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### ER-anchored protein sorting controls the fate of two proteasome activators for intracellular organelle communication during proteotoxic stress

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1 ER-anchored protein sorting controls the fate of two proteasome activators

#### 2 for intracellular organelle communication during proteotoxic stress

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#### 17 Summary

Proteotoxic stress, characterized by the accumulation of damaged proteins, poses a significant 18 19 challenge to cellular homeostasis. To mitigate proteotoxicity eukaryotes employ the proteasome 20 that is regulated by proteasome activators, e.g. transcription factors that promote gene expression of proteasome subunits. As proteotoxicity originates in different compartments, cells 21 22 need to perceive signals from various locations. Understanding which components integrate 23 signals to address proteotoxicity is essential to develop strategies to cope with proteotoxicity 24 but remain elusive. Here, we identify that the proteasome autoregulatory feedback loop acts as 25 a gatekeeper to facilitate the communication between nucleus and chloroplast. We reveal that 26 the ER-anchored protein sorting system (ERAPS) controls the proteasomal degradation or nuclear translocation of proteasome activators NAC53 and NAC78. While both transcription 27 28 factors activate the proteasome gene expression, they repress photosynthesis-associated nuclear genes during proteotoxicity through association with a conserved cis-element. Our data 29 30 implicate a general trade-off between proteasome function and energy metabolism unravelling an unprecedented mechanism of how eukaryotic cells cope with proteotoxicity. Collectively, 31 32 our discoveries provide a novel conceptual framework in which the proteasome autoregulatory 33 feedback loop coordinates subcellular proteostasis and the trade-off between growth and 34 defence.

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#### 36 Main

37 Protein homeostasis, hereafter proteostasis, is defined as the synthesis of proteins and their regulated degradation. This intimate balance between protein synthesis and degradation is 38 tightly regulated in all organisms to respond to several environmental stimuli<sup>1,2</sup>. Various 39 perturbations can alter this equilibrium, leading to proteotoxic stress, the accumulation of 40 41 aberrant proteins that cause ultimately irreversible cellular damage. These perturbations can 42 range from ageing to pathological diseases such as Parkinson and Alzheimer as well as infection by pathogens<sup>1,3</sup>. The latter is a consequence of the onset and maintenance of defense responses 43 44 during microbial infections and microbes' ability to manipulate proteostasis to their own benefit<sup>3,4</sup>. 45

46 To mitigate deleterious effects due to excessive proteotoxic stress, cells employ various protein 47 quality control machineries. One of the major protein quality control machineries across the 48 tree of life is the ubiquitin-proteasome system (UPS). The UPS controls proteostasis through 49 selective elimination of defective proteins and short-lived regulatory proteins<sup>5</sup>. To rapidly react 50 to proteotoxic stress, all the constituents of the 26S proteasome are under a tight transcriptional 51 control. All eukaryotic kingdoms possess transcription factors (TFs) required for proteasome 52 gene activation<sup>6</sup>. Yeast utilizes Rpn4, mammals Nrf1/2 and plants the pair of NAC TFs (NAM, 53 ATAF and CUC) NAC53/NAC78 as a conserved mechanism to cope with proteotoxicity. In yeast and animals, proteasome activators Rpn4 and Nrf1/2 have been shown to be UPS targets 54 55 themselves, being degraded by the proteasome in the cytosol or ER<sup>6</sup>. Consequently, proteasome 56 malfunction impairs their degradation leading to their stabilization and subsequent nuclear translocation to activate proteasome gene expression<sup>6</sup>. In plants, NAC78 mediates the 57 transcriptional activation of proteasome promoters through the association with the proteasome 58 regulatory cis element PRCE [TGGGC]<sup>7</sup>. In addition, loss of the NAC53/78 TF pair rendered 59 plants hypersensitive to proteasome inhibition<sup>8</sup>. Therefore, a similar proteasome regulatory 60 61 feedback loop has been proposed in plants<sup>6</sup>; however, to date, no evidence supports proteasomal removal of NAC53/78. 62

The involvement of the proteasome in various cellular pathways<sup>9–11</sup>, suggests that the autoregulatory feedback loop is a central mechanism to precisely integrate and coordinate stress responses through proteotoxic stress sensing. Indeed, a growing body of evidence suggests that localized proteotoxic stress in organelles causes the activation of respective proteasomemediated degradation pathways<sup>12–16</sup>. This implies a multi-layered signaling role of the proteasome in governing subcellular proteostasis. This aspect is particularly relevant for semiautonomous organellar nuclear-encoded proteins, as their regulation also involves cytosolic proteasomal degradation prior to organellar import<sup>17,18</sup>. As such, proteasome activators could act as gatekeepers of the communication between the nucleus and energy-producing organelles to maintain subcellular proteostasis. This could link the proteasome directly to energy metabolism as well as to the trade-off between growth and defense. However, to date it is unknown whether proteasome activators exert these functions.

Here we report that proteasome activators, TFs NAC53 and 78, orchestrate the communication between the nucleus and chloroplast during various stress conditions. We reveal that ERanchored protein sorting (ERAPS) controls the proteasomal degradation and nuclear translocation of NAC53 and NAC78. While both TFs activate the proteasome gene expression, they repress photosynthesis-associated nuclear genes during proteotoxicity. The trade-off between proteasome activation and photosynthesis downregulation seems to be a general feature as it occurs in response to various environmental and developmental cues.

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#### 83 NAC53/78 are central integrators of various proteotoxic stress conditions

84 Proteotoxicity is triggered by a wide range of physicochemical stresses at different subcellular locations often leading to accumulation of proteins that are not imported into these organelles 85 86 <sup>17,19</sup>. To understand whether proteasome activators might play a role in integrating signals from different locations, we used the proteasome activator mutant nac53-1 78-18 to disrupt cellular 87 proteostasis through a variety of approaches (Fig.1a): (i) inhibiting the proteasome via 88 89 bortezomib (BTZ) and bacterial infection with *Pseudomonas syringae pv. tomato DC3000* 90 (Pst)<sup>20,21</sup>, (ii) inhibiting the segregase CDC48 (CB-5083)<sup>16,22</sup>, (iii) targeting HSP90 (GDA)<sup>23</sup>, which plays a critical role in the import and degradation of chloroplastic/mitochondrial 91 precursors, (iv) inducing ER stress with DTT and TM, and (v) targeting semi-autonomous 92 organelles such as chloroplasts and mitochondria (Lin, Cml, MV)<sup>23</sup>. 93

Perturbation of the proteasome by chemicals, bacteria, CDC48 inhibition and induced ER stress
resulted in an increased susceptibility of the *nac53-1 78-1* mutant (Fig.1b), suggesting that both
proteasome activators are involved in a general regulation of subcellular proteostasis.
Strikingly, all other treatments perturbing organelle function (Lin, Cml, MV, GDA) resulted in
increased tolerance of *nac53-1 78-1* (Fig.1b). Overall, our phenotypic screen implies that
NAC53 and NAC78 integrate signals from different compartments to adjust their impact on
plant response to stress.

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#### 104 NAC53/78 are part of an autoregulatory proteasome feedback loop

105 To elucidate how both TFs might integrate signals from different compartments and organelles, we aimed to decipher their regulation through interactome analysis. Transgenic Arabidopsis 106 107 GFP-NAC53 and 78 lines (under the control of UBQ10 promoter) were only detectable for the 108 GFP fusion proteins upon proteasome inhibition by BTZ (Fig. 2,b), implying a high 109 proteasomal turnover of both NACs. Thus, we used Agrobacterium-mediated transient 110 expression in Nicotiana benthamiana to boost the expression of NACs for our interactome 111 studies. Transient expression of GFP-NAC53 and 78 (sTag construct) decorated the ER, being 112 consistent with the predicted transmembrane domain (TM) at the C-terminus of both proteins 113 (Fig. 2c and Extended Data Fig.1a). We additionally employed a chimeric construct (dTag-114 NACs) tagged with fluorophores at the N and C-terminus following the TM domain (Extended Data Fig.1a) to monitor subcellular interaction partners, as the RFP signal remained strictly in 115 116 the ER (Fig. 2d). BTZ treatment, stabilized all NAC constructs, supporting the notion of 117 NAC53/78 as proteasome targets and validating the functionality of the system in N. 118 benthamiana (Extended Data Fig.1b,c).

119 Next, we performed immuno-precipitation (IP) followed by tandem mass spectrometry 120 (MS/MS) with both constructs. We processed a total of 4 IP-MS/MS conditions for both NACs 121 among two experiments. We performed IP of sTag-NACs in mock conditions and at 8 hours 122 after bacterial infection, to induce proteotoxic stress (Extended data Fig.1D). In parallel, we 123 performed IP of dTag-NACs enriching for the N- or C-terminus end of the proteins, which 124 allowed us to characterize the interactome at a subcellular resolution (Extended data Fig.1E). 125 For our downstream analysis, we focused on the common interactors for both NACs. Out of the 4 conditions we identified 245 unique A. thaliana orthologous proteins as interactor 126 127 candidates (Fig. 2e, Supplementary Table 1). The Gene Ontology (GO) annotations for cellular components and the displayed enrichment for terms mainly associated with the proteasome 128 129 complex and plastid components (Extended Data Fig.1f). Investigating the SUBA5 annotation of NAC53/78 interacting candidates, we found enrichment for following compartments: plastid, 130 131 peroxisome, Golgi, and ER (Extended Data Fig.1g). Further GO analysis for biological processes revealed that possible candidates were enriched in proteins related to three main 132 133 biological process: proteolysis, trafficking and translation (Fig. 2f). We extracted the 134 corresponding proteins and performed a protein network analysis (Fig. 2g), highlighting the 135 presence of UPS components (Fig. 2g; Extended Data Fig.1h-k). However, proteasome 136 components were strongly reduced by bacterial infection and mainly enriched when targeting 137 the ER-anchored end of the TFs (C-ter IP) (Extended Fig.1h), indicating that both proteasome

regulators are degraded at the ER. The latter was further supported by the presence of several ER-associated degradation (ERAD) components in the interactome (Fig.2g and Extended Data Fig.1i). In summary, our interactome analysis validates the role of NAC53/78 as integrators of subcellular proteostasis disruptions, affirming their regulation by the proteasome autoregulatory feedback loop in plants.

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144 ER-anchored protein sorting (ERAPS) regulates the subcellular fate of NAC53 & NAC78 145 Our interactome approach revealed that NAC53/78 associated with candidates that belong to 146 the ERAD machinery, including membrane bound E3 ligase HRD1 and CDC48 (Fig. 2g and 147 Extended data Fig.1i). To test whether ERAD is required to turnover the NACs, we took 148 advantage of pharmacological drugs targeting individual steps in ERAD: we treated the GFP-NAC53 and GFP-NAC78 transgenic lines with CB-5083 and LS-102, inhibitors of CDC48 and 149 HRD1 proteins, respectively<sup>22,24</sup>. We confirmed that previously uncharacterized LS-102 can 150 151 efficiently inhibit both A. thaliana orthologs AtHRD1a and AtHRD1b in vitro (Extended data 152 Fig. 2a,b). Similar to BTZ and CDC48, HRD1 inhibition led to a stabilization of both proteins; 153 confirmed by confocal microscopy and immunoblotting (Fig. 3a,b). Collectively, these results 154 indicate that NAC53/78 are targets of ERAD. Interaction studies of NACs with HRD1 isoforms 155 revealed that GFP-NACs strongly associated with RFP-HRD1a/b in localization and co-IP 156 experiments (Fig. 3c,d). The association was largely reduced upon deletion of the TM domain of both NAC TFs ( $\Delta$ TM, Fig. 3c). Interestingly, loss of ER association led to a strong 157 158 stabilization of the proteins, supporting ERAD-dependent turnover for both TFs (Fig. 3e). 159 Subsequent in vitro ubiquitination assays using purified GST-HRD1a/b isoforms and MBP-160 NAC53/78 proteins confirmed that both NACs are ubiquitinated by HRDs, indicated by the 161 appearance of higher molecular weight bands of MBP-NAC proteins (Fig. 3f). We could 162 confirm the presence of ubiquitination marks at different sites via MS/MS analysis of the in 163 vitro ubiquitination reactions (Extended Data Fig. 2c,e). In addition, ubiquitin marks were consistently inhibited by the addition of the inhibitor LS-102 to the in vitro ubiquitination assay 164 165 (Extended data Fig.2f). The in vitro data was corroborated by the identification of several ubiquitination sites on NAC53 and 78 using in vivo IP-MS/MS analysis (Extended data Fig. 166 167 2d,e). This could be confirmed by performing ubiquitin IP on *N. benthamiana* leaves transiently expressing the NAC constructs (Extended data Fig. 2g). Mutation of identified lysine residues 168 169 substantially reduced the association with anti-ubiquitin beads in planta (Extended data Fig. 170 **2h,i**). Altogether, our *in vivo* and *in vitro* analysis demonstrate that NAC53 and NAC78 are 171 directed to proteasomal degradation via ERAD through HRD1 ubiquitination.

During our analysis we observed that NAC53 and 78 responded different to the tested 172 173 inhibitors: while BTZ led to a strong signal in the nucleus, CDC48 inhibition led to the appearance of cytoplasmic dot-like structures (Fig. 2a) that was not due to differences in drug 174 175 potency depending on the concentration of the drugs (Extended data Fig. 3a). Co-expression of CDC48 isoform AtCDC48c with both NACs revealed that both proteins associated in dot-like 176 177 structures (Extended data Fig. 3b), suggesting that NAC53/78 accumulate at sites of CDC48 178 action upon CDC48 inhibition. Given the role of CDC48 in retro-translocation of ER proteins 179 to the cytosol we tested whether inhibition of its action can also perturb the subcellular sorting 180 of NACs comparing the GFP signal intensity in the nucleus and cytoplasm upon BTZ or CB-181 5083 treatment. The proportion of the GFP signal in the nucleus was significantly lower when 182 plants were treated with CB-5083, supported by the lowered ratio of nucleus/cytoplasm GFP 183 intensity of plants subjected to the same treatment (Extended data Fig. 3c-f).

Together, our findings demonstrate the recycling of both NACs by the ERAD machinery,
regulated by HRD1 and CDC48, with the latter facilitating their nuclear translocation (Fig. 3g).
Thus, we propose to refer to this mechanism as ERAPS (ER-anchored protein sorting).

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#### 188 The transcriptional landscape of proteotoxic stress

189 To understand why the nac53-1 78-1 mutant is more tolerant to organelle-directed proteotoxic 190 stress while it is hypersensitive to general proteotoxic stress we undertook a transcriptomic 191 analysis. To this end we exploited the Pst-mediated proteotoxic stress caused by the suppression 192 of proteasome activity and broad modification of chloroplast proteome<sup>20,21,25</sup>. Our analysis 193 revealed that Pst induced proteotoxic stress hallmarks were dependent on NAC53/78 (Fig. 194 4a,b,c, Extended data Fig.4a) and stabilized both NAC TFs in N. benthamiana and A. thaliana 195 (Fig. 4e,f). As such, this system is ideal to unveil the function of the proteasome autoregulatory 196 feedback loop in response to environmental perturbation. Next, we analysed the transcriptomic 197 profile of nac53-1 78-1 infected with Pst, which revealed 945 differentially expressed genes (DEGs). Strikingly, apart from the proteasome cluster most of the DEGs displayed a positive 198 199 Log2FC (Fig. 4g,h and Extended data Fig. 4a). This suggests that NAC53/78 are major 200 transcriptional repressors upon bacteria-induced proteotoxic stress. Further investigation of 201 which biological processes are enriched within the DEGs revealed photosynthesis, 202 glucosinolates, cell wall and auxin signalling as major terms (Fig. 4i). To obtain a global view 203 of the transcriptional landscape during proteotoxicity, we included two other datasets: (i) 204 transcriptome analysis of proteasome mutant *rpt2a-2* in response to *Pseudomonas syringae pv*. 205 maculicola (Pma) infection (Extended data Fig.4b-e) and (ii) previously characterized

transcriptome analysis on Col-0 plants upon MG132 treatments<sup>8</sup>. The comparison of all three 206 datasets allowed us to extract a core set of 538 DEGs present in the nac53-1 78-1 dataset and 207 208 at least one of the other datasets (Fig. 4j and Supplementary Table 2). Extracting the genes 209 associated with these processes from the nac53-1 78-1 945 DEGs, we could define the core 210 transcriptional network associated with proteotoxic stress (Fig. 41). This network is comprised 211 of 4 modules: the proteasome genes, a major cluster of photosynthesis associated nuclear genes 212 (PhANGs), glucosinolate and auxin signalling pathways (Fig. 4k,l). Only the proteasome and 213 photosynthesis cluster were present in all three datasets (Extended data Fig.4f). This suggests 214 that PhANGs transcriptional repression is a recurrent response to proteotoxic stress. Altogether 215 our analysis revealed a so far unidentified link between NAC53/78 and the transcriptional 216 regulation of PhANGs in response to proteotoxic stress.

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# 218 NAC53 and NAC78 coordinate the regulation of proteasome and photosynthesis gene 219 expression through the same cis-element

220 The global transcriptional profile during proteotoxic stress indicates a strong link between 221 proteasome activation and repression of photosynthesis. Thus, we hypothesized NAC53/78 can 222 act as novel repressors of PhANGs. Detailed analysis of distinct photosynthetic processes 223 revealed that while many processes were downregulated during proteotoxic stress, loss of 224 NAC53/78 had a general effect on PhANGs transcriptional regulation (Extended data Fig. 5a). 225 Enrichment analysis for specific promoter cis-elements in the 4 gene clusters of the core 226 transcriptional network (Fig. 4k,l) revealed that the proteasome genes and PhANGs share a 227 common cis-element characterized by the PRCE-like [TGGGC] core motif (Fig. 5a). In line 228 with this, PRCE-like elements appeared homogeneously distributed among PhANGs promoters 229 and enriched close to the transcription starting site (Extended data Fig. 5b,c). We then extracted 230 the promoter regions of selected candidates, LHCa3 and PSAD1, (Fig. 5b), members of the light harvesting complex I and photosystem I<sup>26</sup>. Performing electromobility shift assay 231 (EMSA), we could confirm that NAC53 and NAC78 directly bind to these elements (Fig. 5c), 232 233 dependent on the presence of their respective DNA recognition motif (Fig. 5c). Furthermore, 234 we confirmed the TGGGC motif as being the driver of this association, as mutated oligos were 235 unable to outcompete the association of NAC53/78 with the probes (Fig. 5d). Chromatin IP 236 followed by qPCR corroborated our findings, as both TFs were only able to associate to the 237 selected promoter regions when plants were challenged with bacteria-induced proteotoxic stress (Fig. 5e). Performing luciferase reporter assays in protoplasts <sup>27</sup>, we revealed that the PSAD1 238 239 promoter activity was consistently repressed by NAC53 and 78, which did not occur in nac53-

1 78-1 mutant protoplasts (Extended data Fig. 5d). Deletion of the PRCE motif in PSAD1 240 241 abolished NAC78-mediated repression, while NAC53 led to an increase in promoter activity (Extended data Fig. 5e). In contrast, LHCa3 promoter activity appeared to be rather induced by 242 243 NAC53 and unchanged by NAC78 (Extended data Fig. 5d), with NAC53-mediated activation 244 being dependent on the PRCE motif (Extended data Fig. 5e). This difference might be due to 245 the fact that both TFs are expressed without a TM domain, bypassing ERAPS, in a rather 246 artificial system. To circumvent this, we analysed the gene expression of LHCa3 and PSAD1 in 247 the transgenic NAC53 and 78 lines upon BTZ treatment. Proteasome inhibition in the 248 transgenic NAC53 and 78 lines partially enhanced the repression of PhANGs, supporting the 249 notion that NAC53/78 act as repressors of photosynthesis during proteotoxic stress (Extended 250 data Fig. 5f). In addition, both lines showed an increase in PBA1 and RPT2a mRNA (Extended 251 data Fig. 5g), confirming that NAC53/78 can act concomitantly as transcriptional repressor and 252 activator. These results confirmed the importance of the PRCE-like element in PhANGs 253 promoters, suggesting that NAC53 and NAC78 possess the ability to modulate each other's 254 activity and highlights the complexity of the NAC53/78-PRCE regulatory module mediating 255 transcriptional repression of PhANGs and activation proteasome genes. To confirm that 256 NAC53/78 act indeed as novel repressors of photosynthesis we monitored the abundance of 257 photosynthesis proteins, PSII activity and photosynthetic pigment content upon bacteria-258 induced proteotoxicity. To summarize, we detected that bacterial infection had a significantly 259 lesser impact on all measured photosynthesis readouts in the nac53-1 78-1 double mutant 260 compared to Col-0 (Fig. 5f-j). Consistently with these findings, subjecting the NAC53/78 261 transgenics to constant proteasome inhibition resulted in strong developmental defect 262 (Extended data Fig.5h,i), suggesting that the role of NAC53/78 on photosynthesis repression is 263 not restricted to bacterial infection. Altogether, we demonstrate the unique ability of NAC53 264 and NAC78 to mediate activation and repression of target genes through the same PRCE 265 element. This makes them novel repressors of photosynthesis during proteotoxic stress and explains our previous findings that the NAC mutant was more tolerant to organelle-directed 266 267 proteotoxic stress.

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# 269 The proteasome autoregulatory feedback loop monitors photosynthesis homeostasis 270 during stress responses

Our results suggest that photosynthesis repression by NAC53/78 is a general feature of the proteasome autoregulatory feedback loop to cope with proteotoxicity. In line with this, recent evidence indicates that loss of proteasome function in a chloroplast import deficient mutant

improved photosynthesis due to a lack of chloroplast precursor protein degradation<sup>28</sup>. To 274 275 analyze to which extent NAC53/78 are involved in the response to chloroplast perturbation, we 276 investigated photosynthesis readouts upon BTZ and Lincomycin treatments in nac53-1 78-1 277 plants. All readouts (pigment content, PhANGs expression, PSII activity) were significantly 278 less impaired in nac53-178-1 (Fig. 6a, b and Extended data Fig. 6a), suggesting that NAC53/78 279 are indeed monitoring PhANGs expression during chloroplast stress. We then repeated the phenotypic screen introduced in Fig. 1 using the hrd1a 1b double mutant<sup>29</sup> to investigate the 280 direct involvement of the proteasome autoregulatory feedback loop in coordinating cellular 281 282 proteostasis and photosynthesis. Proteasome inhibition rendered the hrd1a 1b more tolerant in 283 comparison to Col-0 (Fig. 6c), which is consistent with its role in negatively regulating NAC53/78 stability (Fig. 3g). In contrast, all other tested drugs or bacterial infection rendered 284 285 the hrdla 1b mutant more susceptible (Fig. 6c,d). The reduced growth of hrdla 1b upon 286 chloroplastic perturbations (Cml and Lin) are consistent with our previous results (Fig. 1b), as 287 stabilisation of NACs would lead to increased repression of PhANGs and growth reduction 288 (Extended data Fig. 5a, h,I). The impaired performance of hrd1a 1b upon CDC48 inhibition, pathogen infection or ER stress induction might be due to the general function of HRDs in 289 290 ERAD<sup>30</sup>. Analysis of the photosystem II activity revealed a stronger reduction in the hrd1a 1b 291 double mutant (Extended data Fig. 6b) upon infection corroborating our findings that ERAPS 292 coordinates proteasome and photosynthesis through NAC53/78 stability. In addition, these data 293 suggest that organellar perturbation would lead the NAC53/78 activation. Indeed, subjecting 294 GFP-NACs transgenics to several organellar perturbators led to stabilization and nuclear 295 localization of both TFs (Extended data Fig. 6c). Taken together our analysis illustrates the 296 importance of the proteasome autoregulatory feedback loop in maintaining the fine equilibrium 297 of proteostasis to ensure normal response to proteotoxic stress and suggests that it is involved 298 in other biological contexts. To answer this, we performed a large-scale meta-transcriptomic 299 analysis using the recently developed Arabidopsis RNAseq data base<sup>31</sup>. Using our pipeline (see 300 methods and Supplementary Table 3) we investigated the correlation of transcript abundance 301 between the 54 genes encoding 26S proteasome subunits and the 68 PhANGs associated with 302 proteotoxic stress. We could observe that genes within the same cluster displayed a strong positive correlation ( $\rho \approx 0.6$  and  $\rho \approx 0.5$  for 26S Proteasome and PhANGs, respectively). 303 304 Strikingly, analysing the relationship between the two clusters Proteasome vs. PhANGs showed a significant negative correlation ( $\rho \approx -0.4$ ) (Fig. 6e), supporting the notion that this is a general 305 phenomenon. To investigate this in more detail, we extracted 43 projects in which 26S 306 307 Proteasome and PhANGs appear transcriptionally co-regulated (Supplementary Table 3). From

308 these 43 projects, 35 displayed an opposite regulation for 26S Proteasome and PhANGs mRNA 309 level (Extended data Fig. 6d), confirming that both clusters undergo recurrent contrasting coregulation. Amongst them pathogen attack, abiotic stress (cold, dark, drought), circadian 310 311 rhythm, nutrient stress, hormone treatment (SA, auxin) as well as treatment with phytotoxins (trans-chalcone) resulted in a negative correlation. It suggests that coordination of 312 313 PhANGs/proteasome is a general feature of plant stress responses and developmental processes. 314 Thus, the coordination of proteasome activation and PhANGs repression is a key feature of the 315 A. thaliana transcriptome in response to multiple biotic, abiotic, and developmental cues.

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#### 317 Discussion

318 Understanding how the proteasome can degrade proteins from different compartments and how 319 these different subcellular signals are integrated upon external cues and to maintain overall 320 proteostasis was a long-standing question. Our study discovered that the NAC53/78 module 321 acts as a gatekeeper to facilitate the communication between the chloroplast-proteasome-322 nucleus during proteotoxic stress (Extended Data Fig. 7). We have identified ERAPS as the key regulatory node of the proteasome autoregulatory feedback loop that shapes and maintains 323 324 subcellular proteostasis in plants. Considering this loop is mechanistically conserved across 325 kingdoms, similar control mechanisms of subcellular proteostasis are possible in yeast and in 326 animals<sup>6</sup>. Interestingly, connections between ER-anchored TFs and proteasome activation have 327 previously been observed in animals, whilst separate studies identified an indirect link between 328 proteasomal, and mitochondrial transcriptional regulation in yeast<sup>19</sup>. However, a unified 329 mechanism that explains the direct crosstalk between these seemingly disparate processes has 330 been lacking. This implies a divergence between yeast and plants on subcellular proteostasis 331 coordination.

332 Our findings highlight that subcellular proteostasis is governed by the ERAPS-dependent 333 autoregulatory pathway permitting a rapid integration of proteotoxic signals from various 334 compartments. As such, it allows the communication between different organelles such as chloroplast or mitochondria with the nucleus, a process referred as retrograde signaling<sup>32</sup>. This 335 336 communication is essential to respond to sudden changes in the environment. Retrograde signaling from mitochondria or chloroplast to the nucleus involves massive transcriptional 337 changes that in turn influence organelle function<sup>33</sup>. Some nuclear communicators have been 338 identified to mediate retrograde signaling<sup>33</sup>. However, to date, transcription factors GLK1 and 339 2, are the only known PhANGs activators<sup>34</sup>, while putative repressors ABI4 and PTM could not 340 be verified as retrograde signaling components $^{35-38}$ . Here we have identified that plants have 341

evolved a dual-regulatory system through the proteasome autoregulatory feedback loop: the NAC53/78-PRCE module acts as a novel retrograde signaling component, repressing PhANGs expression and activating proteasome genes via the same cis-element during proteotoxic stress. In this scenario, (i) repression of PhANGs by NAC53/78 may be a strategy to cope with the excess of precursor accumulation due to proteasomal stress (Extended Data Fig. 7) which is corroborated by our findings that the nac53-1 78-1 mutant is more tolerant to photosynthesis stress and HSP90 inhibition which activate NAC53/78 nuclear localization but also (ii) to counteract the overactivation of PhANGs by GLK1 that is degraded by the proteasome<sup>39</sup>. To our knowledge, a similar system has not been found in other multicellular organisms such as animals. However, findings in animals that connect proteasome activators and mitochondrial biogenesis<sup>40</sup>, strongly suggest a tight link between proteoasome and energy metabolism in animals.

Various environmental perturbations impede the balance between protein translation, sorting and degradation resulting in proteotoxicity. Mounting evidence highlights a role for the proteasome in clearing organelle-associated proteins and immune components, mediating the growth-defense trade-off. Thus, it is essential to decipher how signals from different compartments and organelles are integrated to maintain proteostasis during various stress conditions. Our findings provide a new conceptual framework for understanding the integral role of the proteasome complex as a *bona fide* signalling hub, to manage cellular proteostasis under environmental stress. This framework could be potentially universally employed across various organisms to adapt to internal and external disruptions.

#### **374 FIGURE LEGENDS**

375 Fig. 1. NAC53/78 are central integrators of various proteotoxic stress conditions.

(A) The role of the 26S Proteasome in degradation of proteins from distinct subcellular
compartments. Prime site of action of the several treatments used in this study: Blunt head
arrows indicate enzymatic activity suppression; pointed head arrows indicate trigger of
substrate accumulation.

380 (B) Fresh weight of seedlings grown under the indicated treatments at 10-14 day after

- 381 germination (dag). Fresh weight is expressed as a percentage of Col-0 mock conditions.
- Boxplots colors refer to the genotype. Statistical significance is assessed by a Wilcoxon-Mann-Whitney test (p values: n.s. > 0.05; \* < 0.05; \*\* < 0.01). Every treatment has been repeated at least twice with similar results.
- 385 (C) Bacterial density in Log10 Colony-Forming-Unit (CFU) per leaf cm<sup>2</sup>. Statistical
- 386 significance is assessed by a Wilcoxon-Mann-Whitney test (p values: n.s. > 0.05; \*\* < 0.01).
- Boxplots colors refer to the genotype as in panel B. The treatment has been repeated three timeswith consistent results.
- (B-C) Representative phenotypic pictures of Col-0 and *nac53-1* 78-1 plants under several
   treatments are included.
- 391

#### 392 Fig. 2. NAC53/78 are part of an autoregulatory proteasome feedback loop.

- (A) Confocal microscopy pictures of roots of GFP-NAC53/78 *Arabidopsis thaliana* transgenics
   at 7dag exposed to mock or BTZ 10µM treatment for 3h. Treatments were repeated at least
   three times with similar observations.
- (B) Immunoblot analysis against GFP on crude extract of seedlings related to panel A. Ponceaustaining is used as loading control.
- 398 (C) Confocal microscopy pictures of transiently co-expressed sTag-NAC53/78 with 399 prom35S::RFP-HDEL or together in *N. benthamiana* epidermis cells. Pearson index ( $\rho$ )
- 400 represents co-localization index. Scale bars =  $10\mu m$
- 401 (D) Confocal microscopy pictures of transiently co-expressed sTag-NAC53/78 together or 402 dTag-NAC53/78 alone in *N. benthamiana* epidermis cells. Scale bars =  $10\mu m$
- 403 (E) Bar plots representing the number of significantly enriched proteins in the 4 IP-MS/MS
  404 conditions for NAC53 only, NAC78 only and NAC53+NAC78 (Common).
- 405 (F) Cladogram of top 30 gene ontology (GO) terms for biological process (BP) enriched in the
  406 list of *A. thaliana* ortholog proteins found from NAC53/78 common interactome.
- 407 (G) Protein network of the interacting candidates related to the GO BP terms annotated as408 "Transport" and "Proteolysis" in panel F.
- 409

#### 410 Extended data Fig. 1. Deciphering the details of NAC53/78 interactome.

- (A) Chimeric NAC53/78 constructs generated in this study. Upstream promoter, fluorescent tag
   and protein domain of interests are represented.
- 413 (B) Immunoblot against GFP on crude extracts of *N. benthamiana* leaves transiently expressing
- 414 sTag-NACs in addition to mock or 6h BTZ  $(10\mu M)$  treatment. Ponceau staining is used as 415 loading control.
- 416 (C) Immunoblot against GFP or RFP on crude extracts of *N. benthamiana* leaves transiently
- 417 expressing dTag-NACs in addition to mock or BTZ  $10\mu$ M for 6h. Ponceau staining is used as
- 418 loading control.
- 419 (D) Immunoblot against GFP after immunoprecipitation (IP) with anti-GFP agarose beads on
- 420 *N. benthamiana* leaf extracts transiently expressing sTag-NACs after mock or  $Pst \Delta HopQ$ 421 infection for 8h. The same samples were used for subsequent MS/MS analysis.
- 421 (E) Immunoblot against GFP or RFP after IP with anti-GFP or anti-RFP agarose beads on *N*.
- 422 (E) initiation of a second of the second
- 424 subsequent MS/MS analysis.

- 425 (F) Cladogram of top 30 GO terms for cellular components enriched in the list of *A. thaliana* 426 ortholog proteins found in NAC53/78 common interactome.
- 427 (G) Subcellular compartments enrichment in the list of *A. thaliana* proteins from NAC53/78
- 428 common interactome based on their SUBA5db annotation. Statistical significance is provided
- 429 through a chi-square testing.
- (H-K) Heatmap representing the peptide enrichment (Log2FC) of the protein clusters from thenetwork Fig. 2G.
- 432

### Fig. 3. ER-Anchored Protein Sorting (ERAPS) coordinates the subcellular fate of NAC53 & NAC78.

- 435 (A) Confocal microscopy pictures of GFP-NAC53/78 expressing A. thaliana roots. 7 dag
- 436 seedlings were exposed to mock treatment, BTZ  $10\mu$ M, CB-5083  $10\mu$ M or LS-102  $100\mu$ M for
- 437 3h. The treatments were repeated at least three times with similar observations. Scale bars =  $10 \mu m$
- (B) Immunoblot against GFP on crude extracts of GFP-NAC53/78 A. thaliana adult plants
- 440 infiltrated with mock treatment, BTZ 10μM, CB-5083 10μM or LS-102 100μM for 6h. Ponceau
- 441 staining is used as loading control.
- 442 (C) Confocal microscopy pictures of N. benthamiana transiently co-expressing GFP-
- 443 NAC53/78 full-length (FL) or deleted for the transmembrane domain ( $\Delta$ TM) with RFP-HRD1a 444 or RFP-HRD1b. Pearson index ( $\rho$ ) indicates correlation index at the orange lines.
- 45 (D) Co-IP of RFP-HRD1a/b with GFP-NAC53/78. IP was performed with anti-GFP beads.
- 446 GFP-HDEL and free-GFP were used as negative control. The experiment was repeated twice 447 with similar results.
- 448 (E) Immunoblot against GFP on crude extract of *N. benthamiana* leaves transiently expressing
- 449 GFP-NAC53/78FL or  $\Delta$ TM. Ponceau was used as loading control. Boxplots represent the 450 relative  $\Delta$ TM abundance compared FL.
- 451 (F) In vitro trans-ubiquitination assays of GST-HRD1a/b against MBP-NAC53/78. Removal of
- 452 E1 and ATP from the reaction was used as negative control. The experiment was repeated twice453 with similar results.
- 454 (G) Representation of the ER-Anchored Protein Sorting (ERAPS) mechanism. NAC53/78 are
- 455 ER-anchored proteins recognized and ubiquitinated by the HRD1 complex. The CDC48
- 456 complex subsequently extracts NAC53/78 from the ER to mediate ER-Associated Degradation
- 457 or allowing nuclear translocation in the context of proteasomal stress to mitigate proteotoxicity.
- 458

#### 459 Extented data Fig. 2. NAC53/78 are subjected to high level of poly-ubiquitination.

- 460 (A) *In vitro* analysis of LS-102 effect on HRD1a/b ubiquitination activity. Removal of E1 and461 ATP from the reaction was used as negative control.
- 462 (B) Quantification of the inhibitory effect of LS-102 (100µM) on HRD1 activity. Bar plots
- represent the mean of three independent replicates with error bars representing the standarddeviation.
- 465 (C) Heatmaps representing the mean Log10 intensity of peptides bearing a di-glycine mark on
- 466 the indicated lysin residue identified by MS/MS analysis of *in vitro* trans-ubiquitination 467 reactions (see Figure 3F).
- 468 (D) Heatmaps representing the mean Log10 intensity of peptides bearing a di-glycine mark on
- the indicated lysin residue identified by MS/MS analysis of IP samples generated for Figure 2.
- 470 (E) Alignments of NAC53 and NAC78 peptide sequence regions around the identified lysin
- 471 residues in panel C and D. Colors represent the residue conservation (green: conserved, shade
- 472 of red: not conserved). All the identified lysins in one protein are conserved in the other.
- 473 Underlined residues correspond to the mutated residues generated for the panel H and I.

- 474 (F) In vitro trans-ubiquitination assays of GST-HRD1a/b against MBP-NAC53/78 in the
- 475 presence of LS-102 inhibitor. Removal of E1 and ATP from the reaction was used as negative
   476 control.
- 477 (G) Immunoblot analysis of *N. benthamiana* transiently expressing free-GFP, RFP-HDEL or
- dTag-NAC53/78 subjected to IP with anti-ubiquitin beads. The experiment was repeated twicewith similar results.
- 480 (H) Immunoblot analysis of *N. benthamiana* transiently expressing GFP-NAC53/78 mutated 481 for 5 lysing (Mut. see negal E) or not (WT) subjected to ID with anti-which in back
- for 5 lysins (Mut., see panel E) or not (WT) subjected to IP with anti- ubiquitin beads.
- 482 (I) Quantification of the relative ubiquitination levels assessed by the ratio between GFP signal
- and ubiquitin signal after IP as in panel H. Barplots represent the mean ratio of two replicates,
- anormalized to the ratio of WT constructs with error bars representing the standard deviation.
- 485

## 486 Extended data Fig. 3. NAC53/78 association with CDC48 is required for their nuclear 487 translocation.

- 488 (A) Confocal microscopy pictures of transgenic GFP-NAC53/78 root seedlings exposed to BTZ 489 10 $\mu$ M, BTZ 20 $\mu$ M, CB-5083 10 $\mu$ M or CB-5083 20 $\mu$ M for 3h. Scale bars = 10 $\mu$ m
- 490 (B) Confocal microscopy pictures of N. benthamiana leaves transiently co-expressing
- 491 AtCDC48c-GFP with RFP-NAC53/78. Co-localization is visible in aggregate-like structures.
  492 Scale bars = 1µm
- 493 (C) Confocal microscopy representative pictures (used for panel D-E) of transgenic GFP-494 NAC53/78 roots treated with BTZ 10 $\mu$ M and CB-5083 10 $\mu$ M followed by Propidium Iodide 495 staining. Scale bars = 10 $\mu$ M
- 496 (D-F) Boxplot representing the GFP signal quantification after confocal microscopy imaging
- 497 of transgenic GFP-NAC53/78 roots exposed to BTZ 10μM or CB-5083 10μM. Black dots
- 498 represent one cell. Statistical difference is assessed by a Wilcoxon-Mann-Whitney test.499

#### 500 Fig. 4. The transcriptional landscape of proteotoxic stress

- (A) Log2FC mRNA levels of *NAC53* and *NAC78* transcripts after *Pst* infection of adult Col-0
   plant leaves. The heatmap represent the mean of 4 biological replicates.
- (B) Log2FC mRNA levels of 26S proteasome transcripts after *Pst* infection of Col-0 or *nac53 1 78-1* adult plant leaves. The heatmap represent the mean of 4 biological replicates. The experiment has been repeated 3 times with consistent results.
- 506 (C) Immunoblot against multiple 26S proteasome subunits on crude extracts of Col-0 or *nac53*-
- 507 *1 78-1* adult plant leaves inoculated with *Pst* or mock solutions for 24h. Ponceau staining is used as loading control.
- 509 (D) Relative Log2FC abundance of representative 26S proteasome subunits in Col-0 or *nac53*-
- 510 1 78-1 adult plant leaves. The abundance is calculated relative to the mock conditions.
- 511 Statistical differences are assessed via a Welch t.test (p values: n.s > 0.05, \* < 0.05, \* < 0.01). (E) Immun chlot a gainst CEP on and a sutnot of *N* houth main a lagrant transiently approximately for the supression of the supression of
- 512 (E) Immunoblot against GFP on crude extract of *N. benthamiana* leaves transiently expressing
  513 GFP-NAC53/78 after mock treatment or *Pst∆HopQ* infection for 8h. Ponceau staining is used
- 514 as loading control.
- 515 (F) Immunoblot against GFP on extract of adult A. thaliana transgenics GFP-NAC53/78
- 516 subjected to mock treatment or *Pst* infection 8h by vacuum infiltration followed by IP with anti-
- 517 GFP beads.
- 518 (G) Number of differentially expressed genes (DEGs) in the different conditions of the *nac53*-
- 519 *1 78-1 Pst* RNAseq analysis. Total number of DEGs per condition is indicated. Colors indicate
- 520 the proportion of DEGs up-regulated and down-regulated. DEGs are considered when |Log2FC|
- 521 > 1.5. Genot. mock: *nac53-1* 78-1 mock vs. Col-0 mock; WT *Pst*: Col-0 *Pst* vs. Col-0 mock;
   522 Mut. *Pst*: *nac53-1* 78-1 *Pst* vs. *nac53-1* 78-1 mock; Genot. *Pst*: *nac53-1* 78-1 *Pst* vs. Col-0 *Pst*.
- 522 Iviut. *Fst. nac55-1 /o-1 Fst vs. nac55-1 /o-1* mock; Genot. *Fst: nac55-1 /o-1 Fst vs.* Col-0 *Pst.*523 (H) Level of differential expression (Log2FC) of the 945 DEGs found in Genot. *Pst* (see panel)
- 524 G) for the several conditions analyzed.

- 525 (I) Cladogram of top 40 GO terms for BP enriched in the list of 945 DEGs. Colors represent526 broad GO clusters.
- 527 (J) Venn diagram representing the overlap between the 945 DEGs and DEGs from rpt2a-2 Pma 528 (|Log2FC| > 0.5) or Col-0 MG132 24h (|Log2FC| > 0.5).
- (K) Cladogram of top 30 GO terms for BP enriched in the list of 538 DEGs extracted from the
   combined analysis (panel J). For conciseness terms are hidden and grouped in 4 processes.
- 531 (L) Protein network of the genes from the 945 DEGs list related to the GO BP terms from panel
- 532 K. Node colors refers to the 4 GO clusters from panel K.
- 533

### Extended data Fig. 4. Transcriptional analysis of proteotoxic stress reveals a trade-off between 26S proteasome and other biological process.

- (A) mRNA level Log2FC of all 26S proteasome genes and associated interactors/chaperones in
  several conditions of the *nac53-1 78-1 Pst* RNAseq.
- 538 (B) Number of differentially expressed genes (DEGs) in the different conditions of the *nac53*-
- 539 *1 78-1 Pst* RNAseq analysis. Total number of DEGs per condition is indicated. Colors indicate
- 540 the proportion of DEGs up-regulated and down-regulated. DEGs are considered when |Log2FC|
- 541 > 1.5. Genot. mock: rpt2a-2 mock vs. Col-0 mock; WT *Pma*: Col-0 *Pma* vs. Col-0 mock; Mut.
- 542 *Pma: rpt2a-2 Pma* vs. *nac53-1 78-1* mock; Genot. *Pma: rpt2a-2 Pma* vs. Col-0 *Pma*.
- 543 (C) mRNA levels Log2FC of the 981 DEGs found in Genot. *Pma* (see panel D) for the several544 conditions analyzed.
- 545 (D) mRNA level Log2FC of all 26S proteasome genes and associated interactors/chaperones in
   546 the several conditions of the *rpt2a-2 Pma* RNAseq.
- 547 (E) Cladogram of top 40 GO terms for BP enriched in the list of 945 DEGs. Colors represent548 the broad GO clusters.
- (F) mRNA level Log2FC of the genes present in the 4 network modules from Figure 4L in the3 Transcriptome used.
- 551

## Fig. 5. NAC53 and NAC78 coordinate the regulation of proteasome and photosynthesis gene expression through the same cis-element.

- (A) Top 3 motifs found by STREME software in the promoter regions from the 4 network
  clusters (Figure 4L). PRCE-like elements from proteasome and PhANGs clusters are
  highlighted.
- 557 (B) 25bp region comprising the PRCE-like elements from LHCa3 and PSAD1 promoters used
- in this figure. The region corresponds to the probe sequence used in panel C and D. The PRCEis highlighted in red.
- 560 (C) Electro-Mobility Shift Assay (EMSA) of MBP-NAC53/78 with LHCa3/PSAD1 probes (see
- 561 panel B). As a negative control, probes were incubated without MBP-NAC53/78 (-) or with
- 562 MBP-NAC53/78 deleted for their DNA recognition motif ( $\Delta$ RM). Experiment was repeated 563 twice with similar results.
- 564 (D) EMSA competition assay of MBP-NAC53/78 association with LHCa3/PSAD1 probes.
- 565 Competitor is applied at a concentration gradient (25X, 50X, 75X and 100X) indicated by the
- shade of grey. WT and mutated competitors are labelled TGGGC and AAAAA, respectively.Experiment was repeated twice with similar results.
- 568 (E) Boxplot representing association of GFP-NAC53/78 with LHCa3/PSAD1 PRCE in adult
- 569 *A. thaliana* transgenics lines infected with Pst or mock for 8h after Chromatin
- 570 Immunoprecipitation. The association is quantified as % of input and amplification of a region 571 in the CDS is used as negative control.
- 572 (F) Immunoblot against mature PhANGs proteins in Col-0 or *nac53-1* 78-1 adult plant leaves
- 573 inoculated with *Pst* or mock solutions for 24h. Ponceau staining is used as loading control.

- 574 (G) Relative Log2FC abundance of PhANGs mature proteins immunoblotted in panel F. The 575 abundance is calculated relative to the mock conditions. Statistical differences are assessed via 576 a Welch t.test (p values: n.s > 0.05, \*< 0.05, \*\* < 0.01).
- 577 (H) Photosystem II (PSII) activity measurement on Col-0 or nac53-1 78-1 adult plants
- 578 inoculated with *Pst* or mock solutions for 24h. Letters indicate the statistical group assessed by
- 579 pairwise Welch t.test (p value < 0.05). The experiment was repeated 3 times with consistent 580 results.
- 581 (I) Representative pictures of measurements from panel I. Fv/Fm values are represented by582 false color as indicated by the color gradient legend.
- 583 (J) Total chlorophylls and carotenoids from Col-0 or *nac53-1* 78-1 adult plant leaves inoculated
- with *Pst* or mock solutions for 24h. Letters indicate the statistical group assessed by pairwise
- 585 Wilcoxon-Mann-Whitney test (p value < 0.05). The experiment was repeated 3 times with 586 consistent results.
- 587

### 588 Extended data Fig. 5. Characterization of the NAC53/78-PRCE module in PhANGs 589 promoters.

- (A) mRNA level Log2FC of the PhANGs clusters from Figure 4L in the 3 transcriptomes used.
   Genes are separated based on their associated process in the photosynthetic pathway.
- (B) Mapping of the PRCE-like motifs in PhANGs promoter regions from panel A. Promoter
- regions are organized according to the order of the genes from panel A. Purple marks represent the position of the motifs based on their distance from the transcription starting site (TSS). The two red arrows highlight LHCa3 and PSAD1 promoter regions.
- 596 (C) Histograms representing the number of occurrences of the PRCE-like motifs across the 4 597 genes cluster from the network Figure 4L. Each bar represents a 20bp region, frequency f of 598 promoters bearing at least one motif is indicated as a percentage.
- promoters bearing at least one motif is indicated as a percentage.
  (D) LHCa3/PSAD1 promoter activity measured by luminescence in Col-0 or *nac53-1 78-1*protoplasts extract transiently co-expressing LHCa3/PSAD1 reporter constructs and
- 601 NAC53/78 $\Delta$ TM. Log2FC is calculated from the luminescence level of protoplasts expressing
- the reporter construct alone. Replicates are a pool of at least two independent experiments.
   Letters indicate the statistical group assessed by pairwise Wilcoxon-Mann-Whitney test (p
- 604 value < 0.05).
- (E) Promoter activity as calculated in panel D for LHCa3/PSAD1 promoters deleted for their
   respective DNA regions from Figure 5B. Replicates are a pool of at least two independent
   experiments. Letters indicate the statistical group assessed by pairwise Wilcoxon-Mann Whitney test (p value < 0.05).</li>
- 609 (F-G) Boxplot representing the Log2FC mRNA level the indicated transcripts in Col-0, GFP-
- 610 NAC53 or GFP-NAC78 seedlings treated with BTZ 10µM or mock solutions for 6h. Log2FC
- 611 is calculated from Col-0 mock. Letters indicate the statistical group assessed by pairwise
- 612 Wilcoxon-Mann-Whitney test (p value < 0.05).
- 613 (H) Fresh weight of seedlings from indicated genotypes grown under the indicated treatments
- 614 at 10 days after germination. Letters indicate the statistical group assessed by pairwise
- 615 Wilcoxon-Mann-Whitney test (p value < 0.05). Experiment was repeated 3 times with 616 consistent results.
- 617 (I) Representative phenotypic pictures related to panel D.
- 618

## Fig. 6. The proteasome autoregulatory feedback loop monitors photosynthesis homeostasis during stress responses

- 621 (A) Photosynthetic pigment content from Col-0 or *nac53-1* 78-1 adult plant leaves infiltrated
- 622 with Lin 200 $\mu$ M, BTZ 2 $\mu$ M or mock solutions for 24h. Content is expressed in percentage
- 623 relative to Col-0 mock. Letters indicate the statistical group assessed by pairwise Wilcoxon-

- 624 Mann-Whitney test (p value < 0.05). The experiment was repeated 3 times with consistent 625 results.
- 626 (B) Log2FC mRNA level of the indicated transcripts in Col-0, *nac53-1 78-1* adult plants leaves
- $Lin 200 \mu M$ , BTZ  $2\mu M$  or mock solutions for 24h. Log2FC is calculated from Col-0 mock and
- 628 *PDH1* is used as negative control. Statistical differences are assessed with a Welch t-test (p
- values: n.s. > 0.05, \* < 0.05, \*\* < 0.01). The experiment was repeated twice with consistent</li>
  results.
- 631 (C) Fresh weight of seedlings grown under the indicated treatments at 10-14 day after
- 632 germination. The fresh weight is represented as a percentage of Col-0 mock conditions. Letters
- 633 indicate the statistical groups assessed by pairwise Wilcoxon-Mann-Whitney test (p value <
- 634 0.05). Every treatment has been repeated at least twice with consistent results. Representative635 phenotypic pictures are included.
- 636 (D) Bacterial density shown as Log10 Colony-Forming-Unit (CFU) per leaf cm<sup>2</sup> in Col-0 and 637 *hrd1a hrd1b* plants. Statistical significance is assessed by a Welch t-test (p values: \* < 0.05).
- 638 The experiment has been repeated three times with consistent results.
- (E) Correlation heatmap of the 54 26S proteasome subunit genes and the expression of 68
  PhANGs from publicly available 1223 RNAseq libraries identified in this study.
- 641

### Extended data Fig. 6. The proteasome autoregulatory feedback loop transcriptional signature is a systemic response to environmental cues.

- 644 (A) PSII activity measurement on Col-0 and *nac53-1 78-1* treated as indicated in Figure 6A.
  645 Replicates are a pool of 3 independent experiments. Letters indicate the statistical group
  646 assessed by pairwise Wilcoxon-Mann-Whitney test (p value < 0.05).</li>
- (B) PSII activity measurement on Col-0 and *hrd1a hrd1b* adult plant leaves inoculated with *Pst*or mock solutions for 24h. Letters indicate the statistical group assessed by pairwise Welch
  t.test (p value < 0.05). The experiment was repeated 2 times with consistent results.</li>
- 650 (C) Confocal microscopy pictures of transgenic GFP-NAC53/78 *A. thaliana* roots exposed to 651 mock treatment, MV 100 $\mu$ M, GDA 100 $\mu$ M and Lin 100 $\mu$ M for 2h. The treatments were 652 repeated at least three times with similar observations. Scale bars = 10 $\mu$ m
- 653 (D) Density functions of the 54 26S proteasome genes and 68 PhANGs transcripts (Log2FC) 654 in the different transcriptome projects. Log2FC limits are -10 to +10. Treatment types and 655 relevant condition information are provided in the figure. Plots are separated by treatment 656 groups and subdivided by experiments.
- 657

# Extended data Fig. 7. ER-anchored protein sorting (ERAPS) controls the fate of two proteasome activators for intracellular organelle communication during proteotoxic stress.

- 661 In steady state conditions, the ER-anchored protein sorting system (ERAPS) promotes the constitutive degradation of NAC53/78 via the 26S proteasome. Meanwhile, PhANGs 662 expression is activated and subsequent chloroplastic import permits the maintenance of active 663 photosynthesis. Upon proteotoxic stress, the chloroplast import machinery is targeted for 664 proteasomal degradation. This leads to an accumulation of PhANGs precursors which are 665 therefore subjected to proteasomal degradation, inducing proteotoxicity. Thus, to avoid 666 667 proteotoxicity, the ERAPS system facilitates the nuclear translocation of NAC53/78, to activate the production of a new proteasome complex and to repress PhANGs expression to mitigate 668 substrate accumulation. 669
- 670
- 671
- 672

#### 673 Methods

#### 674 Plant materials and growth conditions

A. thaliana Columbia-0 (Col-0) ecotype was considered as WT. T-DNA insertion mutants for 675 676 NAC53 (nac53-1, SALK 009578C) and NAC78 (nac78-1, SALK 025098) were previously 677 described in Gladman et al., 2016. T-DNA insertion mutants for RPT2a was previously 678 described<sup>20</sup>. T-DNA insertion mutants for *HRD1a (hrd1a*, SALK 032914) and *HRD1b (hrd1b*, 679 SALK 061776) were obtained from Yasin Dagdas. promUBQ10::GFP-NAC53 and promUBO10::GFP-NAC78 transgenics lines were obtained by Agrobacetrium tumefaciens 680 681 floral dipping of Col-0 plants. For experiments on seedlings, seeds were surface sterilized 682 10min with 1.3% sodium hypochlorite. Seeds were sown on <sup>1</sup>/<sub>2</sub> Murashige and Skoog (MS) 683 medium plus 1% sucrose and stratified for 2 days. Plants were grown under long day conditions (light/dark cycles: 16h 22°C/8h 20°C, 130µmol.mm<sup>2</sup>.s<sup>-1</sup> light intensity, 70% relative humidity). 684 685 For experiments on adult plants, plants were grown under short day conditions (light/dark cycles: 12h 22°C/ 12h 20°C, 90µmol.mm<sup>2</sup>.s<sup>-1</sup> light intensity, 70% relative humidity). Plants 686 687 were grown 4-5 weeks until use.

688

#### 689 Nicotiana benthamiana growth

690 N. benthamiana plants were grown under long day conditions (light/dark cycles: 16h/8h, at

- 691 21°C and 70% humidity) and typically used 4 weeks after germination for confocal microscopy
  692 experiments and 5 weeks after germination for co-immunoprecipitation experiments.
- 693

#### 694 **Bacterial strains**

695 For Pseudomonas syringae pv. tomato DC3000 wild-type and Pseudomonas syringae pv. 696 maculicola strains ES4326 were grown on King's B medium containing rifampicin 100µg.mL<sup>-</sup> 697 <sup>1</sup> at 28°C. For floral dipping and transient expression in *Nicotiana benthamiana* leaves, Agrobacterium tumefaciens C58C1 strain was grown in LB broth high salt (Duchefa L1704) 698 699 containing the required antibiotics at 28°C. For molecular cloning, Escherichia coli Top10 700 strain was grown in LB broth high salt (Duchefa L1704) containing the required antibiotics at 701 37°C. For protein purification, Escherichia coli BL21 (DE3) was grown in LB broth high salt (Duchefa L1704) containing the required antibiotics at 37°C or 16°C. 702

703

#### 704 Gene Accession

Arabidopsis genome initiative (AGI) locus identifier of the principal genes investigated in this
study are the following: *NAC53* (AT3G10500), *NAC78* (AT5G04410), *HRD1a* (AT3G16090),

707 *HRD1b* (AT1G65040), *CDC48c* (AT5G03340), *RPT2a* (AT4G29040), *LHCa3* (AT1G61520),
708 *PSAD1* (AT4G02770).

709

#### 710 Molecular cloning

711 For chimeric protein constructs generated in this study, protein coding sequence (CDS) of the 712 desired genes were amplified from A. thaliana cDNA. For Golden Gate based cloning<sup>41</sup>, CDS 713 were amplified with addition of flanking regions including Bpil/BsaI sites and final constructs 714 were generated using the LI CDS module of interest and desired other modules and assembled in the LIIa F 1-2 vector. For GATEWAY<sup>™</sup> based cloning, CDS were amplified with addition 715 716 of attb1/attb2 flanking regions and final constructs were generated through BP clonase<sup>TM</sup> II 717 enzymatic reaction and LR Clonase<sup>TM</sup> II enzymatic reaction into the desired destination vectors. 718 All LI and GATEWAY<sup>TM</sup> entry clones were verified by sanger sequencing.

719

#### 720 Seedlings phenotyping

For phenotyping assay, seeds were sown on ½ MS 1% sucrose round petri dish supplemented with the indicated concentration of drug or a mock treatment. After 10 to 14 days of growth fresh weight was measured. For one replicate, 5 representative seedlings were scaled together on an analytical scale (resolution 0.0001g). Typically, 5 replicates were measured for subsequent statistical analyses.

726

#### 727 Transient expression in *N. benthamiana* leaves

Bacterial suspension was centrifuged for 4min at 4000g after overnight incubation of *A*. *tumefaciens* strain carrying the desired expression construct. Supernatant was removed and
pellet was resuspended in 500µl of agroinfiltration buffer (10mM MgCl<sub>2</sub>, 10mM MES pH 5.7).
OD600 was measured and infiltration solutions were diluted to reach final 0.5 OD600 in
agroinfiltration buffer supplemented with 200µM acetoseryringone. Solution were incubated in
dark at least 1h prior to infiltration. Infiltrated tissues were used 30h post infiltration for
subsequent experimental procedures.

735

#### 736 **Protein purification**

Recombinant proteins were expressed in E. coli BL21(DE3) by IPTG induction. Bacterial
solution was centrifuged 20min at max speed and pellet was subjected to sonication after
resuspension in 1mL MBP-buffer (20 mM TRIS-HCl pH 7.5, 200mM NaC, 1mM EDTA) or
1mL IPP50 (10 mM Tris-HCl, pH 8.0. 150mM NaCl, 0.1% NP40). Sonicated samples were

- visual result results and results of the result of the res
- for MBP-NAC53/78 or glutathione sepharose 4B (cytiva) for GST-HRD1a/b. For recombinant
- 743 His-UBA1 and His-UBC8 protein were purified using Ni-Ted resin (Macherey-Nagel).
- 744

#### 745 In vitro ubiquitination assay

In vitro ubiquitination assay was performed as described previously<sup>42</sup>. Samples were separated
by SDS–PAGE electrophoresis using 4–15% Mini-PROTEAN® TGX<sup>™</sup> Precast Protein Gels
(BioRad) followed by detection of the ubiquitinated substrate by immunoblotting using antiMBP (New England Biolabs), anti-GST and anti-ubiquitin (Santa Cruz Biotechnology)
antibodies.

751

#### 752 Co-immunoprecipitation

753 Plant tissue was homogenized in a mortar with liquid nitrogen to keep the sample frozen during 754 the process. And extracted in 1ml/g of extraction buffer (10% glycerol, 25 mM Tris pH 7.5, 1 755 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.5% Triton X, 1X protease cocktail inhibitor (Sigma)). 756 Solution was vortexed for 3min and incubated in cold room on rotator for 10min following a 757 30min centrifugation at 4000g, 4°C. Supernatant was filtered. 50µl of filtrate was sampled as 758 input, supplemented with 12.5µL laemmli buffer 4X (Biorad) and boiled for 10min at 95°C. 759 Next, 10µl/g of ChromoTek GFP-Trap® or RFP-Trap Agarose beads were added and tubes 760 were incubated 2-3h in cold room. Tubes were then centrifuge at 800g for 1min; supernatant 761 was carefully removed and pelleted beads were transferred to 1.5mL microcentrifuge tube in 762 1mL of washing buffer (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5% 763 Triton X-100, 1X protease cocktail inhibitor (Sigma)). Washing was performed by 764 centrifugation at 800g for 1min using 1mL washing buffer. After last washing, Laemmli buffer 765 2X (Biorad) was added to equal amounts of beads and sample was boiled 10min at 95°C.

766

#### 767 RNA isolation & RT-qPCR

Total RNA isolation was performed using RNeasy Plant Mini Kit (Qiagen 74904) according to
manufacturer instructions. To exclude potential contaminant DNA, RNA samples were
subjected to DNase I treatment (Thermo scientific<sup>TM</sup>) following provider instructions. For RNA
sequencing, integrity and RNA concentration was determined (2100 Bioanalyzer, Agilent). For
RT-qPCR sample analysis was performed as described previously<sup>43</sup>; cDNA synthesis was
performed using LunaScript® RT SuperMix Kit (NEB) following provider recommendation.
qPCR was performed using MESA BLUE qPCR 2X MasterMix Plus for SYBR®

Assay (Eurogentec) using a 2-step reaction protocol for 40 cycles with systematic evaluation of primer melting curve. mRNA level was quantified based on the  $\Delta\Delta$ Ct method followed by Log2 transformation.

778

#### 779 NanoLC-MS/MS analysis and data processing

780 For purification, proteins were subjected to a NuPAGE 12% gel (Invitrogen) and in-gel trypsin digestion was done on Coomassie-stained gel pieces with a modification: chloroacetamide was 781 782 used instead of iodoacetamide for carbamidomethylation of cysteine residues to avoid 783 formation of lysine modifications isobaric to two glycine residues left on ubiquitinylated lysine 784 after tryptic digestion. Next, peptides mixture were desalted using C18 Stage tips and run on an 785 Easy-nLC 1200 system coupled to a Q Exactive HF mass spectrometer (both Thermo Fisher Scientific) as described elsewhere<sup>44</sup> with some modifications: separation of the peptide 786 787 mixtures was done using a 87-min or 127-min segmented gradient from 10-33-50-90% of 788 HPLC solvent B (80% acetonitrile in 0.1% formic acid) in HPLC solvent A (0.1% formic acid) 789 at a flow rate of 200 nl/min. The seven most intense precursor ions were sequentially 790 fragmented in each scan cycle using higher energy collisional dissociation (HCD) 791 fragmentation. In all measurements, sequenced precursor masses were excluded from further selection for 30 s. The target values were  $10^5$  charges for MS/MS fragmentation and  $3 \times 10^6$ 792 793 charges for the MS scan.

- Acquired MS spectra were processed with MaxQuant software package version 1.5.2.8 with an
- integrated Andromeda search engine. For *in vivo* IP samples, database search was performed against a *N. benthamiana* database containing 74,802 protein entries<sup>45</sup>, the sequences of eGFP-
- 797 NAC53/78, mCerrulean3-NAC53/78-mCherry and 285 commonly observed contaminants.
- For *in vitro* ubiquitination samples, database search was performed against a Uniprot *E. coli*database (4,403 entries, downloaded on 7th of October 2020), the sequences of MBPNAC53/78, GST-HRD1a/b and 285 commonly observed contaminants.
- Endoprotease trypsin was defined as a protease with a maximum of two missed cleavages.
  Oxidation of methionine, phosphorylation of serine, threonine, and tyrosine, GlyGly dipeptide
  on lysine residues, and N-terminal acetylation were specified as variable modifications.
  Carbamidomethylation on cysteine was set as a fixed modification. Initial maximum allowed
  mass tolerance was set to 4.5 parts per million (ppm) for precursor ions and 20 ppm for fragment
  ions. Peptide, protein, and modification site identifications were reported at a false discovery
  rate (FDR) of 0.01, estimated by the target-decoy approach (Elias and Gygi). The iBAQ

808 (Intensity Based Absolute Quantification) and LFQ (Label-Free Quantification) algorithms
 809 were enabled, as was the "match between runs" option<sup>46</sup>.

810

#### 811 Photosynthesis monitoring

812 For photosynthetic pigment concentration, 2 leaf discs (6mm) were sampled and incubated 813 over-night in 1mL acetone 100% on a rotating wheel in cold room. Tubes were next centrifuged for 3min at 400g. 200µl of was then pipetted in a transparent 96-well plate and absorbance was 814 815 measured at 470nm, 646nm and 663nm using a plate reader (Tecan Infinite 200 PRO®). 816 Acetone 100% was used as blank. Concentration was calculated as following the obtained from 817 Karlsruher Institut für Technologie (KIT, https://www.jkip.kit.edu/molbio/998.php, 818 "Chlorophyll and Carotenoid determination in leaves"). Values were then then expressed as 819 ng.mm<sup>2</sup>.For photosynthetic efficiency, photosystem II (PSII) activity was measured by 820 quantifying the maximum photosystem II yield, Fv/Fm via the saturation pulse methods on dark 821 acclimated plants (Schreiber 2004), a Imaging-PAM chlorophyll fluorometers: Maxi version 822 v2-46i, for measurements or Dual-PAM100 (Walz GmbH, Effeltrich, Germany).

823

#### 824 Bacterial Infection

*Pst* overnight liquid culture was centrifuged for 15min at 4000g. Bacterial pellet was
resuspended in 5mL of MgCl<sub>2</sub> 10mM prior to OD600 measurement.

- 827 For qPCR, RNAseq and western blot analysis; a *Pst* solution at OD600=0.2 in 10mM MgCl<sub>2</sub> 828 was used. Adult plant leaves were syringe infiltrated with the Pst solution or a 10mM MgCl<sub>2</sub> 829 solution for 24h until sampling. For IP and Chromatin-IP on A. thaliana, Pst infiltration was prepared as explained above and detached whole rosettes of adult plants were vacuum 830 infiltrated 2 times 2 min at < 15mbar. For mock, control solution (10mM MgCl<sub>2</sub>) was used 831 832 similarly. Rosettes were put back in the growth chamber for 8h on multiple layers of wet tissue. 833 For assessment of bacterial density, Pst infiltration solution was prepared in 10mM MgCl<sub>2</sub> at an OD600 of 0.0001. Adult plant leaves were syringe infiltrated with the Pst infiltration solution 834 835 for 72h in high humidity conditions. Next, 2 leaf disks per replicates were sampled and homogenized in 200µl 10mM MgCl<sub>2</sub> and a serial dilution from 10<sup>-1</sup> to 10<sup>-6</sup> in 200µL was done. 836 For bacterial counting, 20µL of 10<sup>-5</sup> and 10<sup>-6</sup> dilutions were plated. Growing colony-forming 837
- units were counted and Log10CFU/cm<sup>2</sup> was calculated.
- 839

#### 840 Chromatin Immuno-Precipitation

For Chromatin-IP, after Arabidopsis rosettes were vacuum infiltrated for 30min with a fixation 841 842 buffer solution (1% formaldehyde, 10mM KH2PO4 pH 7, 50mM NaCl, 0.1M sucrose, 0.01% 843 Triton X-100). Plant material (1g) was dried and frozen in liquid nitrogen. Tissue was ground 844 in 5mL nuclei isolation buffer (20mM Hepes pH8, 250mM sucrose, 1 mM MgCl<sub>2</sub>, 5mM KCl, 845 40% Glycerol, 0.25% Triton X-100, 0.1mM PMSF, 0.1% 2-mercaptoethanol, 1µM BTZ). The 846 solution was filtered and subjected to 10min centrifugation at 4°C, 3000g. Pellet was resuspended in 1mL nuclei isolation buffer and centrifuged 5min, at 4°C, 3000g, two. Pellet 847 was resuspended in 200µl M3 buffer (10mM KH2PO4 pH7, 0.1mM NaCl, 10mM 2-848 849 mercaptoethanol) and centrifuged 5min, 4°C, 3000g. Nuclei pellet was resuspended in 1mL 850 sonication buffer (10mM KH2PO4 pH7, 0.1mM NaCl, 0.5% Sarkosyl, 10mM EDTA). 851 Sonication was performed with an Active Motif Sonicator (Amp 25%; Pulse: 30sec ON – 30sec 852 OFF; Timer: 300sec). Sonicated sample was centrifuged for 5min, 4°C at max speed. 853 Supernatant was transferred to a new 1 tube and 10% (v/v) Triton X-100 was added to neutralize 854 the sarkosyl. 5% (v/v) of this solution was saved as input sample. For IP, 15µL ChromoTek 855 GFP-Trap® were activates in 15µL IP buffer (50mM Hepes pH 7.5, 150mM NaCl, 5mM MgCl<sub>2</sub>, 10uM ZnSO4, 1% Triton X -100, 0.05% SDS), added to the tube and incubated 3h on 856 857 a rotating wheel at 4°C. Beads were washed 4 times by centrifuging for 30sec, at 1000g and 858 subsequent incubation on rotating wheel in 500µL buffer for 3 min; twice in IP buffer, once in 859 LNDET buffer (0.25 LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA pH 8, 10mM Tris pH 8) 860 and once in TE buffer (10mM Tris pH 8, 1mM EDTA pH 8). After the last washing step, beads 861 were centrifuged for 30sec at 1000g, resuspended in 200µL EB buffer (50mM Tris pH 8, 10mM 862 EDTA pH 8, 1% SDS) supplemented with 8µL 5M NaCl to reverse cross-linking and incubated 863 at 65°C for at least 6h. At this step input sample was added and treated similarly. For protein digestion, 1µL of Proteinase K (Thermo Scientific) was added and incubated for 1h at 45°C. 864 865 Finally, DNA was purified.

For DNA quantification, qPCR was performed using MESA BLUE qPCR 2X MasterMix Plus
for SYBR® Assay (Eurogentec) in technical triplicates. For quantification, % of input was
calculated.

869

- 870 Immunoblot analysis
- 871 For immunoblot analysis, sample processing was performed as described previously<sup>43</sup>.
- 872
- 873 Electromobility Shift Assay

For EMSA assays, complementary DNA oligos were synthesized by EuroFins Genomics with ATTO565 dye linked to the 5' end of minus strand for probes. To generate double stranded probes, complementary oligos were incubated at  $2\mu$ M in annealing buffer (25mM HEPES-KOH pH 7.8, 40mM KCl) at 70°C for 5min and cool down to room temperature.

878 EMSA reactions were performed in 20µL reaction buffer (25 mM HEPES-KOH pH 7.8, 40

mM KCl, 1 mM DTT, 10% Glycerol) using 1µg purified MBP-NAC53/78 and 50ng DNA probe

for 30min at 25°C. Reactions were subjected to native polyacrylamide migration with TGX

FastCast 7.5% Acrylamide gels (Biorad) in TAE (Tris-base 40 mM pH 8.3, 20mM acetic acid,

- 1mM EDTA). After migration, probes were imaged using a Typhoon FLA 9000 Gel Scanner.
- 883

#### 884 Luciferase reporter assay in Protoplasts

For promoter activity analysis, *Arabidopsis* mesophyll protoplasts were generated as described
 perviously<sup>27</sup>. Luciferase quantification was performed according to Dual-Luciferase® Reporter

887 Assay System from Promega recommendation (see link).

888

#### 889 Data analysis

For all statistical analysis, R programming language was used in the Rstudio environment. For non-omics data, the statistical test and associated p value threshold for each analysis are indicated in the figure legends. Data dispersion, medians and quantiles are represented with boxplots and replicates displayed in every figures. For bar plots in Extended Data Fig. 2, number of replicates are indicated in the figure legend, error bars represent the standard deviation from the mean.

896

#### 897 RNA sequencing

For *rpt2a-2 Pma* transcriptome, sequencing was performed by ATLAS Biolabs, Berlin. For *nac53-1 78-1 Pst* transcriptome, sequencing was performed by the NGS Competence Center, Tübingen. After quality control, reads were mapped to the *A. thaliana* TAIR10 reference genome. The mapped reads were counted with *htseq-count* for subsequent analysis. Log2FC and false discovery rate (FDR) were determined using R package DEseq2<sup>47</sup> with default settings. A gene is considered significantly differentially expressed when its FDR < 0.05. Log2FC threshold is indicated in the figure legends according to the analysis.

905

#### 906 IP-MS/MS analysis

For IP-MS/MS analysis, Log2FC and false discovery rate (FDR) for each protein group were determined as described previously<sup>42</sup>. For downstream analysis, to each protein group a unique Agi-code was assigned by blasting every *N. benthamiana* protein present in the protein group against *A. thaliana* proteome and taking the most frequent Agi-code with the best E-value. An E-value threshold of e-10 was used, in case of absence of *A. thaliana* the protein group was not considered for downstream analysis. For heatmap representation, in case multiple protein groups correspond to the same *A. thaliana* orthologs the mean Log2FC is represented.

914

#### 915 Meta-Transcriptomic analysis

916 For meta-transcriptomic analysis in Fig. 6 and Extended data Fig. 6, fragments per kilobase per 917 million counts were retrieved from the data base (http://ipf.sustech.edu.cn/pub/athrna/). 918 Log2FC for individual genes in every project was calculated based on the control RNAseq 919 libraries as annotated in the original publication<sup>31</sup>. Filtering of relevant projects for subsequent 920 correlation analysis was done based on the median |Log2FC|. Median|Log2FC| > 0.5 was used 921 for identification of projects with at least one gene cluster transcriptionally impacted and 922 median |Log2FC| > 0.3219 was used for identification of projects in which 26S proteasome and 923 PhANGs clusters were co-transcriptionally impacted. Pearson correlations were calculated. 924 Only correlations with p-value < 0.05 were considered significant and used for calculation of 925 mean/median.

926

# 927 Omics downstream analysis: Gene ontology, Protein Network, subcellular enrichment & 928 cis-element analysis

929 For Gene Ontology enrichment, the list of Agi-code was uploaded into ShinyGO software
930 (<u>http://bioinformatics.sdstate.edu/go/</u>). Cladogram of the indicated number of top terms for the
931 indicated type of terms was used for graphical representation. Table of annotations was
932 extracted for further analysis.

For network creation, list of Agi-code was provided to the string database (https://stringdb.org/). Table of interactions was extracted and uploaded to cytoscape software (https://cytoscape.org/) for network generation, design, and annotation. For purposes of clarity, network nodes were manually arranged. Edge transparency relate to the strength of interaction with opaque edges corresponding to strong interactions and transparent edges to weak interactions. Node color corresponds to manual annotation inferred based on GO annotations and further curated from the literature. 940 For subcellular compartment enrichment, the list of Agi-code was uploaded into SUBA5db 941 (https://suba.live/). Compartment annotation was extracted, and occurrence of every 942 compartment was counted to estimate the list distribution. For theorical distribution 20 Agi-943 code lists of the same length were randomly generated and processed similarly. The mean 944 distribution of the 20 lists was used as theorical distribution. Enrichment for every compartment 945 was calculated as a Log2FC of the ratio between the observed proportion and the theorical one.

946

#### 947 Cis-Regulatory Element analysis

For cis-regulatory element analysis, promoter sequences of 1000bp upstream of the
transcription starting site were retrieved from plant ensembl database using biomart browser
(https://plants.ensembl.org/biomart/). For identification of novel cis-regulatory elements in
Figure 5A, promoter sequences of the several gene clusters were analyzed using the STREME
software (https://meme-suite.org/meme/tools/streme) with default settings. For mapping and
counting, promoter sequences were analyzed in R.

954

#### 955 Immunoblot semi-quantitative analysis

956 Raw image of the immublot signals against the protein of interest and the corresponding loading 957 control were processed using Fiji software (<u>https://imagej.net/software/fiji/downloads</u>). Image 958 was converted to 8-bit format, background was subtracted. Individual lanes were quantified by 959 measurement of the peak area after circumscription with the rectangular ROI selection and 960 "Gels" function. The obtained values for individual bands were normalized by the 951 corresponding loading control values. Log2FC was calculated using the corresponding mock 962 sample of the same genotype.

963

#### 964 Confocal microscopy imaging

965 Confocal microscopy was done using an inverted Zeiss LSM 880 microscope, an upright Leica SP8 microscope or an inverted Leica Stellaris 8 microscope. For mCerrulean3 imaging, 966 967 excitation = 458nm and emission window = 465nm-500nm; for eGFP or sGFP imaging, 968 excitation = 488nm and emission window = 510nm-540nm; for mRFP or mCherry imaging, excitation = 561nm and emission window = 590nm- 630nm; in addition, chlorophyll auto 969 970 fluorescence was vizualized in the far-red wavelength. Images were acquired with a 40X water 971 immersion objective, pinhole set to 1 airy unit, resolution of acquisition >1024x1024 with a 972 line average of 4.

973 After acquisition images were processed in Fiji Software. For images obtained from Leica
974 microsystem devices, a gaussian blur (radius 0.75) was applied. For clarity purposes, contrast
975 on the individual channels was manually adjusted.

- 976 For subcellular quantification of GFP signal, all images used were acquired on the Zeiss LSM
- 977 880 with a 16bits depth. Prior to imaging seedlings were incubated for 10min in a 10 $\mu$ M 978 propidium iodide (PI) ½ MS solution followed by a brief washing in clear ½ MS. For PI
  - 978 propidium iodide (PI)  $\frac{1}{2}$  MS solution followed by a brief washing in clear  $\frac{1}{2}$  MS. For PI
  - 979 fluorescence, was excitation = 488nm and emission window = 590nm-660nm. Each cell and
    980 corresponding nucleus were segmented using the polygon ROI selection tool in Fiji software.
  - 981 Signal proportion in the nucleus was quantified by dividing integrated density of nucleus ROI
  - 982 by integrated density of whole cell ROI and ratio of signal between nucleus and cytosol was
  - 983 calculated dividing nucleus ROI mean intensity by cytosolic ROI mean intensity.
  - 984 For calculation of co-localization index the image J plugin "co-localization finder" was used to
  - 985 calculate pearson index or plot profile of GFP and RFP signals was extracted at the indicated
  - 986 line using Fiji software and the pearson index was calculated between the two profiles.
  - 987

#### 988 **RESOURCE AVALAIBILITY**

- Source code of R and Python3 scripts used for the several computational analysis done in this
  study will be put available on <a href="https://github.com/Gogz31">https://github.com/Gogz31</a> or can be directly requested.
- 551

#### 992 DATA AVAILABILITY

- 993 The mass spectrometry data from this publication will be made available on the PRIDE archive
  994 (https://www.ebi.ac.uk/pride/archive/) and with the identifier (PXDXXXXX; and
  995 (PXDXXXX). All relevant proteomics data are made available in the supplemental information.
- 996

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- 1012

#### **1013** Author Contribution

1014 S.Ü. conceived the project. G.L. performed most experiments, analyzed the data, and performed

all computational analysis. M.R. performed the *in vitro* ubiquitination assays. D.B performed

1016 the EMSA, assisted with the Luciferase reporter assay and the molecular cloning. D.S.

1017 generated the rpt2a-2 Pma transcriptome under the supervision of F.B..M.F.W and B.M.

1018 performed LC-MS/MS analysis. S.Ü. wrote the manuscript together with G.L..

1019

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1123		

### **Figures**



#### Figure 1

NAC53/78 are central integrators of various proteotoxic stress conditions. (A) The role of the 26S Proteasome in degradation of proteins from distinct subcellular compartments. Prime site of action of the several treatments used in this study: Blunt head arrows indicate enzymatic activity suppression; pointed head arrows indicate trigger of substrate accumulation. (B) Fresh weight of seedlings grown under the indicated treatments at 10-14 day after germination (dag). Fresh weight is expressed as a percentage of Col-0 mock conditions. Boxplots colors refer to the genotype. Statistical significance is assessed by a Wilcoxon-Mann- Whitney test (p values: n.s. > 0.05; \* < 0.05; \*\* < 0.01). Every treatment has been repeated at least twice with similar results. (C) Bacterial density in Log10 Colony-Forming-Unit (CFU) per leaf cm<sup>2</sup>. Statistical significance is assessed by a Wilcoxon-Mann-Whitney test (p values: n.s. > 0.05; \*\* < 0.01). Boxplots colors refer to the genotype as in panel B. The treatment has been repeated three times with consistent results. (B-C) Representative phenotypic pictures of Col-0 and nac53-1 78-1 plants under several treatments are included.



#### Figure 2

NAC53/78 are part of an autoregulatory proteasome feedback loop. (A) Confocal microscopy pictures of roots of GFP-NAC53/78 Arabidopsis thaliana transgenics at 7dag exposed to mock or BTZ 10µM treatment for 3h. Treatments were repeated at least three times with similar observations. (B) Immunoblot analysis against GFP on crude extract of seedlings related to panel A. Ponceau staining is used as loading control. (C) Confocal microscopy pictures of transiently co-expressed sTag-NAC53/78

with prom35S::RFP-HDEL or together in N. benthamiana epidermis cells. Pearson index ( $\rho$ ) represents co-localization index. Scale bars = 10µm (D) Confocal microscopy pictures of transiently co-expressed sTag-NAC53/78 together or dTag-NAC53/78 alone in N. benthamiana epidermis cells. Scale bars = 10µm (E) Bar plots representing the number of significantly enriched proteins in the 4 IP-MS/MS conditions for NAC53 only, NAC78 only and NAC53+NAC78 (Common). (F) Cladogram of top 30 gene ontology (GO) terms for biological process (BP) enriched in the list of A. thaliana ortholog proteins found from NAC53/78 common interactome. (G) Protein network of the interacting candidates related to the GO BP terms annotated as "Transport" and "Proteolysis" in panel F.



#### Figure 3

ER-Anchored Protein Sorting (ERAPS) coordinates the subcellular fate of NAC53 & NAC78. (A) Confocal microscopy pictures of GFP-NAC53/78 expressing A. thaliana roots. 7 dag seedlings were exposed to mock treatment, BTZ 10 $\mu$ M, CB-5083 10 $\mu$ M or LS-102 100 $\mu$ M for 3h. The treatments were repeated at least three times with similar observations. Scale bars = 10 $\mu$ m (B) Immunoblot against GFP on crude extracts of GFP-NAC53/78 A. thaliana adult plants infiltrated with mock treatment, BTZ 10 $\mu$ M, CB-5083

10μM or LS-102 100μM for 6h. Ponceau staining is used as loading control. (C) Confocal microscopy pictures of N. benthamiana transiently co-expressing GFP443 NAC53/78 full-length (FL) or deleted for the transmembrane domain (ΔTM) with RFP-HRD1a or RFP-HRD1b. Pearson index ( $\rho$ ) indicates correlation index at the orange lines. (D) Co-IP of RFP-HRD1a/b with GFP-NAC53/78. IP was performed with anti-GFP beads. GFP-HDEL and free-GFP were used as negative control. The experiment was repeated twice with similar results. (E) Immunoblot against GFP on crude extract of N. benthamiana leaves transiently expressing GFP-NAC53/78FL or  $\Delta$ TM. Ponceau was used as loading control. Boxplots represent the relative  $\Delta$ TM abundance compared FL. (F) In vitro trans-ubiquitination assays of GST-HRD1a/b against MBP-NAC53/78. Removal of E1 and ATP from the reaction was used as negative control. The experiment was repeated twice with similar results. (G) Representation of the ER-Anchored Protein Sorting (ERAPS) mechanism. NAC53/78 are ER-anchored proteins recognized and ubiquitinated by the HRD1 complex. The CDC48 complex subsequently extracts NAC53/78 from the ER to mediate ER-Associated Degradation or allowing nuclear translocation in the context of proteasomal stress to mitigate proteotoxicity.



#### Figure 4

The transcriptional landscape of proteotoxic stress (A) Log2FC mRNA levels of NAC53 and NAC78 transcripts after Pst infection of adult Col-0 plant leaves. The heatmap represent the mean of 4 biological replicates. (B) Log2FC mRNA levels of 26S proteasome transcripts after Pst infection of Col-0 or nac53- 1 78-1 adult plant leaves. The heatmap represent the mean of 4 biological replicates. The heatmap represent the mean of 4 biological replicates. The heatmap represent the mean of 4 biological replicates. The heatmap represent the mean of 4 biological replicates. The heatmap represent the mean of 4 biological replicates. The heatmap represent the mean of 4 biological replicates. The heatmap represent the mean of 4 biological replicates. The

proteasome subunits on crude extracts of Col-0 or nac53-1 78-1 adult plant leaves inoculated with Pst or mock solutions for 24h. Ponceau staining is used as loading control. (D) Relative Log2FC abundance of representative 26S proteasome subunits in Col-0 or nac53-178-1 adult plant leaves. The abundance is calculated relative to the mock conditions. Statistical differences are assessed via a Welch t.test (p values: n.s > 0.05, \* < 0.05, \*\* < 0.01). (E) Immunoblot against GFP on crude extract of N. benthamiana leaves transiently expressing GFP-NAC53/78 after mock treatment or Pst∆HopQ infection for 8h. Ponceau staining is used as loading control. (F) Immunoblot against GFP on extract of adult A. thaliana transgenics GFP-NAC53/78 subjected to mock treatment or Pst infection 8h by vacuum infiltration followed by IP with anti- GFP beads. (G) Number of differentially expressed genes (DEGs) in the different conditions of the nac53-1 78-1 Pst RNAseg analysis. Total number of DEGs per condition is indicated. Colors indicate the proportion of DEGs up-regulated and down-regulated. DEGs are considered when |Log2FC| > 1.5. Genot. mock: nac53-1 78-1 mock vs. Col-0 mock; WT Pst: Col-0 Pst vs. Col-0 mock; Mut. Pst: nac53-1 78-1 Pst vs. nac53-1 78-1 mock; Genot. Pst: nac53-1 78-1 Pst vs. Col-0 Pst. (H) Level of differential expression (Log2FC) of the 945 DEGs found in Genot. Pst (see panel G) for the several conditions analyzed. (I) Cladogram of top 40 GO terms for BP enriched in the list 525 of 945 DEGs. Colors represent broad GO clusters. (J) Venn diagram representing the overlap between the 945 DEGs and DEGs from rpt2a-2 Pma (|Log2FC| > 0.5) or Col-0 MG132 24h (|Log2FC| > 0.5). (K) Cladogram of top 30 GO terms for BP enriched in the list of 538 DEGs extracted from the combined analysis (panel J). For conciseness terms are hidden and grouped in 4 processes. (L) Protein network of the genes from the 945 DEGs list related to the GO BP terms from panel K. Node colors refers to the 4 GO clusters from panel K.



#### Figure 5

NAC53 and NAC78 coordinate the regulation of proteasome and photosynthesis gene expression through the same cis-element. (A) Top 3 motifs found by STREME software in the promoter regions from the 4 network clusters (Figure 4L). PRCE-like elements from proteasome and PhANGs clusters are highlighted. (B) 25bp region comprising the PRCE-like elements from LHCa3 and PSAD1 promoters used in this figure. The region corresponds to the probe sequence used in panel C and D. The PRCE is

highlighted in red. (C) Electro-Mobility Shift Assay (EMSA) of MBP-NAC53/78 with LHCa3/PSAD1 probes (see panel B). As a negative control, probes were incubated without MBP-NAC53/78 (-) or with MBP-NAC53/78 deleted for their DNA recognition motif ( $\Delta RM$ ). Experiment was repeated twice with similar results. (D) EMSA competition assay of MBP-NAC53/78 association with LHCa3/PSAD1 probes. Competitor is applied at a concentration gradient (25X, 50X, 75X and 100X) indicated by the shade of grey. WT and mutated competitors are labelled TGGGC and AAAAA, respectively. Experiment was repeated twice with similar results. (E) Boxplot representing association of GFP-NAC53/78 with LHCa3/PSAD1 PRCE in adult A. thaliana transgenics lines infected with Pst or mock for 8h after Chromatin Immunoprecipitation. The association is guantified as % of input and amplification of a region in the CDS is used as negative control. (F) Immunoblot against mature PhANGs proteins in Col-0 or nac53-1 78-1 adult plant leaves inoculated with Pst or mock solutions for 24h. Ponceau staining is used as loading control. (G) Relative Log2FC abundance of PhANGs mature proteins 574 immunoblotted in panel F. The abundance is calculated relative to the mock conditions. Statistical differences are assessed via a Welch t.test (p values: n.s > 0.05, \* < 0.05, \*\* < 0.01). (H) Photosystem II (PSII) activity measurement on Col-0 or nac53-1 78-1 adult plants inoculated with Pst or mock solutions for 24h. Letters indicate the statistical group assessed by pairwise Welch t.test (p value < 0.05). The experiment was repeated 3 times with consistent results. (I) Representative pictures of measurements from panel I. Fv/Fm values are represented by false color as indicated by the color gradient legend. (J) Total chlorophylls and carotenoids from Col-0 or nac53-1 78-1 adult plant leaves inoculated with Pst or mock solutions for 24h. Letters indicate the statistical group assessed by pairwise Wilcoxon-Mann-Whitney test (p value < 0.05). The experiment was repeated 3 times with consistent results.



#### Figure 6

The proteasome autoregulatory feedback loop monitors photosynthesis homeostasis during stress responses (A) Photosynthetic pigment content from Col-0 or nac53-1 78-1 adult plant leaves infiltrated with Lin 200 $\mu$ M, BTZ 2 $\mu$ M or mock solutions for 24h. Content is expressed in percentage relative to Col-0 mock. Letters indicate the statistical group assessed by pairwise Wilcoxon Mann-Whitney test (p value < 0.05). The experiment was repeated 624 3 times with consistent results. (B) Log2FC mRNA level of the indicated transcripts in Col-0, nac53-1 78-1 adult plants leaves Lin 200 $\mu$ M, BTZ 2 $\mu$ M or mock solutions for 24h. Log2FC is calculated from Col-0 mock and PDH1 is used as negative control. Statistical differences are assessed with a Welch t-test (p values: n.s. > 0.05, \* < 0.05, \*\* < 0.01). The experiment was repeated twice with consistent results. (C) Fresh weight of seedlings grown under the indicated treatments at 10-14 day after germination. The fresh weight is represented as a percentage of Col-0 mock conditions. Letters indicate the statistical groups assessed by pairwise Wilcoxon-Mann-Whitney test (p value < 0.05). Every treatment has been repeated at least twice with consistent results. Representative phenotypic pictures are included. (D) Bacterial density shown as Log10 Colony-Forming-Unit (CFU) per leaf cm<sup>2</sup> in Col-0 and hrd1a hrd1b plants. Statistical significance is assessed by a Welch t-

test (p values: \* < 0.05). The experiment has been repeated three times with consistent results. (E) Correlation heatmap of the 54 26S proteasome subunit genes and the expression of 68 PhANGs from publicly available 1223 RNAseq libraries identified in this study.

### **Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Figure2S1.png
- Figure3S2.png
- Figure3S3.png
- Figure4S4.png
- Figure5S5.png
- Figure6S6.png
- FinalModelOnOff.png
- TableS1.xlsx
- TableS2.xlsx
- TableS3.xlsx
- TableS4.xlsx