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## Light changes promote distinct responses of plastid protein acetylation marks

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**Abstract**

Protein acetylation is a key co- and post-translational modification. However, how different types of acetylation respond to environmental stress is still unknown. To address this, we investigated the role of a member of the newly discovered family of plastid acetyltransferases (GNAT2), which features both lysine- and N-terminal acetyltransferase activities. Our study aimed to provide a holistic multi-omics acetylation-dependent view of plant acclimation to short-term light changes. We found that both the yield and coverage of the N-terminal acetylome remained unchanged in wild-type and *gnat2*-knockout backgrounds after two hours of exposure to high light or darkness. Similarly, no differences in transcriptome or adenylate energy charge were observed between the genotypes under the tested light conditions. In contrast, the lysine acetylome proved to be sensitive to the changes in light conditions, especially in the *gnat2* background. This suggests unique strategies of plant acclimation for quick responses to environmental changes involving lysine, but not N-terminal, GNAT2-mediated acetylation activity.

## Introduction

Protein acetylation is the most frequent protein modification after phosphorylation in eukaryotes, including plants (1). N-acetylation refers to the addition of an acetyl moiety on amino groups of lysine side chains (acK) or on N-termini (NTA) of proteins through an amide bond. While a majority (>75%) of eukaryotic proteins have their N-terminus entirely modified, not all lysine chains display acK. As proteins have an average of 32 lysines (2), many proteins are nevertheless expected to display either one or both acetylation marks, i.e. NTA or acK. Acetylation marks have been demonstrated to play crucial roles in all cellular processes. For instance, acK was initially observed on histone proteins where it is involved in the regulation of transcription (3). Since then, proteomic studies have revealed thousands of non-histone proteins containing acK sites (4), where acetylation affects protein functions and can block lysine residues from other protein modifications such as ubiquitination (5). For an increasing number of these proteins, acK was shown to occur in diverse subcellular compartments, including the cytosol and chloroplast, and it was demonstrated that acK can regulate the activity of certain enzymes such as RuBisCO and malate dehydrogenase for example (6-8). In most cases, acK and NTA are supported by distinct families of acetyltransferases (9). All N-terminal acetyltransferases identified so far belong to the GCN5-family (GNAT) of acetyltransferases (NATs), whereas lysine acetyltransferases (KATs) are found in at least three families: GNAT, MYST and p300/CBP (10). Unlike acK, which occurs post-translationally and is reversibly controlled by specific deacetylases, NTA is considered an irreversible modification occurring mostly co-translationally (1). In a few cases, NTA was described to occur post-translationally on hormone peptides or on exported proteins of apicomplexa (11). Only recently it was shown that NTA occurs both co-translationally on plastid-encoded proteins, and post-translationally on a significant fraction of nuclear-encoded, plastid-imported proteins after cleavage of the transit peptide (2, 12). A new family of nuclear-encoded acetyltransferases localized in plastids was identified and revealed to display unique features including unexpectedly dual NTA and acK activities (2). Because of their unique mode of action, all these plastid-localized acetyltransferases were named GNATs, and two subfamilies, NAA70 and NAA90 were identified (1). To date, the *in planta* dual activity of the plastid GNATs was demonstrated only for AtGNAT2 – one of the three members of the NAA90 subfamily - by showing a global reduction of both plastid NTA and acK

in *gnat2* knockout plants (2, 13). The protein acetylation activity of GNAT2 was also reported for the rice homolog (14). For rice and *A. thaliana* GNAT2 weak serotonin acetyltransferase (SNAT) activities were reported as well (15, 16). However, the melatonin content of the Arabidopsis knockout plants was decreased only upon challenging of the plant with avirulent pathogens or high light (17, 18). Moreover, the *gnat2* plants showed changes in the accumulation of various other compounds, such as ascorbate and N $\alpha$ -acetyl-L-arginine (19). Interestingly, *gnat2*-knockout plants are more susceptible to pathogen infection, which was suggested to depend on its SNAT activity (17). Still, many open questions remain regarding this new GNAT family, such as why there are so many plastid GNATs, why they display dual KA and NTA activities, and what the physiological role of each of them is?

Plants regularly face adverse conditions, such as rapid changes in light intensity, temperature, and water or nutrient availability. Upon perturbation of cellular homeostasis, protein-based signaling networks are activated to orchestrate physiological responses, by regulating gene expression, translation, protein function and turnover. Signal-responsive protein modifications are emerging as a key mechanism for controlling plant stress responses (20). Protein acetylation is one such modification. Recent studies have shown that protein acetylation in plants is highly dynamic in response to many different stresses, particularly when dealing with cytosolic NATs (21, 22). Loss of AtGNAT2 causes a defect in the dynamics of photosynthetic light harvesting, and *gnat2* mutants are unable to balance the distribution of excitation energy between the two photosystems (PS) in a process called state transitions (13, 23, 24). While this causes relatively subtle growth defects under standard laboratory growth conditions, the *gnat2* plants show severely retarded growth under fluctuating light (24). Moreover, although both acK and NTA were affected in the *gnat2* plants, the most sensitive acK and NTA targets in these mutant lines were different (4, 13). Several proteins involved in photosynthesis were only affected in their acK status but not in overall abundance, and therefore it is likely that GNAT2-catalysed acetylation plays a role in the light response. Decreased acetylation level of specific photosynthesis-related proteins suggested that there may be defects in the interaction of PSI and Light Harvesting Complex II (LHCII), strengthened binding of LHCII to PSII, or more indirect effects on thylakoid macrostructure (13, 23, 24). It

has also been suggested that the identified extensive NTA of several LHC proteins might be involved in membrane folding of grana stacks (25).

Light is one of the major regulatory factors controlling germination, growth and general fitness of plants, and therefore plants must be able to acclimate to short- and long-term changes in light quality and quantity (26). Here, we performed a multi-omics analysis including deep qualitative and quantitative characterization of N-terminal and lysine acetylation of proteins in the context of WT versus *gnat2* plants to understand the function of GNAT2 in light acclimation. To this end, we treated mature Arabidopsis plants, both WT and *gnat2* mutants, in high light, under standard growth conditions or in darkness for two hours. The extensive data set revealed that both WT and *gnat2* mutants responded similarly to changes in light conditions, since no major differences in transcriptome, proteome or energy metabolite composition were revealed between the two genotypes. Further characterization of the impact of these different light conditions revealed that the N-terminal acetylome remained widely unchanged. A strong light-independent reduction on specific plastid proteins was observed in *gnat2* compared to WT. The lysine acetylome proved to be very sensitive to the changes in light conditions especially in the *gnat2* background, suggesting that acK sites change within the investigated timeframe during acclimation, while N-terminal acetylation changes might be associated with long-term responses. Taken together, our data reveals unique strategies of plant acclimation to the different light treatments involving specific PTMs and suggests that plastid lysine and N-terminal acetylations may respond differently to environmental or developmental stimuli.

## Experimental procedures

### Experimental Design and Statistical Rationale

Experiments were carried out in four replicates. Wild type plants were used to compare *gnat2* knock-out plants with. They were treated for two hours in the dark under standard growth conditions (as control) or under high light illumination in parallel. For the respective multi-omics experiments, different, well established analysis pipelines were chosen. As a result, cutoffs for significance were chosen to best fit the respective nature of the data. The analytical and statistical approaches are outlined in detail in the respective method sections.

## Plant growth and light treatments

*Arabidopsis thaliana* ecotype Col-0 and *gnat2* T-DNA knock-out (SALK\_033944) plants were grown for 4.5 weeks in short day light regime (8 h light/16 h darkness) at PPFD of  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$  (light source: Osram Powerstar HQI-BT 400W/D daylight), 50 % humidity, and 23°C. Two hours after the onset of light, plants were treated for two hours in the dark (D), under standard growth conditions (GL) or under high light illumination (HL;  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ , 23°C). Immediately after the treatments, the rosettes were harvested and frozen in liquid nitrogen (**Figure 1**). Four distinct biological replicates were assessed. The same material from each treatment was subjected to transcriptome, metabolome, acetylome, and proteomic analyses.

## Transcriptomics

The peqGOLD Total RNA kit (PepLab) was used to extract RNA from 100 mg plant powder. A global transcriptome analysis was performed using the Affymetrix AraGene-1\_0-st arrays (Thermo Fisher Scientific, Waltham, MA, USA) as described in detail by (21). Biotinylated antisense cDNA was then prepared according to the standard labelling protocol with the GeneChip® WT Plus Reagent Kit and the GeneChip® Hybridization, Wash and Stain Kit (both from Thermo Fisher Scientific, Waltham, MA, USA). Afterwards, 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 3000. All equipment used was from the Affymetrix-Company (Affymetrix, High Wycombe, UK).

Arrays were annotated with a Custom CDF Version 22 with TAIR based gene definitions (27). The raw fluorescence intensity values were normalized applying quantile normalization and RMA background correction. OneWay-ANOVA was performed to identify differential expressed genes using a commercial software package SAS JMP15 Genomics, version 10, from SAS (SAS Institute, Cary, NC, USA). A false positive rate of  $\alpha=0.05$  with FDR correction was taken as the level of significance.

An enrichment score of  $\log_{2} \text{FC} * p\text{-values}$  was calculated and used for functional enrichment in the binary comparisons of either *gnat2* vs WT under different light

conditions or growth light vs light alteration in the different genotypes *via* STRINGdb's "Proteins with Values/Ranks - Functional Enrichment Analysis" (28).

### **Analysis of metabolites via UPLC-FLD**

The adenylate status (ATP and ADP) and the NADH/NAD<sup>+</sup> redox status were analyzed by metabolite fingerprinting using an ultra-performance liquid chromatography (UPLC) system. Approximately 25 mg ground shoot material was extracted with 1 mL 0.1 M HCl by vortexing at 4°C for 15 min. The resulting homogenates were centrifuged for 10 min at 4°C and 16.400 g to remove cell debris. Adenosines were derivatized with chloroacetaldehyde as described in (29) and separated by reversed-phase chromatography on an Acquity HSS T3 column (100 mm x 2.1 mm, 1.7 µm, Waters) connected to an Acquity H-class UPLC system. Prior to separation, the column was heated to 43°C and equilibrated with 5 column volumes of buffer A (5.7 mM TBAS, 30.5 mM KH<sub>2</sub>PO<sub>4</sub> pH 5.8) at a flow rate of 0.6 mL min<sup>-1</sup>. Separation of adenosine derivatives was achieved by increasing the concentration of buffer B (2/3 acetonitrile in 1/3 buffer A) in buffer A as follows: 1 min 1 % B, 2 min 8 % B, 3.2 min 14 % B, 9.5 min 50 % B, and return to 1 % B in 1.5 min. The separated derivatives were detected by fluorescence (Acquity FLR detector, Waters, excitation: 280 nm, emission: 410 nm) and quantified using ultrapure standards (Sigma). Data acquisition and processing was performed with the Empower3 software suite (Waters).

### **Quantitative proteomics and lysine acetylation**

Frozen leaf tissue was ground in liquid nitrogen to a fine powder and subsequently processed with a modified filter-aided sample preparation protocol using 30k MWCO Amicon filters (Merck Millipore, [www.merckmillipore.com](http://www.merckmillipore.com)) as described in (30). After the tryptic digest, peptides were labeled with TMT according to (31). One channel of a 6plex reagent kit was assigned to a condition (three light conditions (control, high light, darkness) for two genotypes (WT, *gnat2*)). The assignment was randomized within each replicate / kit (**Supplemental Data Set 6**). The samples were resuspended in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) and 10 µg peptide of each sample stored for whole proteome analysis. Enrichment for lysine-acetylated peptides was performed as described in (30) using anti-acetyl lysine antibody bound to agarose beads first from CellSignaling (CS) and subsequently from ImmuneChem (IC,



Burnaby, Canada). All samples were divided into two equal portions. They were desalted on SDB-RPS stagetips as described in detail in (30). One-half was eluted directly and the other half eluted from the tips as three fractions with 20  $\mu$ L elution buffer each. Eluted samples were dried in a vacuum concentrator.

### **LC-MS/MS measurement of proteome and lysine acetylome peptide samples**

Dried peptide samples were resuspended in 2 % acetonitrile, 0.1 % trifluoroacetic acid and subsequently measured on LC-MS/MS using an EASY-nLC 1200 system coupled to an Exploris 480 mass spectrometer with a FAIMS interface (Thermo Scientific, [www.thermofisher.com](http://www.thermofisher.com)). Separation of peptides took place on 20 cm fritless silica emitters with 0.75  $\mu$ m inner diameter (New Objective, [www.newobjective.com](http://www.newobjective.com)) in-house packed with reversed-phase 1.9  $\mu$ m ReproSil-Pur C<sub>18</sub>-AQ (Dr. Maisch GmbH, [www.dr-maisch.com](http://www.dr-maisch.com)). Peptides were separated on 115-minute gradients, using 0.1 % formic acid as buffer A and 80 % acetonitrile, 0.1 % formic acid as buffer B. MS<sup>1</sup> scans were acquired at an Orbitrap Resolution of 120 000 with a Scan Range (m/z) of 350-1200, a maximum injection time of 100 ms and a normalized AGC Target of 300 %. For fragmentation only precursors with charge states 2-5 were considered. Up to 20 dependent scans were taken. For dynamic exclusion the exclusion duration was set to 40 sec and a mass tolerance of +/- 10 ppm. The isolation window was set to 0.7 m/z with no offset. A normalized collision energy of 36 was used. MS<sup>2</sup> scans were taken at an Orbitrap resolution of 30 000, with a fixed first mass (m/z) = 100. Maximum injection time was 86 ms and a normalized AGC Target of 50 %. Turbo TMT was enabled. For full proteome analysis, CVs of -45, -60 and -80 were used, while peptides enriched for acK were measured at CVs -40, -55 and -75 in one run. The FAIMS MzXML Generator was used to pre-process raw files with data recorded at several CVs and convert the raw files to mxml files (32).

### **MS data analysis of the proteome and lysine acetylome**

Interpretation of MS raw data was done using the MaxQuant (MQ) software with the embedded Andromeda search engine (v 2.0.2.0, [www.maxquant.org](http://www.maxquant.org)) (33), searching against the latest version of the Araport11 database containing 48,266 entries (v2016/06, [www.bar.utoronto.ca/thalemine](http://www.bar.utoronto.ca/thalemine)). Samples (proteome and acetylome) were

searched at once, however with dedicated parameter groups with the following settings: A list of common contaminants and a reverse decoy database was enabled for the search. Carbamidomethylation was used as fixed modification. Oxidation of methionine and tryptophan, deamidation of asparagine and glutamine and N-terminal acetylation were set as variable modification. In addition, lysine acetylation was enabled as variable modification for enriched samples in the respective parameter group. MS<sup>2</sup> based quantification for TMT 6plex was enabled for all parameter groups. Trypsin was set as protease and maximal 2 missed cleavages (proteome) or 4 missed cleavages (acetylome) were accepted. Minimum length of valid peptides was set to 7 amino acids. The re-quantify feature was enabled. PSM and protein FDR was 0.01. Minimum scores were set to 0 and 30 for unmodified and modified peptides, and delta score to 0 and 6, respectively. A tolerance of peptide of 20 / 4.5 ppm were allowed for first / main search, while the MS/MS peak tolerance was 20 ppm.

MaxQuant output tables (protein groups and acetyl K sites) were imported to R. The six TMT reporter intensities from all 4 replicates were log<sub>2</sub> transformed and each channel was median normalized prior to differential expression analysis via Limma. The differences of the log<sub>2</sub> transformed TMT reporter intensities were tested for 4 vs. 4 replicates.

The full MaxQuant search results, including all files and folders produced by the software as well as the respective raw data are available via ProteomeXchange repositories, providing all necessary information for in depth assessment of the results. Result tables and assigned spectra can be viewed either with MaxQuant's viewer or PDV (34).

### **N-terminal acetylome**

For the N-terminal acetylome analysis, samples were prepared and processed as previously described (35) with few exceptions. Briefly, the proteins extracted from the biological material were labelled on their N-termini and lysine ε-amino groups using N-acetoxy-[2H3] succinimide, before being subjected to trypsin digestion. The peptides obtained were then fractionated on a strong cation exchange chromatography, and the fractions were analyzed, without pooling, on an LTQ-Orbitrap mass spectrometer coupled to a liquid chromatography. The data obtained were processed using Mascot

Distiller version 2.6.2 with “Acetyl:2H(3) (K)”, “Carbamidomethyl (C)” as fixed modifications and “Oxidation (M)” as variable modification, “Acetyl (N-term)” and “Acetyl:2H(3) (N-term)” being included in the “Acetylation” quantitation method of Mascot Distiller. Along with these parameters, searching against the Araport-11 protein database, with the parent and fragment mass tolerance respectively defined to 10 ppm and 0.5 Da. The quantitation results, along with each Mascot search output, were then parsed using the EnCOUNTER script to obtain the final N-ter acetylome datasets (36). The complex datasets were manually consolidated to allow for individual result comparison between all the different experimental conditions. Statistical analysis was then performed in the Excel spreadsheets using available built-in formulas and tools. Briefly, all biological replicates of each specific condition were combined, averaging the N-terminal acetylation levels and providing standard deviation across those replicates. After that, NTA levels ratios were calculated and, when possible, p-values were determined using a two-tailed t-test applied to the comparison of two sample groups of similar variances. This allowed to account for both decreases and increases in acetylation levels. Because of the complex experimental design, two distinct types of comparisons were made, either looking for differences related to light treatments in one of the genotypes, or for the effect of the *gnat2* knock-out compared to the WT in one of the three light conditions groups.

## Results

In order to study the plant responses to fluctuations in light intensity with multi-omics approaches (e.g. transcriptome, proteome and acetylome), the *gnat2* and WT plants were grown under the same experimental conditions. Two hours after the onset of control light, the one-month-old plants were subjected to three light regimes for two hours: control light (C), high light (HL) or darkness (D). Immediately after the treatment, rosettes of individual plants were harvested and split for the multi-omics analysis (**Figure 1**).

### Energy status of *gnat2* and WT plants upon short-light stress conditions

First, we focused on the plant energy status by determining ATP, ADP, and AMP levels in WT and *gnat2*, and calculating the adenylate energy charge under C as well as under short-term HL and D stress (**Supplemental Data Set 1**). We found that ATP, ADP, and AMP abundances increased in both WT and *gnat2* after two hours of

treatment with HL and decreased under D independently of the genotype (**Supplemental Figure 1**). Nonetheless, the energy charge was not affected by HL and increased slightly upon short-term D in both genotypes (**Supplemental Figure 1**). From these findings, we conclude that both genotypes were able to acclimate their metabolism to HL or D comparably.

### **Comparison of WT and *gnat2* transcriptomes**

Under control conditions, only 39 transcripts (including *GNAT2*) were differentially regulated between WT and *gnat2* (**Supplemental Data Set 2**). Of those 39 gene products, eight were down- and 31 were upregulated in *gnat2*. Whereas the downregulated transcripts were not enriched in any specific pathways, the upregulated transcripts were mainly involved in translation-related pathways (RNA transport, ribosome biogenesis, aminoacyl-tRNA biosynthesis), suggesting that *GNAT2* might be involved in the control of translation.

While the global transcriptomes of the WT and *gnat2* plants differed only weakly under control conditions, pronounced changes in transcript abundances in both genotypes in response to the altered light conditions were detected (**Supplemental Data Set 2A** and **Supplemental Figure 2**). However, both genotypes show a similar response to the change in light conditions. This is indicated by high Pearson correlation coefficients, which are 0.90 and 0.89 when comparing the WT vs. *gnat2* response to darkness or high light on transcriptome level, respectively (**Supplemental Figure 3, A and B**). Of 27,826 quantified transcripts, only 70 and 71 show a  $\log_2$  FC > 1 or < -1 in response to the respective light change. No functional pathway enrichment could be detected for either altered gene sets using STRINGdb.

### **High light-dependent transcriptome changes in WT and *gnat2***

In WT, the expression of 1,808 of 27,826 analyzed transcripts was significantly (filter: >2-fold,  $p < 0.05$ ) altered after HL treatment. Of those 1,808 transcripts, 943 were up- and 865 were downregulated. A similar response to HL was observed in *gnat2*, where 2,083 transcripts (1,126 up and 957 down) were significantly regulated upon HL stress (**Figure 2A and B**). When comparing the transcripts regulated in WT and *gnat2* in response to HL, a substantial overlap of 1,489 commonly regulated transcripts was identified (82 % of regulated transcripts in WT, 71 % of regulated transcripts in *gnat2*). The remaining significantly altered transcripts were exclusively regulated in one of the

two genotypes (319 in WT and 594 in *gnat2*). Interestingly, there were no antagonistically regulated transcripts between the two genotypes.

The unfiltered data matrix was subjected to an unbiased functional enrichment analysis to identify biologically relevant processes affected by the HL treatment. For this purpose, an enrichment score ( $ENS = -\log_{10}(p\text{-value}) \cdot \log_2(FC)$ ) was calculated for each transcript and the full list of transcripts was analyzed with the functional enrichment tool STRING (27). The analysis revealed that upon HL treatment, 14 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were upregulated (**Figure 2C** right and **Supplemental Data Set 2B**) and 18 KEGG pathways were downregulated (**Figure 2C** left and **Supplemental Data Set 2B**) in the WT. Out of these 32 pathways, 24 were also regulated in *gnat2* (**Figure 2C** and **Supplemental Data Set 2B**), demonstrating that a substantial proportion of the HL response is independent of GNAT2. In both genotypes, the HL treatment decreased transcripts related to photosynthesis, antenna protein biosynthesis, porphyrin/chlorophyll turnover and lipid metabolism. Processes upregulated in both genotypes were associated with translation, RNA processing and flavonoid biosynthesis. The specific response of *gnat2* to HL included downregulation of diverse anabolic reactions like carbon fixation, biosynthesis of unsaturated fatty acids and amino acids as well as sulfur incorporation (**Figure 2C** and **Supplemental Data Set 2B**), whereas the base excision repair pathway was the only one specifically upregulated in *gnat2* (**Figure 2C**, and **Supplemental Data Set 2B**). Our findings indicate that apart from minor changes that specifically occur in *gnat2* the overall transcriptional response to short-term high-light treatment is very similar between WT and *gnat2*.

### **Transcriptome response to darkness in WT and *gnat2***

Next, we analyzed the response from control light to darkness. This treatment altered the expression of 2,042 transcripts in the WT, of which 979 were up- and 1,063 were downregulated, respectively (**Figure 2A and B**, and **Supplemental Data Set 2A**). The *gnat2* mutant displayed a transcriptional response of similar severity (1,195 up (**Figure 2A**) and 1,166 down (**Figure 2B**)). 1,701 of these differentially expressed transcripts were commonly regulated in both genotypes (83 % of regulated transcripts in WT, 72 % of regulated transcripts in *gnat2*). Like in the high-light response, no transcript was antagonistically regulated between both genotypes upon darkness.

Again, a minor set of transcripts (341 in the WT and 660 in *gnat2*) was regulated in a genotype-specific manner.

The functional enrichment analysis showed that upon darkness, only transcripts associated with the spliceosome, endocytosis, and autophagy were upregulated in the WT (**Figure 2 D**, and **Supplemental Data Set 2C**). Upregulation of the latter suggests significant nutrient recycling in response to darkness. Loss of GNAT2 did not alter these transcriptional responses and caused an additional upregulation of SNARE-mediated vesicle transport (**Figure 2D** pink bar, and **Supplemental Data Set 2C**). In both genotypes, most metabolic processes were downregulated upon darkness, including DNA replication, carbon fixation, sulfur incorporation, and porphyrin/chlorophyll biosynthesis (**Figure 2D** left, and **Supplemental Data Set 2C**). The downregulation of sulfur assimilation can be explained by its dependency on photosynthetic reduction equivalents and its importance for light acclimation (37). The downregulation of DNA replication can be interpreted as a hallmark of decelerated cell division. The dampening of metabolic core pathways is a canonical response of plants to unexpected darkness and is required to maintain the energy charge of leaf cells. Besides these general responses, the *gnat2* mutant also displayed downregulation of transcripts involved in translation-related pathways (RNA transport, ribosome biogenesis, aminoacyl-tRNA biosynthesis) (**Figure 2D** and **Supplemental Data Set 2C**). Interestingly, these pathways were upregulated in the WT upon illumination with high light; hence our results suggest that GNAT2 might be involved in the light-dependent control of translation.

### High light-dependent proteome regulations in WT and *gnat2*

The transcriptome results led us to elucidate the effect of light on the proteomes of WT and *gnat2* plants. For this a TMT-6plex labeling approach followed by quantitative mass spectrometry analyses and MS<sup>2</sup>-based quantification was used and allowed to quantify 5,620 protein groups in total (**Supplemental Data Set 3**).

Upon high-light treatment, both WT (**Figure 3A**) and *gnat2* mutants (**Figure 3C**) showed a strong accumulation of heat shock response proteins, such as the HSP20 family proteins HSP17.4 (#8 in **Figure 3**) and HSP17.6II (#13), HSP70 (#7), HSP81.4

(#32), HSP101 (#24), and the co-chaperone ROF2 (#16), which is a carboxylate clamp-tetratricopeptide protein, predicted to interact with HSP70 and HSP90 proteins (38). Several of these proteins are involved in the cytosolic response to heat as well as high light stress to protect translation factors, prevent protein aggregation, and enable proteasomal degradation of damaged proteins (39-41). Both genotypes also showed a twofold up-regulation in HY5 (#12 in **Figure 3**) protein level. HY5 is a basic leucine zipper transcription factor, which is critical for protection from high light-associated DNA damage by anthocyanin accumulation (42). Among the down-regulated proteins upon high-light treatment, the ribosomal protein S6 A (RPS6A, #11) was identified in both genotypes. RPS6A is important for light- and photosynthesis-enhanced translation by its association with polysomes in its phosphorylated state and connected to the TOR pathway (43). Interestingly, two proteins involved in oxidative stress tolerance, the chromatin-associated factor OXS3 (#17) and a 2-alkenal reductase (#10, AT4G17085.1) involved in NADPH-dependent detoxification of reactive carbonyls (44, 45), were found more than two-fold up-regulated in the WT upon high-light stress (**Fig. 3A**), but not in the *gnat2* mutant (**Fig. 3C**). In contrast, a hydroxyproline-rich glycoprotein family protein of unknown function (#22, AT1G14710.2) was systematically down-regulated by more than 2-fold in *gnat2* but not in WT upon high-light treatment.

### Darkness-dependent proteome regulations

Upon 2h of darkness, six proteins were significantly and exclusively up-regulated in their abundance in WT (**Figure 3B** and **3C**). Among them, we identified TOUCH 3 (#3 in **Figure 3**), a calmodulin-like 4 protein already known to be transcriptionally induced by darkness (46), and the two oxidative stress-related proteins OXS3 (#17) and 2-alkenal reductase (#10). The nucleosome assembly protein 1;3 (NFA3, #18), which is vital for the protection of DNA damage, was only downregulated in WT plants. The plastid methionine synthase 3 protein (#31), the ribosomal protein S6 A (RPS6A, #11) and the granule bound starch synthase 1 (GBSS1, #23) were exclusively up-regulated in the *gnat2* mutant upon darkness, while the S phase kinase-associated protein 1 (UIP1, #25) was significantly down-regulated in its abundance. Only one protein, the chlorophyll binding protein LHCA3 (#1) of photosystem I, already known to be involved in the response to light stimuli (47), was decreased in abundance in both genotypes.

### Differences in protein abundances between WT and *gnat2*

Changes in protein abundances between WT and *gnat2* were mostly independent of the stress treatment (**Figure 3E, 3F and 3G**). The most striking differences were detected in the more than 16-fold up-regulation of the nuclear-encoded photosystem II 5kDa protein (PsbTn, #48 in **Figure 3**) in *gnat2*, which was found to be constitutively up-regulated in its abundance. PsbTn was recently shown to be critical for light acclimation (48). In addition, the protein TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 62 (TIC62, #47), which is fundamental for the anchoring of the chloroplast ferredoxin-NADPH reductase to the thylakoid membrane (49), was more than 2-fold up-regulated in *gnat2* compared to WT under all growth conditions. Generally, the transcriptome changes were more pronounced than those related to the proteome. Due to the short 2h light treatments, the transcriptome changes were not yet propagated to the proteome except for a few gene products (**Supplemental Figure 4 and Supplemental Data Set 3**).

### The N-terminal acetylome under short-term changes in light conditions

Recently, it has been suggested that plastid NTA might be related to oxidative damage because of the high levels of NTAed subunits of many plastid complexes correlated with oxygenic photosynthesis (25). Moreover, although NTA is a widespread modification in plastids, it was proposed that plastid NTA yield might depend on environmental stimuli, e.g. light (1). Nonetheless, no information was available on the processing of the N-termini of plastid proteins under different light conditions so far. Therefore, to get an insight on the possible dynamics of the N-terminal acetylome in *Arabidopsis*, and to decipher the influence of NTA in response to different light conditions, we performed a global NTA quantitative analysis on the very same sample set as above (**Figure 1**). With the SILProNAQ procedure (35, 50), we identified a total of 1783 non-redundant proteoforms in WT plants (**Supplemental Data Set 4 and Supplemental Table 1**). Amid all identified proteoforms, the acetylation yields were quantified for 761 of them.

Of the 761 N-termini quantified in the leaves of WT under control conditions, only 112 started with the initiator methionine (iMet, 15 %), 280 (37 %) were subject of iMet removal, and 369 were generated by a cleavage of the transit peptide. 62 % of N-termini undergoing removal of iMet were found fully NTAed (NTA yield > 95 %),



whereas 14 % were partially NTAed (NTA yield between 5 and 95 %). Concerning the N-termini retaining the iMet, 79 % were fully NTAed and only 12 % partially NTAed. As previously observed, among the N-termini with processing downstream position 2, only 8 % were fully NTAed and 37 % partially NTAed. When these parameters were analyzed in the samples arising from plants treated with D or HL, no significant difference was observed with the plants from control conditions, and all three conditions of each group followed the same pattern of NTA yield variations, regardless the NTA position (**Figure 4A, 4B**, and **Supplemental Data Set 4**).

Since *gnat2* was recently shown to strongly affect plastid NTA in plants grown in standard conditions (2), we wondered whether this phenotype was intensified or reduced when the plants were treated with D or HL. Hence, we performed a global NTA quantitative analysis of *gnat2* grown in the same light conditions (C, D, and HL) and compared their retrieved N-termini and NTA yield to WT (**Figure 4C-4H** and **Supplemental Data Set 4**). Comparison of *gnat2* with WT plants under C confirmed the decrease of plastid protein NTA when GNAT2 is inactivated (**Figure 4C**, **Supplemental Data Set 4** and **Supplemental Table 2**). More than 20 N-termini with significant decreases of acetylation ( $> 40\%$ ), including about 15 with statistical significance ( $|\log_2(\text{ratio})| > 1$ ,  $p\text{-value} < 0.1$ ) were retrieved in *gnat2* under standard growth light conditions (**Figure 4D** and **Supplemental Table 2**). Identical trends were observed when comparing WT and *gnat2* under D and HL conditions (**Figure 4D-E**, **4G-H** and **Supplemental Table 3** and **4**). Indeed, many entries were quantified in at least two groups with similar NTA variations (**Supplemental Table 2, 3** and **4**) and when comparing each light condition of WT and *gnat2*, no significant NTA variations could be observed. These results indicated that the N-terminal acetylome is not influenced by short-term light changes and that the different light conditions in terms of NTA are as similar as biological replicates. Treating the different light conditions as biological replicates increased the number of identified and quantified N-termini (e.g. yielded 87 % overlap between WT and *gnat2*, **Figure 5A**). This allowed us to get a more profound analysis of the impact of *gnat2* knockout background as we could validate further proteins (**Figure 5B, 5C** and **Supplemental Data Set 4**). Very intriguingly, proteins in which NTA was strongly reduced in the *gnat2* background were involved in photosynthesis, amino acid biosynthesis, plastid translation, and RNA interactions (**Table 1**). The Icelogo representation of the N-terminal sequences with a

40 % decreased NTA yield (with less than a 5 % variation) in *gnat2* compared to WT shows that proteins starting with Thr or Val are the most favored substrates of GNAT2 (**Figure 5D**). NatB-like substrates (MetAsp/Glu) are clearly insensitive to the loss of GNAT2. In addition, residues at positions 1 and 3 seem to play an important role for GNAT2 substrate recognition, with Ala and Ser largely preferred, as well as Val at position 1 (**Figure 5D**). Finally, alterations in NTA level did not lead to significant changes in total protein abundance during the short-term light treatments, as well as in the *gnat2* mutant when compared to the WT (**Supplemental Figure 5**).

### Lysine acetylome analysis under the different light conditions

Previous studies have shown that photosynthetic proteins are overrepresented among the cellular acK proteins next to histones (51). In addition, it was reported that lysine acetylation affects the activities of several Calvin-Benson cycle enzymes, such as RuBisCO and RuBisCO activase (RCA) (6, 7, 51). While a decrease in RuBisCO lysine acetylation on K334 was reported to occur in the darkness within the diurnal cycle of Arabidopsis plants (7), the dynamic response of lysine acetylation to unexpected changes in light conditions has not been investigated so far. Hence, we analyzed the light-dependent response of the lysine acetylome in response to D and HL, as well as the specific role of GNAT2 in this response. For the lysine acetylome analysis, TMT-labelled peptides from each growth condition were pooled and enriched for peptides carrying acetylated lysines (52). The samples were analyzed by mass spectrometry and acK changes were quantified in both genotypes under the different light conditions. In total we detected 2560 acK sites (**Supplemental Data Set 5**). **Figure 6** summarizes the overall response in altered lysine acetylation of the plants. In WT plants, 26 and 17 acK sites showed a significant change in their abundances ( $p$ -value  $< 0.05$ , fold-change  $> 0.75$  or  $< -0.75$ ,  $n > 2 - 4$ ) upon 2 h HL or D treatment, respectively (**Figure 6A** and **6B**). Of those, 8 and 7 acK sites were derived from plastid proteins (indicated in green). In comparison, the *gnat2* plants showed a more pronounced de-regulation in the lysine acetylome after 2 h HL treatment with 50 acK sites up-regulated, while only nine acK sites were also significantly up-regulated in the WT. From the 54 significantly regulated acK sites in total, 16 were derived from plastid proteins (**Figure 6D**, highlighted in green). For example, the large subunit of RuBisCO (#60 in **Figure 6**) and RCA (#41) were increased in lysine acetylation, while their modification status was not significantly altered in WT plants under the different

light conditions. However, proteins from other subcellular localizations, such as phospholipase D Z1 (#14) which resides at the plasma membrane, were also significantly more acetylated in *gnat2* under HL conditions, while it was downregulated in WT. Only 7 acK sites were significantly changed in their abundances after 2h D in *gnat2* plants (**Figure 6C**), among which two were over-lapping with WT plants from the same treatment. In *gnat2*, the light harvesting protein LHCB2.1 (#39) was strongly increased in its acK status under both D and HL compared to C conditions, while in WT much milder (though significant) increases in acK fold-changes were observed. This is interesting in the context that *gnat2* mutants fail to perform state transitions (13). In both genotypes, several proteins involved in transcriptional regulation, such as HY5 (#25 in **Figure 6**), CONSTANS-like 1 (#26), SALT TOLERANCE (STO, #4), histone H2A (#70), and the CLEAVAGE AND POLYADENYLATION SPECIFICITY FACTOR 73-I (#72) were found more acetylated under 2 h HL conditions (**Figure 6B, 6D and 6G**). When transferred to 2 h D, plants of both genotypes respond with a strong acetylation of K55 of acyl carrier protein 2 (ACP2, #28), localized to chloroplasts (**Figure 6A, 6C and 6F**). While the respective site is conserved in ACP4 (#19) no modification is detected on K52. The protein sequences of ACP2 and 4 are 48 % identical however not aligned in the first 48 AA representing the respective transit peptides. The acetylation of the conserved K67 in ACP4 (#19) is more pronounced in WT plants (**Figure 6A**,  $p < 0.05$ ), which might be explained due to the varying sequence context in ACP2 (#28) and 4 for this residue. Both these ACPs are known to be expressed in leaves. Generally, the lysine acetylome was responsive to the light treatment shown in the de-regulation of several acetylation sites on proteins from photosynthesis and other cell functions. While there was little change in protein abundance levels, the lysine acetylation was primarily responsive to the different light treatments (**Supplemental Figure 6A and 6B**).

A direct comparison of the effect of *gnat2* relative to WT shows a more than 30-fold downregulation in acK level of the photosystem II PSBD protein (#117, **Figure 6E, 6F and 6G**), which is dependent on the genotype but independent of the light conditions. This essential photosynthetic protein, forming the core of PSII together with the D1 protein, is not altered in its protein abundance when comparing *gnat2* to WT. This contrasts with PSBTN, which is strongly upregulated in its protein abundance in *gnat2*, while its acK status is slightly decreased in *gnat2* relative to WT (**Supplemental**

**Figure 6**). While the acK PSBD protein has not been identified in our previous study, several other proteins were confirmed as GNAT2 substrate proteins in this lysine acetylome analysis, such as PSBH, KEA1/2 and two small unknown thylakoid membrane proteins (**Supplemental Figure 7**). When correlating changes in acK and NTA status of the different light treatments in WT and *gnat2* mutants also here it was apparent that the lysine acetylome is responsive to light while nearly no variation in NTA levels were observed (**Supplemental Figure 8 A-D**). In response to *gnat2* compared to WT, there is only the PSBD protein which is simultaneously down-regulated in its acK and NTA level (acK down-regulated by 45-fold, NTA down-regulated by 20 %) independent of the light treatment (**Supplemental Figure 8 E-G**).

## Discussion

The rapid development of multi-omics approaches has made it possible to acquire and analyze multidimensional big data (e.g. representing protein and nucleic acid modifications, proteomes, transcriptomes, and metabolomes) of entire organisms. However, several challenges persist before we can fully take advantage of these approaches. When the data are collected from various sources, it can be challenging to connect the data with each other with the aim to provide an integrative overview about the cellular or organism wide responses. Especially co- and post- translational protein modifications provide essential mechanisms for regulating protein activities and functions. However, the systematic evaluation of their relevance is at its infancy, and even less is known about the interplay of different co-occurring protein modifications and how they respond to different stimuli (53). There are only a few studies where the dynamics of different protein modifications are coupled with other omics data in response to specific signals to systemically address cell signaling processes and physiological responses to the environment (41, 54-56).

In this study, we provide the first comprehensive overview of how the transcriptome, proteome, lysine and N-terminal acetylome are simultaneously orchestrated in response to short-term D and HL treatments in *Arabidopsis* WT and a *gnat2* knock-out mutant line. Specifically, we investigate how the two different types of acetylations catalyzed by one catalyst – here GNAT2 - in the chloroplast distinctively respond to short-term changes in light conditions.

Light alterations on the long run, including seasonal variations or night-day duration, are one of the major regulatory factors controlling germination, growth and general fitness of plants. In addition, within a single day, hour after hour, plants need to constantly acclimate to short- and longer-term changes in light intensity. Plants avoid photo-oxidative damage by multiple strategies, including changes in the absorption cross-section of the light-harvesting antenna associated with either PSII or PSI (i.e., state transitions (57)), induction of thermal dissipation of absorbed excess energy (i.e., non-photochemical quenching, NPQ) (58, 59), activation of the malate valve for the export of excess reducing equivalents (60), dissipation of excess energy in mitochondria (61), and induction of the ROS scavenging network (62). Intense illumination also results in alkalization and  $Mg^{2+}$  accumulation in the chloroplast stroma, which activates the Calvin-Benson cycle. All these actions allow transient reconfiguration of primary and secondary metabolism of the plant through retrograde signaling cascades, which coordinate chloroplast function and nuclear gene expression (63). Many studies are available on the acclimation to long-term light changes, but less is known about how the proteome and different PTMs respond in a coordinated manner to short term changes in light availability. A recent study investigated the effect of short-term HL treatment from 2 min to 72h on both the transcriptome and metabolome levels and on the protein redoxome in *Arabidopsis* (41). While strong regulations were observed on the metabolome level, which must be directed by changes in protein activities, no major changes were observed on the dithiol-based regulation of the proteome (41).

In our study, we observed the upregulation of adenosine metabolites after the two hours exposure to HL. The opposite tendency was revealed under the two hours exposure to D. This is in agreement with previous data showing that metabolism of photosynthesizing organisms' changes immediately upon alteration of photosynthetic conditions such as light (41, 54). However, the preservation of the energy status upon HL illumination suggests that our short-term light treatment did not lead to substantial photo-inhibition, which is in line with a recent study showing only a slight decrease in photosynthetic performance of both *gnat2* and WT plants upon 2 h illumination under  $600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (19). The same tendency observed with the *gnat2* mutant line revealed that despite the defects in state transitions (13), the absence of this acetyltransferase does not affect the overall capability of the plants to adjust their

metabolism after their transfer to darkness or high light conditions. This observation agrees with the findings that state transitions are most important for the acclimation of plants to low light rather than high light conditions (64). In contrast to the 4.5-week-old plants analyzed here, eight-day-old *gnat2* seedlings showed a strong decrease in the maximum quantum yield of PSII after 24 hours of constant high light, along with a significant increase in non-regulated photosynthetic quenching (65). Hence, GNAT2 might be important for proper chloroplast development in plants.

High light induced signals lead to early expression responses, which are followed by slower changes in gene expression. It has been shown that the genes encoding AP2/ERF (APETALA2/ETHYLENE RESPONSE FACTOR) transcription factors are upregulated extremely fast in response to high light (66-68). Thereafter, genes responding to abiotic stress, especially the family of heat shock proteins (HSPs), as well as ALTERNATIVE OXIDASE 1a and ascorbate peroxidase APX1 are strongly upregulated (69-71). The genes regulated by hormones are also enriched among the differentially expressed genes (71).

Under our experimental conditions, the transcriptome analysis after 2h HL revealed differential transcript expression of approx. 6.5 % in WT and 7.5 % in *gnat2* plants, respectively. This indicates a profound reprogramming of both genotypes after the light treatment. There was a substantial overlap of commonly regulated mRNAs, with no antagonistically regulated genes between the two genotypes. This data suggests that the global transcriptome adjustment of *gnat2* upon HL treatment occurred early and followed the same pattern as in the WT. This is different to the *stn7* mutant, which has a defect in state transitions and a similar phenotype as *gnat2*, but is incapable to induce genes related to jasmonate signaling, pathogen defense, and anthocyanin accumulation after 1h of HL treatment (72). While jasmonate signaling was not induced in our conditions, the transcription factors MYB75, which is important for anthocyanin accumulation upon HL treatment (73), was significantly induced in both WT and *gnat2*. This might indicate that *stn7* has an additional defect in controlling plastid ROS homeostasis (74), which is not the case in *gnat2*. Like in the HL response, similar responses to D were observed with both WT and *gnat2*. It should be noted, however, that transcriptional changes do not necessarily reflect protein abundance, and the photosynthetic capacity of the plant cannot be predicted based on the transcriptome (71, 75-78). However, our quantitative proteomics analysis revealed

specific protein accumulation regulation, in dependence of the light treatment or genotype. An accumulation of the HSPs was detected both in WT and *gnat2* in response to the intense illumination (**Figure 3, Supplemental Data Set 3**). While we cannot exclude that a raised leaf temperature from the HL treatment was responsible for the induction of the HSPs, recent studies showed that HL itself is probably responsible for the induction of the HSPs (40, 41). Interestingly, a specific accumulation of PsbTn and TIC62 was observed in *gnat2* compared to WT independent of the light condition. PsbTn is an interesting candidate protein since it was recently found to play an important role in the acclimation of PSII to light shifts or intense illumination (48). While TIC62 was not detected in our previous study, PsbTn was previously not found to be significantly de-regulated in its abundance between WT and *gnat2* (13). Intriguingly, it was recently shown that subtle changes in the growth light conditions affect the growth phenotype of *gnat2* (19), pointing to a possibility that the observed abundance alterations may be dependent on harvest time or microclimate since light intensity, photoperiod, and temperature were similar in both studies.

Successful acclimation of plants to light-induced changes requires not only reprogramming of RNA expression and regulation of protein accumulation but also PTMs of proteins (79). For instance, PSII repair cycle and photosynthetic state transitions are dependent on protein phosphorylation modifications (23, 25, 80, 81). During transition from state 1 to 2, LHCII associates with PSI to balance the excitation energy between the two photosystems. This association is regulated through the light-dependent reversible phosphorylation of the LHCII subunits LHCB1 and LHCB2 (49, 82-86). We have recently reported that the loss of GNAT2 results in decreased K- and N-terminal acetylation of chloroplast proteins, as well as in defective state transitions under standard light conditions (2, 13). Upon HL treatment, we observed a stronger de-regulation of K-acetylation on proteins localized in different subcellular compartments in *gnat2* compared to WT (**Figure 6B and 6D**). This might be explained by the action of other acetyltransferases or deacetylases, which compensate for the loss of GNAT2.

Although the high frequency of plastid NTA on subunits of many plastid complexes involved in oxygenic photosynthesis suggested that plastid NTA yield might be dependent on different conditions including light (1), we show here that this does not

occur under short-term light treatments. Interestingly, unlike the N-terminal acetylome, the K-acetylome showed to be very sensitive to the early changes in light conditions especially in the *gnat2* background. This can be explained by the fact that K-acetylation is a reversible PTM that can happen on a faster time scale, while NTA is non-reversible and usually occurs co-translationally or in chloroplasts shortly after protein import and is therefore dependent on protein biosynthesis. In the 2 h time course hardly any changes in protein abundances were observed. This suggests that K-acetylome marks the acclimation in the time regime tested here, while N-terminal acetylation changes might be associated with longer-term responses and/or other signaling responses.

Taken together, our data revealed unique strategies of fast plant acclimation to the different light treatments by lysine acetylation, which suggests that plastid K- and N-terminal acetylations have distinct effects on protein function and therefore mediate different environmental acclimation responses.

#### **Data availability**

Mass spectrometry proteomics data associated to the N-terminal and lysine acetylomes have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomeexchange.org>).

Lysine acetylome and full proteome data with the identifier JPST001810 can be found at <https://repository.jpostdb.org/entry/JPST001810>

In addition to that, spectra are also accessible via MSviewer. The search key for the saved data set is sitefyfimk. The data set can be accessed using the following URL:

[https://msviewer.ucsf.edu/prospector/cgi-bin/mssearch.cgi?report\\_title=MS-Viewer&search\\_key=sitefyfimk&search\\_name=msviewer](https://msviewer.ucsf.edu/prospector/cgi-bin/mssearch.cgi?report_title=MS-Viewer&search_key=sitefyfimk&search_name=msviewer)

N-terminomics data are available with the identifier PXD040235, the Username: [reviewer\\_pxd040235@ebi.ac.uk](mailto:reviewer_pxd040235@ebi.ac.uk) and Password: CqU1BHiZ.

Transcriptome accession number at Gene Expression Omnibus (GEO ; <https://www.ncbi.nlm.nih.gov/geo/>) is GSE178938; the token for reviewers is mjotesucvknjhmp.

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### **Author contributions**

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### **Disclosure and competing interests**

The authors declare no competing interests.

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### Figure 1 - Scheme of the experimental workflow.

4.5-week-old *gnat2* and WT plants were grown on soil under short-day conditions (8 h light/16 h dark) at  $120 \mu\text{mol m}^{-2}\text{s}^{-1}$ . After 4.5 weeks, the plants were subjected to different light regimes interrupting the short-day light cycle. Two hours after the regular onset of light, the plants were treated with high light illumination (HL,  $1000 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) or darkness (D) for two hours. The control group (C) continued growth at  $120 \mu\text{mol m}^{-2}\text{s}^{-1}$ . Treatment and sampling time is indicated in the chart with dashed lines and a black arrow, respectively. Samples were harvested and flash frozen. RNA, metabolites and proteins were extracted from leaf tissue for a multi-omics profiling. Modified peptides were enriched with acK specific antibodies or strong cation exchange chromatography in case of N-terminal acetylation. All experiments were performed on four biological replicates. Created with BioRender.com.

### Figure 2 – Transcriptional regulation in WT and *gnat2* upon high light and darkness.

The leaf material from all genotypes (WT and *gnat2*) and treatments (control, high light and darkness) was subjected to a global transcriptome analysis followed by a functional enrichment analysis with string-db.org. All experiments were performed on four biological replicates. (A + B) UpSet plots, showing number of genes up- (B) or down- (A) regulated in WT (grey bars) and *gnat2* (pink bars) and the respective overlaps upon high light (HL) and darkness (D). Pathways which are differentially regulated between the two genotypes are shown in gray. (C + D) Bar charts showing KEGG pathways regulated in WT (green) and *gnat2* (pink) upon high light (C) and darkness (D). The size of each bar corresponds to the number of genes annotated to this pathway.

### Figure 3 - Quantitative proteome profiling of WT versus *gnat2* under different light conditions.

Protein abundance changes were quantified via TMT labeling and compared for different light conditions (panels A - D) or genotypes (panel E - G). Volcano plots showing expression changes and  $-\log_{10}(p \text{ values})$  from LIMMA analysis for protein groups quantified in at least two replicates.  $\log_2$  fold-changes  $\pm 0.75$  and  $p \text{ value} = 0.05$  are indicated as solid lines. For significantly altered proteins either gene names are given (A - D) or indicated as numbers (E - G) and solid symbols are used. Circles and triangles are scaled in size according to the number of replicates. Plastid localized proteins are indicated in green. Proteins with identical response to the light treatment in either genotype are indicated as circles in A – D, while proteins with potential genotype dependent regulation are indicated as triangles. Protein accessions in this figure from spot #1-68: 1: AT1G61520.3, 2: AT1G64890.1, 3: AT2G41100.7, 4: AT3G07810.1, 5: AT3G10020.1, 6: AT3G10050.1, 7: AT3G12580.1, 8: AT3G46230.1, 9: AT4G15210.2, 10: AT4G17085.1, 11: AT4G31700.1, 12: AT5G11260.1, 13: AT5G12020.1, 14: AT5G16990.1, 15: AT5G43780.1, 16: AT5G48570.1, 17: AT5G56550.1, 18: AT5G56950.1, 19: AT5G61020.2, 20: AT1G01510.1, 21: AT1G13930.3, 22: AT1G14710.2, 23: AT1G32900.1, 24: AT1G74310.1, 25:

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 AT5G28500.1, 66: AT5G66090.1, 67: ATCG00630.1, 68: ATCG01100.1.

**Figure 4: Growth condition dependent NTA analysis of *gnat2* plantlets compared to WT.**

Correlation graphs representing the N-terminal acetylation yields of proteins that have been quantified in both the WT control and WT high light conditions (A) or WT control and WT dark (B), which amount to 487 and 456 N-termini, respectively. These plots show that the NTA levels are similar in WT samples, regardless of the light conditions used during the growth, which means the samples could be combined for further analysis. Panel C-E: Correlation graphs representing the N-terminal acetylation yields of proteins that have been quantified in both the WT and *gnat2* samples in either control (c), dark (d) or high light (e) growth condition, amounting to 450, 435, and 501 N-termini, respectively. All the data points on the lower-right part of the graph represent the proteins with decreased NTA yields in the *gnat2* samples compared to the corresponding WT. Proteins located in the plastid, regardless of NTA levels, are colored in light green. Panel F-H: Volcano plots representing the NTA variations between *gnat2* samples and WT, for each of the three growth conditions. The p-value threshold was set at 0.1, and the log<sub>2</sub> fold-change to +/- 0.75. The N-termini that satisfy both of these conditions and have a decreased NTA level of at least 40 % in *gnat2* are numbered (see Supp. Tables 2-4, respectively) Proteins located in the plastid and displaying a significant decrease of NTA level are colored in light green. Protein accessions (and start positions) for panels D-F of figure 4 from spot #1-17: 1: AT1G64770.1 (19), 2: AT4G27440.1 (68), 3: AT4G24830.1 (75), 4: AT3G57560.1 (51), 5: AT2G24820.1 (51), 6: AT1G03630.1 (69), 7: AT1G16080.1 (45), 8: AT3G54050.1 (60), 9: AT3G03630.1 (57), 10: AT3G54050.1 (61), 11: AT4G17300.1 (60), 12: AT4G35250.1 (65), 13: AT3G63410.1 (51), 14: AT1G45474.1 (33), 15: AT2G29630.1 (55), 16: AT5G45390.1 (66), 17: AT4G30950.1 (70).

**Figure 5: Impact of *gnat2* on *in vivo* NTA substrates.** (A) Venn diagrams representing the N-termini either identified (upper) or quantified (lower) in WT and *gnat2* samples. In each case, more than 80 % of N-termini were shared between the two conditions, allowing for a good comparison. B: Correlation graphs representing the N-terminal acetylation yields of proteins that have been quantified in both WT and *gnat2* samples independently of the growth condition, amounting to 635 N-termini. All



the data points on the lower-right part of the graph represent the proteins with decreased NTA yields in the *gnat2* samples. Proteins located in the plastid, regardless of NTA levels, are colored in light green. (C) Volcano plot representing the NTA variations between *gnat2* and WT grouped samples. The p-value threshold was set at 0.1, and the log<sub>2</sub> fold-change to +/- 0.75. The N-termini that satisfy both of these conditions and have a decreased NTA level of at least 40 % in *gnat2* are numbered (see below). Proteins located in the plastid and displaying a significant decrease of NTA level are colored in light green. (D) IceLogo representation comparing the N-ter sequences with a 40 % decrease minimum of NTA yield to those with less than a 5 % variation, when comparing the *gnat2* mutants to the WT samples, after combining all the entry values from the corresponding light condition groups (see below and Table 1). Protein accessions (and start positions) for panel C of this figure from spot #1-24: 1: AT1G64770.1 (19), 2: AT4G27440.1 (68), 3: AT4G24830.1 (75), 4: AT3G57560.1 (51), 5: AT2G24820.1 (51), 6: AT1G03630.1 (69), 7: AT1G16080.1 (45), 8: AT3G54050.1 (60), 9: AT3G03630.1 (57), 10: AT3G54050.1 (61), 11: AT4G17300.1 (60), 12: AT4G35250.1 (65), 13: AT3G63410.1 (51), 14: AT1G45474.1 (33), 15: AT2G29630.1 (55), 17: AT4G30950.1 (70), 18: AT3G02780.1 (46), 19: AT1G16080.1 (44), 20: AT1G18170.1 (29), 21: AT5G13630.1 (50), 22: AT1G09795.1 (57), 23: AT4G13430.1 (48), 24: AT3G13490.1 (48).

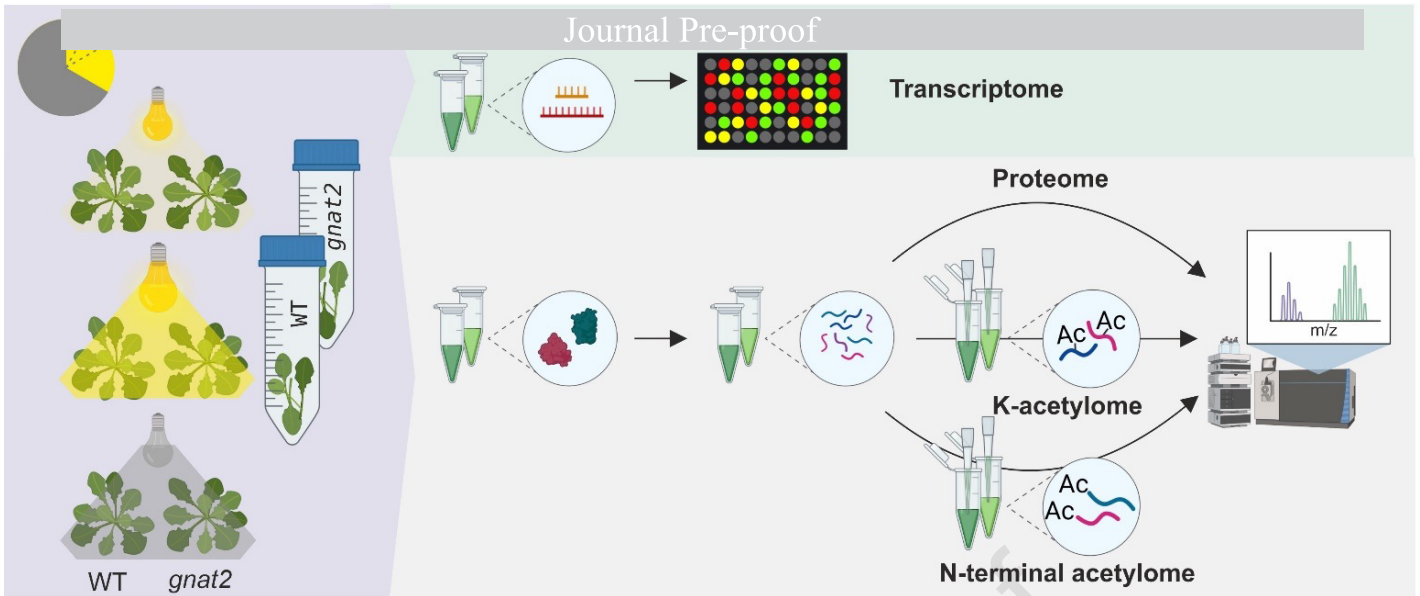
### Figure 6 - Quantitative lysine acetylation (ack) profiling.

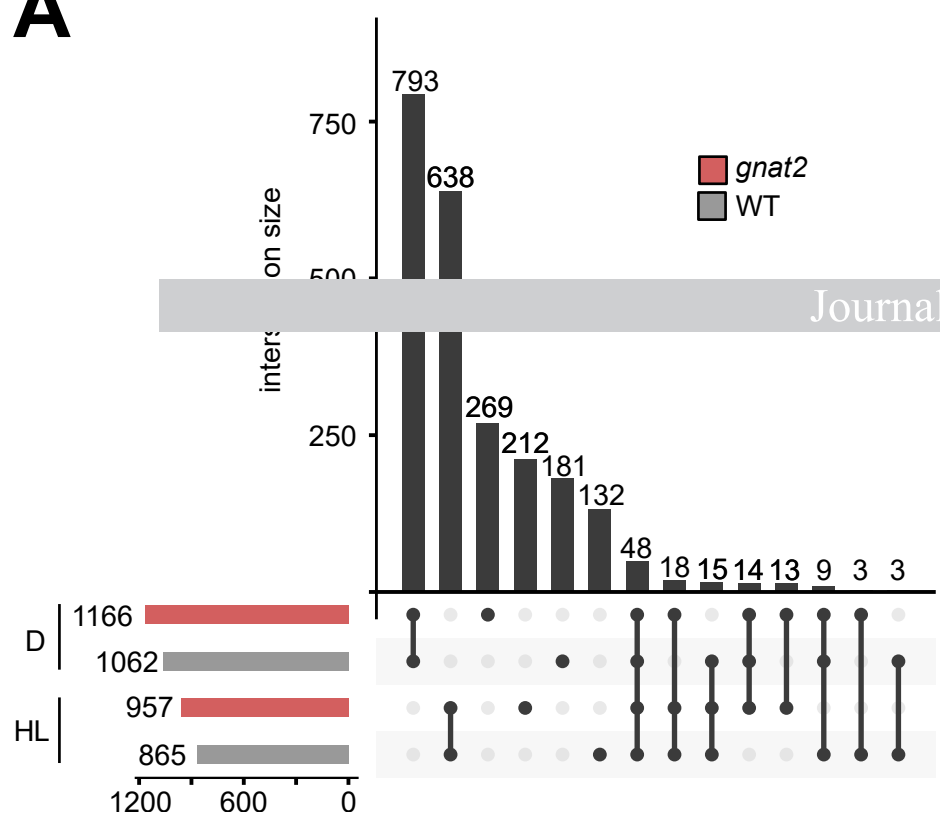
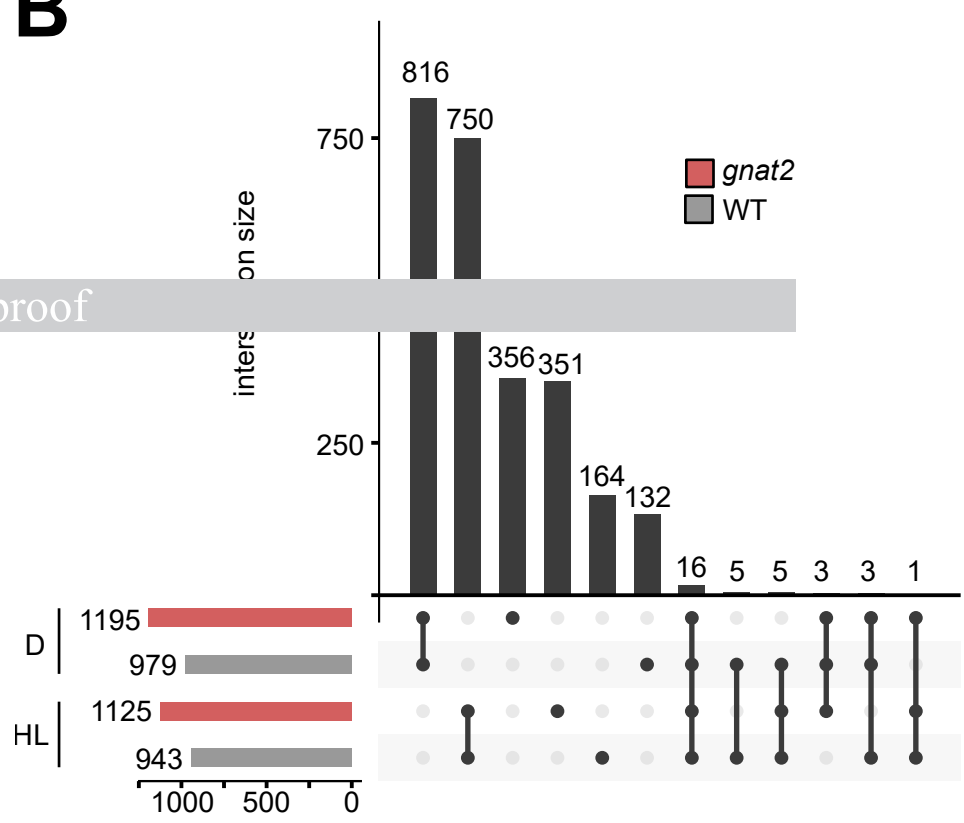
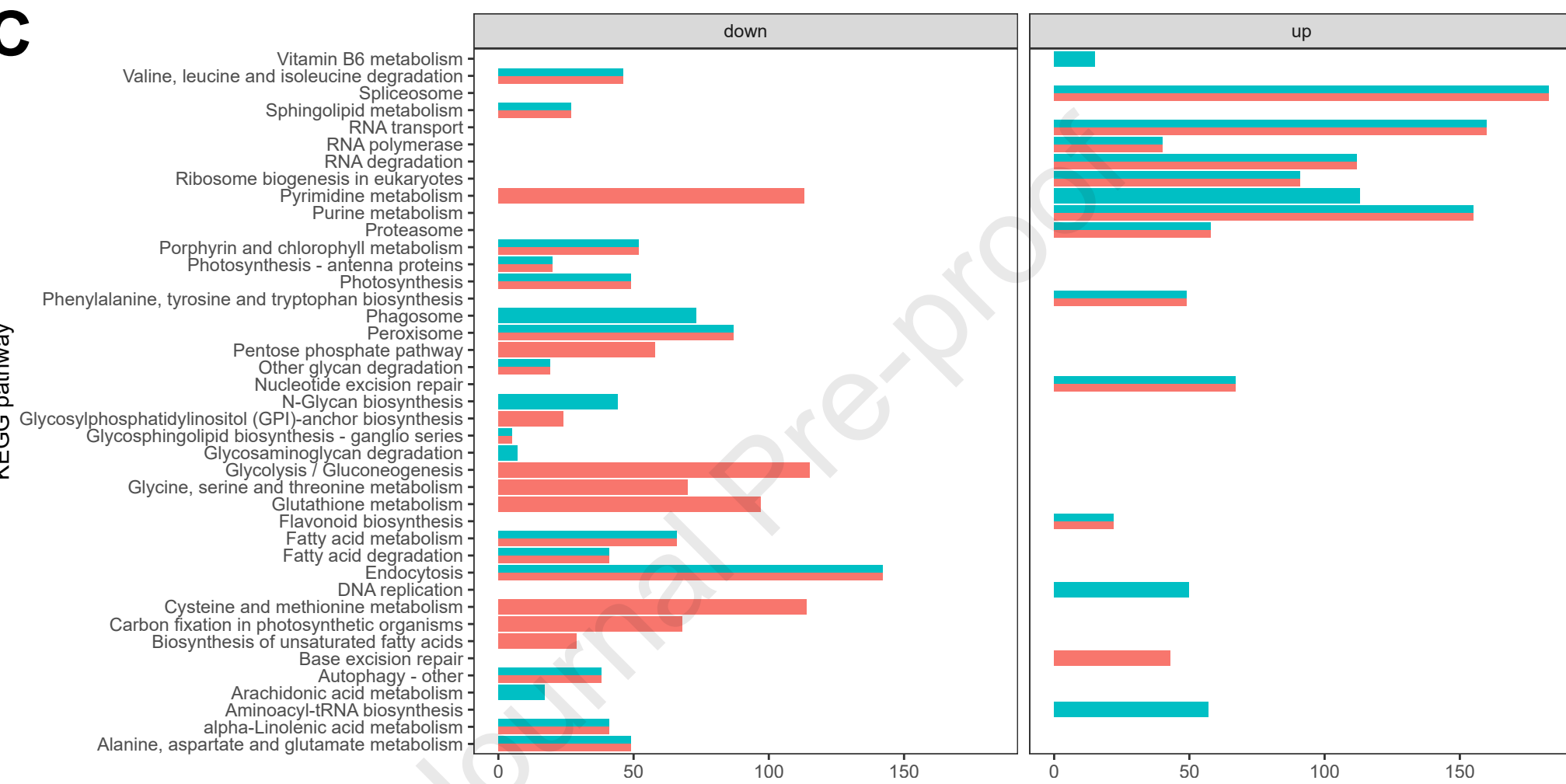
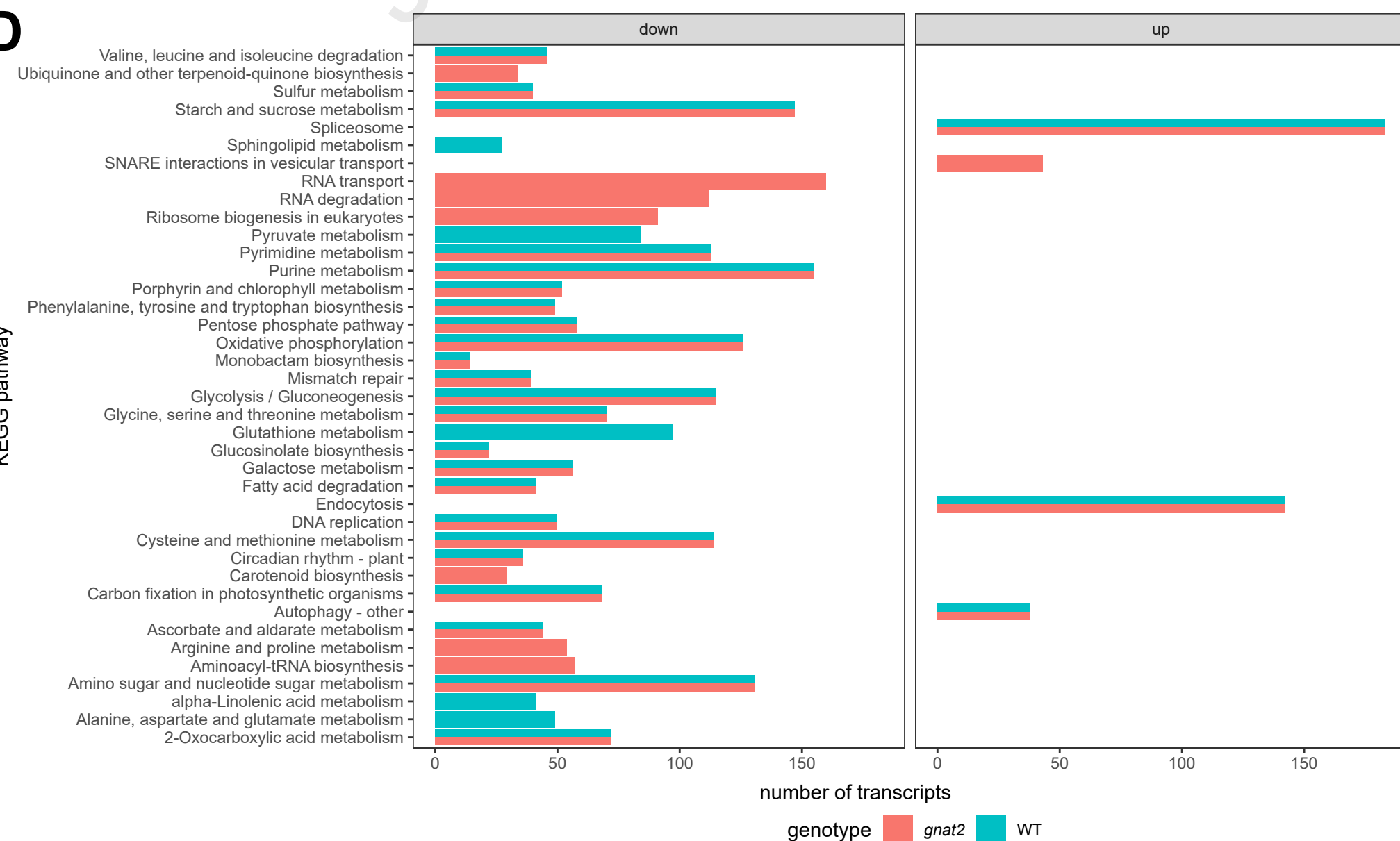
ack abundance changes were quantified via TMT labeling and compared either for different light conditions (panels A - D) or genotypes (panel E - G). Volcano plots showing abundance changes and  $-\log_{10}(\text{values})$  from LIMMA analysis for ack sites quantified in at least two replicates. Log<sub>2</sub> fold-changes +/-0.75 and p-value = 0.05 are indicated as solid lines. For significantly altered ack sites the corresponding gene names are given (A - D) or indicated as numbers, solid symbols are used and both circles and triangles are scaled in size according to the number of replicates. Plastid localized proteins are indicated in green. Sites with identical response to the light treatment in either genotype are indicated as circles in A - D. Protein accessions in this figure from spot #1-136: 1: AT1G66200.1, 2: AT1G70310.1, 3: AT1G74310.1, 4: AT1G06040.1, 5: AT1G77800.7, 6: AT1G79550.2, 7: AT2G20420.1, 8: AT2G27020.1, 9: AT2G28720.1, 10: AT2G30620.1, 11: AT2G30950.4, 12: AT3G10520.1, 13: AT3G14540.2, 14: AT3G16785.6, 15: AT3G22430.1, 16: AT3G47070.1, 17: AT1G12900.1, 18: AT3G55280.3, 19: AT4G25050.1, 20: AT4G29790.1, 21: AT4G32520.2, 22: AT4G34870.1, 23: AT5G02960.1, 24: AT5G07090.3, 25: AT5G11260.1, 26: AT5G15850.1, 27: AT1G20670.1, 28: AT1G54580.1, 29: AT3G55800.1, 30: AT3G62030.1, 31: AT5G14370.1, 32: AT1G26630.1, 33: AT1G32750.1, 34: AT1G44575.3, 35: AT1G52740.1, 36: AT1G56190.1, 37: AT1G65660.1, 38: AT1G79600.1, 39: AT2G05100.1, 40: AT5G60390.3, 41: AT2G39730.2, 42: AT2G45290.2, 43: AT3G04920.2, 44: AT3G09440.4, 45: AT3G15970.2, 46: AT3G17930.1, 47: AT3G44110.1, 48: AT3G62120.3, 49: AT4G01150.1, 50: AT4G20850.1, 51: AT4G38100.2, 52: AT5G01530.1, 53: AT5G07350.1, 54: AT5G13510.1, 55: AT5G15200.1, 56: AT5G26000.1, 57: AT5G27670.1, 58: AT5G47840.2, 59: AT5G59910.1, 60: ATCG00490.1, 61: AT1G51400.1, 62: AT1G75750.1, 63: AT3G46780.1, 64: AT5G13630.1, 65: AT5G20720.4, 66: AT5G66570.1, 67: AT1G29470.2, 68: AT1G30480.1, 69:

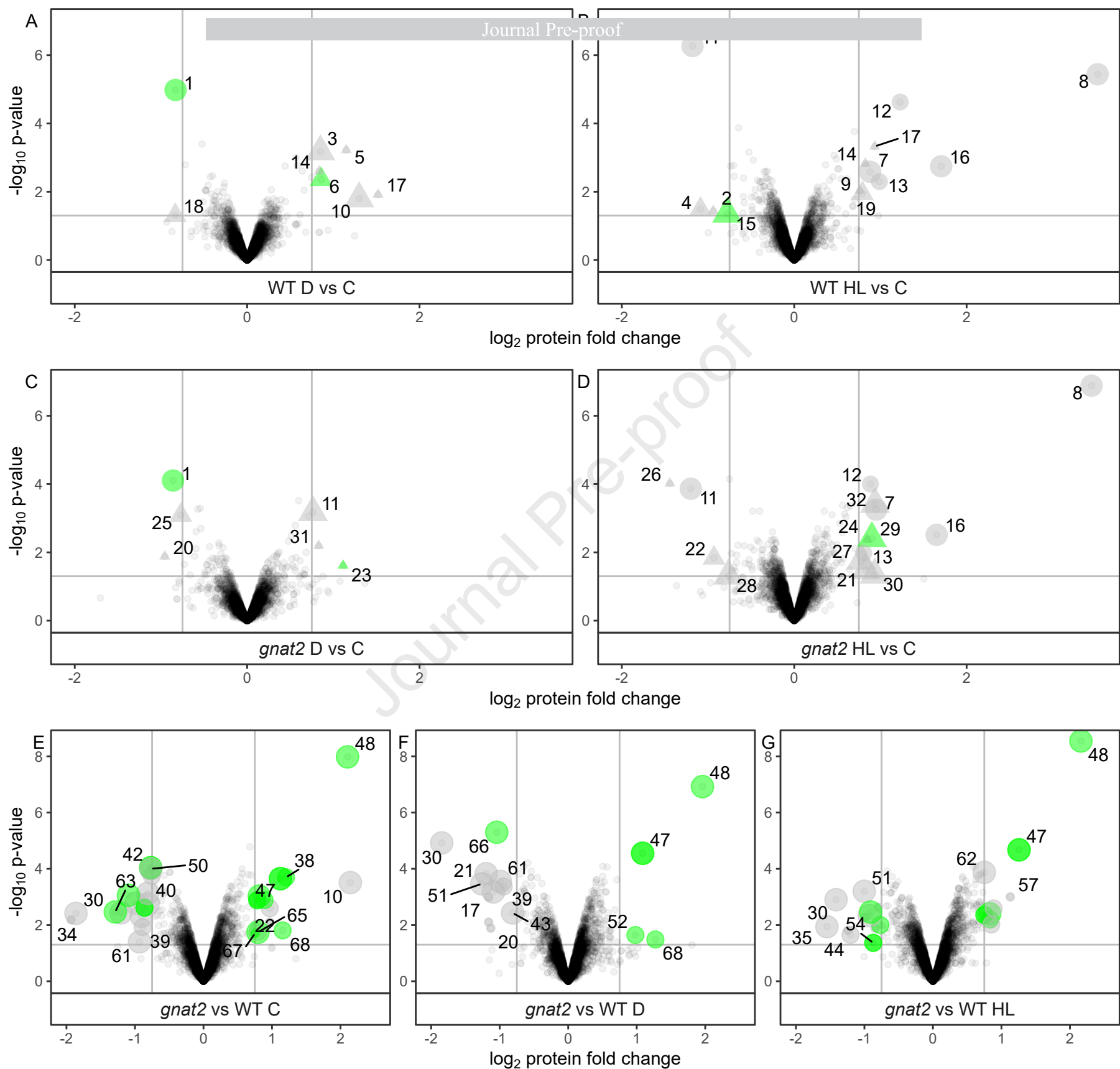
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**Table 1:** Proteins displaying significant NTA decrease (at least 40%) in *gnat2* compared to WT samples, after combining all the entry values from all the corresponding light condition into two global "WT" and "*gnat2*" groups; entries with a plot number were quantified in both replicates of each condition, allowing the calculation of a p-value. Other entries were only quantified in a single replicate of at least one condition. n.i. entries not identified in the experiment. Unless specified otherwise, all acetylated positions are protein N-termini. The plot number (Plot #) is referring to the labels used in Figures 4 and 5.

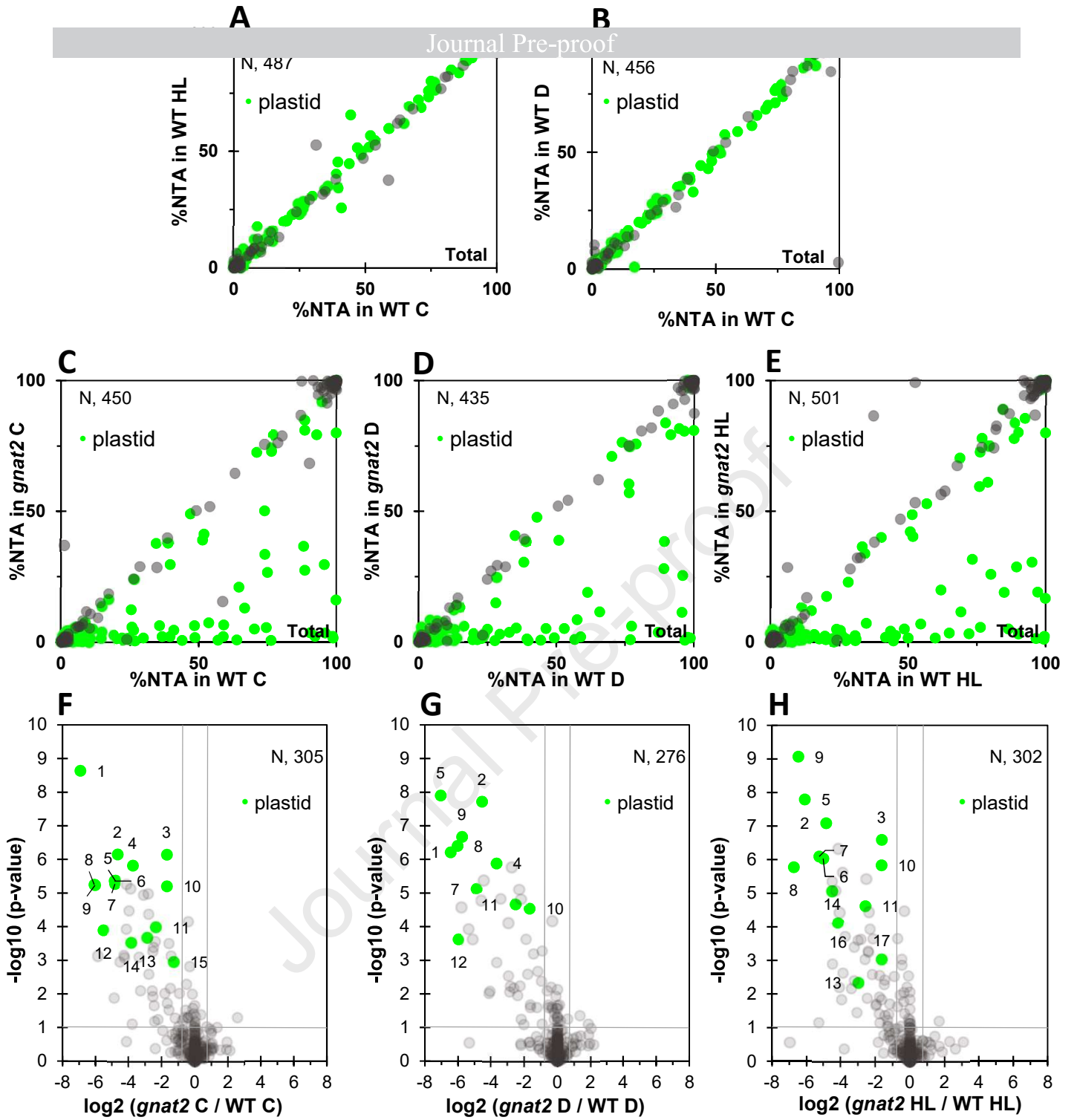
| Plot # | Araport-11 Accession | Entry Name   | Subcellular Localization | Acetylation Position | N-1 residue | N-terminal Sequence | % NTA (WT) | % NTA ( <i>gnat2</i> ) | %NTA Diff. ( <i>gnat2</i> - WT) | Protein Expression Stability | KAcRatio ( <i>gnat2</i> / WT) |
|--------|----------------------|--------------|--------------------------|----------------------|-------------|---------------------|------------|------------------------|---------------------------------|------------------------------|-------------------------------|
| 9      | AT3G03630.1          | CYSK4_ARATH  | plastid                  | 57                   | G           | AISGKSSSTGT         | 98.7 ± 1.5 | 1.5 ± 0.6              | -97.2                           | +                            | n.i.                          |
| 2      | AT4G27440.1          | PORB_ARATH   | plastid                  | 68                   | Q           | TAATSSPTVT          | 89.3 ± 2.2 | 3.5 ± 0.6              | -85.8                           | +                            | n.i.                          |
| 5      | AT2G24820.1          | TIC55_ARATH  | plastid                  | 51                   | S           | AVAGTAVSDQ          | 96.4 ± 1.0 | 1.9 ± 2.4              | -94.6                           | +                            | n.i.                          |
| 8      | AT3G54050.1          | F16P1_ARATH  | plastid                  | 60; 323 (K)          | M           | AVAADAAETK          | 44.3 ± 1.5 | 0.6 ± 0.2              | -43.8                           | +                            | 0.975                         |
| 1      | AT1G64770.1          | PNSB2_ARATH  | plastid                  | 19                   | S           | SISAPQTQTI          | 76.6 ± 1.4 | 0.6 ± 0.3              | -76.0                           | +                            | n.i.                          |
| 7      | AT1G16080.1          | COZ2K9_ARATH | plastid                  | 45; 275 (K)          | A           | AASAATAKKL          | 59.2 ± 2.2 | 1.9 ± 0.7              | -57.4                           | +                            | 1.308                         |
| 3      | AT4G24830.1          | ASSY_ARATH   | plastid                  | 75                   | A           | VLSGDGTALT          | 95.6 ± 0.9 | 29.5 ± 2.0             | -66.1                           | +                            | n.i.                          |
| 10     | AT3G54050.1          | F16P1_ARATH  | plastid                  | 61; 323 (K)          | A           | VAADAAETKT          | 89.1 ± 2.6 | 28.1 ± 1.7             | -60.9                           | +                            | 0.975                         |
| 4      | AT3G57560.1          | NAGK_ARATH   | plastid                  | 51; 91 (K)           | A           | TVSTPPSIAT          | 76.0 ± 1.4 | 5.8 ± 0.8              | -70.2                           | +                            | 0.836                         |
| 6      | AT1G03630.1          | PORC_ARATH   | plastid                  | 69; 334 (K)          | Q           | TVTATPPANE          | 49.2 ± 1.5 | 1.6 ± 0.2              | -47.6                           | +                            | 1.081                         |
| 12     | AT4G35250.1          | HC244_ARATH  | plastid                  | 65                   | C           | SAAAVNLAPG          | 98.9 ± 1.0 | 1.9 ± 0.8              | -97.1                           | +                            | n.i.                          |
| 11     | AT4G17300.1          | SYNO_ARATH   | plastid                  | 60                   | C           | TAVSESLSGSG         | 67.3 ± 2.8 | 12.1 ± 1.4             | -55.2                           | +                            | n.i.                          |
| 14     | AT1G45474.1          | LHCA5_ARATH  | plastid                  | 33                   | K           | AAGGGINPTV          | 70.5 ± 2.7 | 4.1 ± 1.4              | -66.4                           | +                            | n.i.                          |
| 18     | AT3G02780.1          | IDI2_ARATH   | plastid                  | 46; 144 (K)          | R           | AFSGTAMTDT          | 99.8 ± 0.3 | 16.4 ± 0.3             | -83.4                           | +                            | 0.862                         |
| 13     | AT3G63410.1          | BQMT_ARATH   | plastid                  | 51                   | C           | SSSVSSSRP           | 54.8 ± 2.6 | 7.6 ± 1.7              | -47.2                           | +                            | n.i.                          |
| 19     | AT1G16080.1          | COZ2K9_ARATH | plastid                  | 44; 275 (K)          | M           | AAASAATAKAK         | 47.4 ± 1.2 | 5.3 ± 0.5              | -42.1                           | +                            | 1.308                         |
| 20     | AT1G18170.1          | FK172_ARATH  | plastid                  | 29                   | Y           | ASSSNPPEPE          | 62.4 ± 2.1 | 20.2 ± 1.4             | -42.2                           | +                            | n.i.                          |
| 15     | AT2G29630.1          | THIC_ARATH   | plastid                  | 55                   | A           | TLTFDPPTTN          | 89.4 ± 1.7 | 37.3 ± 1.4             | -52.1                           | +                            | n.i.                          |
| 17     | AT4G30950.1          | FAD6C_ARATH  | plastid                  | 70                   | A           | VAAPVAPPSA          | 78.4 ± 2.6 | 26.1 ± 1.1             | -52.3                           | +                            | n.i.                          |
| 21     | AT5G13630.1          | CHLH_ARATH   | plastid                  | 50                   | S           | AVSGNGLFTQ          | 87.8 ± 5.7 | 15.2 ± 6.7             | -72.6                           | +                            | n.i.                          |
| 22     | AT1G09795.1          | HIS1B_ARATH  | plastid                  | 57                   | C           | VSNAQKSVLN          | 72.9 ± 1.2 | 32.6 ± 0.9             | -40.3                           | +                            | n.i.                          |
| 23     | AT4G13430.1          | LEUC_ARATH   | plastid                  | 48                   | S           | VMAPQKDRSP          | 97.1 ± 0.1 | 22.8 ± 3.7             | -74.3                           | +                            | n.i.                          |
| 24     | AT3G13490.1          | SYKM_ARATH   | plastid,mitoch           | 48                   | S           | AASSSSSSAT          | 59.6 ± 3.4 | 6.5 ± 0.0              | -53.0                           | +                            | n.i.                          |
|        | AT1G74970.1          | RR9_ARATH    | plastid                  | 53                   | A           | TVSAPPEEEE          | 96.7       | 6.1                    | -90.6                           | +                            | n.i.                          |
|        | AT1G68590.1          | RRP31_ARATH  | plastid                  | 50; 134 (K)          | L           | AAPELTAET           | 92.3       | 2.0 ± 0.1              | -90.3                           | +                            | 0.949                         |
|        | AT4G33470.1          | HDA14_ARATH  | plastid                  | 45                   | C           | SFSTEKNPLL          | 86.5       | 4.5 ± 1.1              | -82.0                           | +                            | n.i.                          |
|        | AT5G22800.1          | SYAP_ARATH   | plastid                  | 56                   | A           | KSSSVSQPV           | 63.9       | 1.1 ± 0.2              | -62.8                           | +                            | n.i.                          |
|        | AT2G25840.1          | SYWM_ARATH   | plastid                  | 53                   | C           | SVATDDTSPS          | 64.9 ± 2.0 | 3.4                    | -61.6                           | +                            | n.i.                          |
|        | AT3G54050.1          | F16P1_ARATH  | plastid                  | 63; 323 (K)          | A           | ADAAETKTAA          | 57.0       | 0.5 ± 0.2              | -56.5                           | +                            | 0.975                         |
|        | AT3G13490.1          | SYKM_ARATH   | plastid,mitoch           | 46                   | C           | ASAASSSSS           | 56.7       | 1.3 ± 0.3              | -55.4                           | +                            | n.i.                          |
|        | AT1G59840.1          | CCB4_ARATH   | plastid                  | 34                   | A           | SSSSTSQPK           | 69.5       | 19.4                   | -50.1                           | +                            | n.i.                          |
|        | AT2G26670.1          | HMOX1_ARATH  | plastid                  | 57                   | A           | TTAAEKQKKR          | 50.6       | 1.1 ± 0.2              | -49.5                           | +                            | n.i.                          |
|        | AT1G48460.1          | 78W3G7_ARATH | plastid                  | 62                   | C           | SSSQSDSRPE          | 52.9       | 4.4 ± 1.5              | -48.5                           | +                            | n.i.                          |
|        | AT1G31180.1          | LEU33_ARATH  | plastid                  | 35                   | C           | AAASPVKKRY          | 53.9       | 5.5 ± 1.4              | -48.5                           | +                            | n.i.                          |
|        | AT5G16440.1          | IDI1_ARATH   | plastid                  | 56                   | S           | AVTMTDSNDA          | 43.7       | 3.0 ± 1.5              | -40.7                           | +                            | n.i.                          |

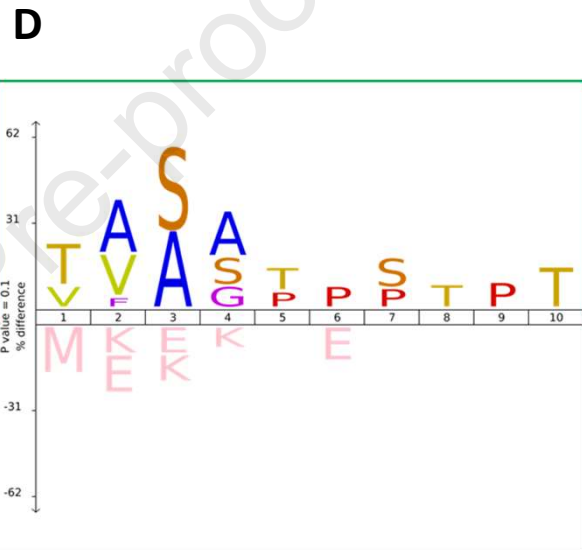
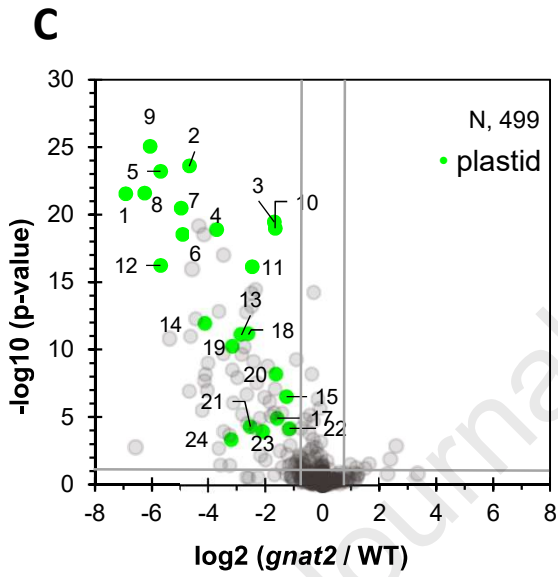
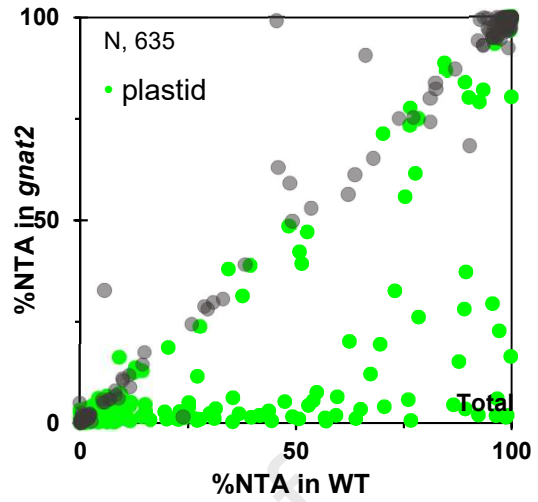
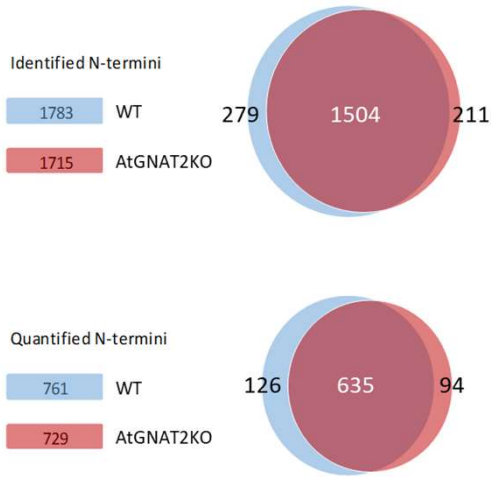


**A** Downregulated genes with respect to control condition**B** Upregulated genes with respect to control condition**C****D**

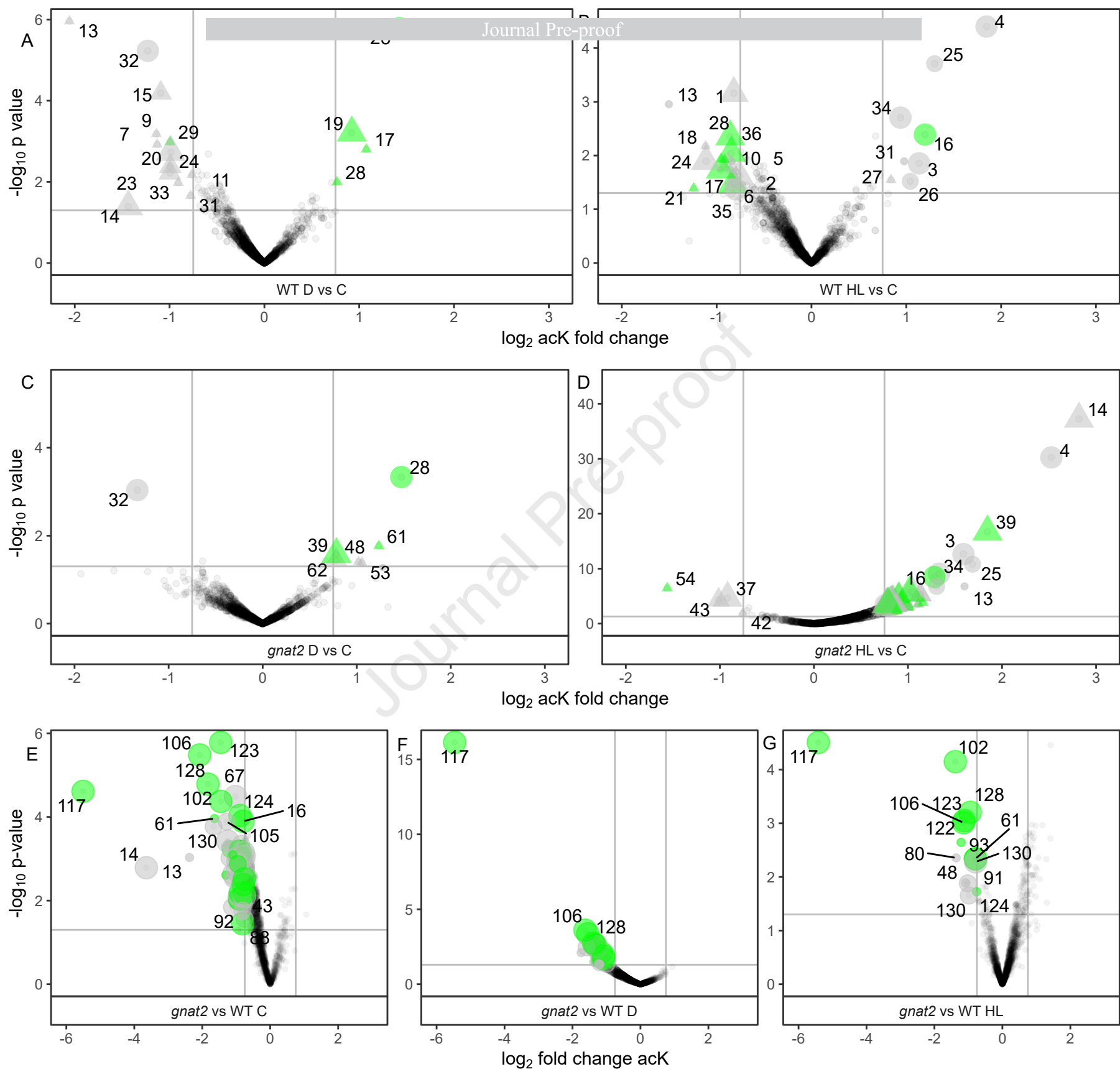


localization • other • plastid replicates • 2 • 3 • 4 genotype dependent • FALSE • TRUE









- Comprehensive multi-omics profiling of plant light response
- Parallel N-terminal and K-acetylation analysis of dual specific GNAT2 and WT
- N-terminal acetylome remained unchanged after two hours change in light conditions
- GNAT2-associated lysine acetylome displayed sensitivity to changes in light
- Distinct protein acetylation responses to light acclimation

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We investigated protein acetylation and the dual-specific plastid acetyltransferase GNAT2 in plant acclimation to short-term light changes. Our multi-omics analysis showed that the N-terminal acetylome remained unchanged in wild-type and *gnat2*-knockout plants after two hours of high light or darkness. However, the lysine acetylome was sensitive to light conditions, especially in the *gnat2* background. This suggests plants use distinct acclimation strategies involving GNAT2-mediated lysine acetylation, but not N-terminal acetylation, for rapid environmental responses.

Journal Pre-proof

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for *[Journal name]* and was not involved in the editorial review or the decision to publish this article.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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