_LP, NAA15_1_RP, NAA15_2_LP, NAA15_2_RP, NAA20_1_LP, NAA20_1_RP) and mutant allele (Wisc_BP, SAIL_BP, SALK_BP). For cloning, DNA was amplified with the high-fidelity DNA polymerase Phusion (New England Biolabs, M0530L). In both cases, the PCR reactions were performed according to the supplier’s instructions manual. Sequences of the respective primers are provided in Supplementary Table 1.

Quantitative real-time PCR. Total RNA from leaf tissue was extracted with the RNeasy Plant Kit (Qiagen, Germany) according to the manufacturer’s protocol. Total RNA was transcribed into complementary DNA (cDNA) and analysed by qRT–PCR as described in ref. 35. For quantification of artificial microRNA (amiRNA), total RNA samples, the amiRNA was polyadenylated, followed by the addition of a reverse primer using the miScript II RT Kit (Qiagen, Germany). The resulting cDNA was analysed by qRT–PCR as described above using actin_7 as reference. The amiNaa10_f and the miScript universal tag primer (supplied with the miScript SYBR II RT Kit, Qiagen) served as primers for specific amplification of amiRNA against NAA10. The sequences of primers used for qRT–PCR are depicted in Supplementary Table 1.

Generation of NAA10 and NAA15 antibodies. The full-length AtNAA10 was PCR amplified and cloned into pET32a (TRX(Thioredoxin)/His-fusion) using BamHI and XhoI restriction sites. A fragment of AtNAA15 including the amino-acid residues 575–668 was PCR amplified and cloned into pET20b (His-fusion) using the Xhol and BamHI restriction sites. Oligospecific primers used to amplify AtNAA10 and AtNAA15: NAA10_BamHI_f, NAA10_XhoI_r, NAA15_BamHI_f and NAA15_XhoI_r. The primer sequences are provided in Supplementary Table 1. The pET32a-AtNAA10 and pET20b-AtNAA15 plasmids were transformed into E. coli HST74 (DE3) cells (Invitrogen) by electrocompetence. Cell cultures (300 ml) were grown in LB (lysogeny broth) medium to an OD₆₀₀nm of 0.3 at 37 °C and protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside. After 5 h of incubation, the cultures were cooled to 4 °C and the cells lysed by sonication and french press in lysis buffer (1 mM DTT, 50 mM Tris-HCl (pH 7.4), 20,000-fold in 1 ml IPH buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM Na₂VO₄, 2 mM pefabloc and 1× complete protease inhibitor cocktail (Roche))). Approximately 1×10⁴ cells were used for each sample. Protein A/G Plus-Agarose slurry (50 μl, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and incubated on ice for 1 h at 4 °C. After centrifugation at 500g for 5 min, the supernatants were incubated with 3.0 μg anti-V5 mouse monoclonal IgG₂b, (Invitrogen), anti-Xpress mouse monoclonal IgG₂b (Invitrogen) or unspecific IgG₂b, (Dako) and incubated for 4 h at 4 °C. Later, 70 μl of Protein A/G Plus-Agarose was added and incubated for 16 h at 4 °C with the samples. The immunoprecipitated samples were collected by centrifugation at 500g for 5 min, washed four times with 1× PBS.

Expression and purification. The pETM-41-AtNAA10 plasmid was transformed into E. coli BL21 Star (DE3) cells (Invitrogen) by heat shock. Cell culture (200 ml) was grown in LB (lysogeny broth) medium to an OD₆₀₀nm of 0.8 at 37 °C and subsequently transferred to 20 °C. After 30 min of incubation, protein expression was induced by isopropyl-β-D-thiogalactoside (1 mM). After 17 h of incubation, the cultures were harvested by centrifugation and the pellets stored at −20 °C. E. coli pellets containing recombinant proteins were thawed at 4 °C and the cells lysed by sonication and french press in lysis buffer (1 mM DTT, 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1 tablet EDTA-free protease inhibitor cocktail per 50 ml (Roche)). The cell extracts were applied on a metal affinity FPLC column (HisTrap HP, GE Healthcare, Uppsala, Sweden). Appropriate fractions containing recombinant protein MBP-His-AtNAA10 were pooled and further purified using size-exclusion chromatography (Superdex 75, GE Healthcare). The purity of the fractions corresponding to purified monomeric recombinant proteins were analysed on Coomassie-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and the protein concentrations determined by OD₂₈₀nm measurements.

In vitro Nats assay. Purified MBP-His-AtNAA10 (0.5 μM) was mixed with selected oligopeptide substrates (200 μM) and 300 μM of acetyl-CoA in a total volume of 60 μl acetylation buffer. The samples were incubated at 37 °C for 60 min. The enzyme activities were quenched by adding 5 μl of 10% TFA. The reaction mixtures were centrifuged and then separated by HPLC on a Symmetry C18 column (250 mm × 4.6 mm, 5 μm). The elution was performed with 0–20% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml min⁻¹ at 40 °C. The elution was monitored at 288 nm.

S.D.S.-PAGE and immunological detection. Soluble protein extracts were subjected to discontinuous S.D.S.–PAGE in Mini-ProteinTM II cells (BioRad) and immunoblotting. For the in vitro Nats assay, anti-V5 monoclonal antibodies and horseradish peroxidase-linked anti-mouse antibodies (Amersham) were diluted 20,000-fold in 1× PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl) before usage. The specific antibodies against OAS-Tl5, the ribosomal protein S14 (Abx112, 2111, Agrisera) and NAA15 were diluted 1,000, 8,000 and 8,000-fold in TBS-T (50 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20) for quantitative of respective proteins in fractions of differential ultra-centrifuged soluble leaf proteins. The abundance of NAA10 and NAA15 in soluble leaf proteins isolated from wild-type, amiNaa10 and amiNaa15 plants were determined with NAA10 diluted 5,000-fold or nNAA15 diluted 8,000-fold in TBS-T. The horseradish peroxidase-linked anti-rabbit antibody (A510 852, Agigrsa) was diluted 25,000-fold in TBS-T. Membranes were developed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) according to the manufacturer’s protocol. The resulting signals were recorded using the ImageQuant LAS 4,000 (GE Healthcare) and subsequently quantified with the ImageQuant TL Software (GE Healthcare). The Original images of the cropped gels/blots shown in the paper are depicted in Supplementary Fig. 7.

BiFC assay. For construction of BiFC vectors, AtNAA10 and AtNAA15 were PCR amplified from total cDNA and cloned into pGFP-C176 (Stratagene) before being subcloned into the final destination vectors (pVENNY-NY or pVENNY-CY). For construction of the pVENNY-NY or pVENNY-CY vectors, AtNAA10 was amplified with NAA10_B_f and NAA10_B_r and AtNAA15 with NAA15_B_f and NAA15_B_r. For all constructs, correct cloning was verified by DNA sequencing. Primer sequences are listed in Supplementary Table 1.
Quantification of N-terminal protein acetylation. Liquid nitrogen-frozen Arabidopsis plant tissues were ground in 2 ml microcentrifuge tubes containing 3 and 5 mm iron beads for 1 min each, using a MM 300 mixer mill at 30 Hz (Qiagen). The resulting fine powder was dissolved in 1 ml of lysis buffer (buffer D) [1]. The homogenates were incubated at 4 °C for 30 min with shaking. The supernatants were separated from the insoluble fraction by centrifugation at 15,000 g at 4 °C for 30 min and used to determine protein concentration using the Bradford protocol. As previously described [4], 1 mg of proteins was denatured in 6 M Guanidine-HCl, 50 mM Tris-HCl (pH 8) and 4 mM DTT, reduced for 15 min at 95 °C and finally alkylated by the addition of iodoacetamide (55 mM) for 1 h at room temperature. Proteins were precipitated by the addition of four times the sample volume of cold acetone followed by 1 h centrifugation at −20 °C. The resulting pellet was resuspended in 50 mM phosphate buffer (pH 7.5) and was measured. The ratio of growth on SD–TL and SD–HLT was calculated for the indicated pair of spots and the average and s.d. of these ratios calculated.

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used in the extraction script, that is, E Val < 0.1, Std. Err. < 0.1, Mascot Score > 30, Correct. > 0.5, Fraction > 0.5. Sc. P = 0.3. NTA yield was determined from the d0/d3 ratio and expressed in% of NTA for the N termini.

**Determination of the global transcriptome.** Total RNA was extracted from leaf tissue from wild-type, amiNaa10 and amiNaa15 plants grown for 52 days on soil under short-day conditions and from wild-type plants grown for 42 days on soil under short-day conditions followed by 10 days of drought using the peqGOLD Total RNA Kits (Peqlab) according to the manufacturer’s protocol. Total RNA was prepared using Trizol (Gibco) followed by additional purification using the RNeasy Mini Kit (Qiagen). RNA was tested by capillary electrophoresis on an Agilent 2100 bioanalyzer (Agilent) and high quality was confirmed.

Gene expression profiling was performed using arrays of Arabagene-1.0-st-type from Affymetrix. Biotinylated antisense cRNA was then prepared following the Affymetrix standard labelling protocol. Later, the hybridization on the chip was performed on a GeneChip Hybridization oven 640, then dyed in the GeneChip Fluidics Station 450 and thereafter scanned with a GeneChip Scanner 3,000.

All of the equipment used was from the Affymetrix-Company (Affymetrix, High Wycombe, UK).

A Custom CDF Version 16 with TAIR-based gene definitions was used to annotate the arrays47. The Raw fluorescence intensity values were normalized applying quantile normalization and RMA background correction. Analysis of variance was performed to identify differential expressed genes using a commercial software package SAS JMP10 Genomics, version 6, from SAS (SAS Institute, Cary, NC, USA). A false positive rate of $a = 0.05$ with false discovery rate correction was taken as the level of significance.

The over-representation analysis is a microarray data analysis that uses predefined gene sets to identify a significant over-representation of genes in data sets36. Pathways belonging to various cell functions such as cell cycle or apoptosis were obtained from public external databases (KEGG, http://www.genome.jp/kegg/ and GO, http://www.geneontology.org/). The analysis was performed using the DAVID Bioinformatics Resources 6.7 (ref. 49).

**Determination of free N termini.** To quantify the free N termini level, soluble proteins were extracted from leaves in 50 mM sodium citrate buffer containing 1 mM EDTA (pH 7.0) and desalted using PD SpinTrap G-25 columns (GE Healthcare) to remove free amino acids. Crude protein extract (2.5 M) was incubated with 0.5 mM NBD-C3 (Sigma–Aldrich) in 50 mM sodium citrate buffer containing 1 mM EDTA (pH 7.0) for 14 h at room temperature. The fluorescence intensity was quantified with a FLUOstar Omega plate reader (BMG Labtech; excitation: 470 ± 10; emission: 520 nm). For the quantification of free N termini in response to ABA (Sigma–Aldrich), leaf discs were floated (30 mg) in ¼ × Hoagland medium supplemented with 50 μM ABA for 2, 4, 6 and 8 h before analysis.

**Determination of ROS in intact guard cells.** ROS accumulation in guard cells was quantified using the H$_2$O$_2$-specific dye H$_2$DCF-DA (Life Technologies). Epidermal peels of wild-type, amiNaa10 and amiNaa15 plants were floated for 120 min on 30 mM KCl, 10 mM MES (pH 6.15). H$_2$O$_2$ was stained for 10 min with 50 μM ABA for 20 min before H$_2$O$_2$ staining. Control samples were treated with 0.1% ethanol. The H$_2$O$_2$-specific fluorescence was recorded in the guard cells with the C2 plus confocal laser microscope (Nikon; excitation 488 nm; emission 525 nm) and evaluated with the Fiji image-processing package.

**Determination of stomatal density.** For the determination of the stomata density, imprints of the abaxial leaf side of 5-week-old wild-type, amiNaa10 and amiNaa15 plants were created on glass microscope slides using super glue and analysed with the DMIIR microscope (Leica).

**Determination of stomatal aperture.** Size of stomatal aperture was determined by imaging the abaxial leaf side of 5-week Fold wild-type, amiNaa10 and amiNaa15 plants with a confocal laser microscope (Zeiss LSM 510). Data analysis was performed with the Fiji image-processing package.

**Determination of primary root length and lateral root density.** After growing for 5 weeks on ¼ × Hoagland or for 14 days on 1 × MS solid medium (Duchela), roots from wild-type, amiNaa10, amiNaa15 and nax20 plants were photographed with a digital camera. The primary root length and the number of lateral roots were measured using the Fiji image-processing package.

**Determination of the transpiration rate.** To measure leaf water loss, fully expanded leaves were detached from 5-week-old plants and placed abaxial side up in open petri dishes at room temperature. The weight of the leaves was monitored during 2 h with a precision balance (Sartorius).

**Quantification of drought stress.** To characterize the drought stress, we determined the water content of the soil by subtraction of the weight of completely dried soil from the weight of the soil taken as the level of significance.

The RWC leaves was determined using the formula: $RWC = \frac{m}{M} \times 100$%. The fresh weight of rosette leaves (N = 3) of individual plants (N = 4) were measured. Subsequently, the leaves were rehydrated for 6 h in 4 °C cold water to determine the rehydrated weight of the sample. The dry weight was determined after drying of leaves for 18 h at 80 °C.

**Basic statistical analysis.** Regression analyses of data sets were performed with SigmaPlot 12.0 that uses the Marquardt–Levenberg algorithm for determination of independent variables. Constant variance and normally distribution of data were carefully checked with SigmaPlot 12.0 before statistical analysis. Comparison of means from different sets of data was analysed for statistical significance with the unpaired t-test or the Mann–Whitney U rank test, if data set was not normally distributed. Significant differences ($P<0.05$) are indicated by asterisks.

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Author contributions

E.L. and I.S. designed and performed the experiments and drafted the manuscript; M.H. and M.W. performed the ribosome association studies and metabolite analyses, respectively. I.M.G., L.M.M., S.G.M. and T.A. designed and performed NatA association and enzymatic activity studies. C.S. performed transcriptome analysis and contributed bioinformatics on large-scale transcriptome data sets. W.V.B., T.M. and C.G. performed and revised the manuscript. E.L., M.H., R.H. and M.W. gratefully acknowledge funding by the Deutsche Forschungsgemeinschaft (SFB 1,036, TP13). T.A. and L.M.M were supported by grants from the Norwegian Cancer Society, The Bergen Research Foundation BFS, the Research Council of Norway (grant 230865), and the Western Norway Regional Health Authority. E.L. and M.H. were supported by the Hartmut Hoffmann-Berling International Graduate School, University of Heidelberg, Heidelberg, Germany. We cordially thank Drs Günter Kramer and Bernd Bukau (University of Heidelberg, ZMBH) for support with ribosome association studies and constructive discussion on the function of NTA for protein quality control, Nikon Imaging Center at the University of Heidelberg and the Metabolomics Core Technology Platform of the Excellence cluster ‘CellNetworks’ (University of Heidelberg).

Additional information

Accession codes: The mass spectrometry proteomics data are deposited in the PRIDE repository with the data set identifier PXD002069. The microarray data sets are uploaded to the NCBI GEO database under the GEO Accession number GSE65414. Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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