1   **Early mannitol-triggered changes in the Arabidopsis leaf (phospho)proteome**

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3   Natalia Nikonorova[1,2,*], Lisa Van den Broeck[1,2,*], Shanshuo Zhu[1,2,3,4], Brigitte van de

4   Cotte[1,2,], Marieke Dubois[1,2,$], Kris Gevaert[1,2,3,4], Dirk Inzé[1,2], and Ive De Smet[1,2,#]

5

6   [1] Ghent University, Department of Plant Biotechnology and Bioinformatics, 9052 Ghent,

7   Belgium

8   [2] VIB Center for Plant Systems Biology, 9052 Ghent, Belgium

9   [3] Ghent University, Department of Biochemistry, 9052 Ghent, Belgium

10   [4] VIB Center for Medical Biotechnology, 9052 Ghent, Belgium

11

12   [$]Present address: Institut de Biologie Moléculaire des Plantes, CNRS, 67084 Strasbourg,

13   France

14

15   [*]Equal contribution

16

17   [#]CORRESPONDING AUTHOR: Ive De Smet, VIB-UGent Center for Plant Systems

18   Biology, Technologiepark 927, 9052 Gent, Belgium, Tel: +3293313930, E-mail:

19   ivsme@psb.vib-ugent.be

20

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23

24 **ABSTRACT**

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26 Drought is one of the most detrimental environmental stresses to which plants are exposed.

27 Especially mild drought is relevant to agriculture and significantly affects plant growth and

28 development. In plant research, mannitol is often used to mimic drought stress and study the

29 underlying responses. In growing leaf tissue of plants exposed to mannitol-induced stress, a

30 highly-interconnected gene regulatory network is induced. However, early signaling and

31 associated protein phosphorylation events that likely precede part of these transcriptional

32 changes are largely unknown. Here, we performed a full proteome and phosphoproteome

33 analysis on growing leaf tissue of *Arabidopsis* plants exposed to mild mannitol-induced stress

34 and captured the fast (within the first half hour) events associated with this stress. Based on

35 this in-depth data analysis, 167 and 172 differentially regulated proteins and phosphorylated

36 sites were found back, respectively. Additionally, we identified H(+)-ATPASE 2 (AHA2)

37 and CYSTEINE-RICH REPEAT SECRETORY PROTEIN 38 (CRRSP38) as novel

38 regulators of shoot growth under osmotic stress.

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40 **HIGHLIGHT**

41 We captured early changes in the *Arabidopsis* leaf proteome and phosphoproteome upon mild

42 mannitol stress and identified AHA2 and CRRSP38 as novel regulators of shoot growth

43 under osmotic stress.

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45 **KEYWORDS**

46 Signalling, Phosphoproteome, Mild osmotic stress, *Arabidopsis thaliana,* AHA2, CRRSP38

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INTRODUCTION

Plants are exposed to a range of disadvantageous environmental conditions, which has led to the development of various molecular coping mechanisms (Wang *et al.*, 2017*a*; Berens *et al.*, 2017; Demarsy *et al.*, 2017). Drought is one of the most detrimental environmental stresses to which plants are exposed (Boyer, 1982; Wang *et al.*, 2003). Plant growth and subsequently plant yield are drastically decreased as a result of drought. In a temperate climate, water scarcity rarely threatens the survival of the plant, but rather reduces the growth and yield of the crop. Undoubtedly, the underlying molecular mechanisms differ depending on how severe the water limitation is, as plant lines that are more tolerant to severe stress rarely perform better under mild stress (Skirycz *et al.*, 2011*c*). Because mild drought is more relevant to agriculture and significantly affects plant growth and development, studies on growth responses of plants exposed to mild drought are of increasing importance (Aguirrezabal *et al.*, 2006; Pereyra-Irujo *et al.*, 2008; Clauw *et al.*, 2015). Several studies have focussed on unravelling the transcriptomic changes associated with the drought response (Harb *et al.*, 2010; Clauw *et al.*, 2015, 2016; Bac-Molenaar *et al.*, 2016; Rasheed *et al.*, 2016; Dubois *et al.*, 2017; Verslues, 2017) and a few studies even addressed drought response on a proteome or phosphoproteome level (Singh and Jwa, 2013; Katam *et al.*, 2016; Vu *et al.*, 2016).

Capturing the early response upon drought, a condition that builds up gradually, is not straightforward. Because drought is associated with osmotic stress, *in vitro* alternatives, such as mannitol, sorbitol or polyethylene glycol (PEG), are used to study the molecular events associated with this stress condition (Verslues *et al.*, 2006*a*). By transferring plants at a desired time point during development to an osmotic compound or by adding such a compound to liquid cultures, the very early signalling mechanisms associated with osmotic

74   stress can be revealed. Low concentrations of mannitol (25 mM) induce mild stress,

75   triggering a decrease in Arabidopsis rosette size of approximately 50% without affecting the

76   plant's development or survival (Claeys *et al.*, 2014). Mannitol can therefore be used as a

77   growth-repressive compound and has been shown to be ideal for studying growth-regulating

78   events (Skirycz *et al.*, 2011*c*; Van den Broeck *et al.*, 2017).

79   The osmotic stress responses in young growing leaves are very different from those in

80   mature leaves. For example, in young leaves, mild stress induces the rapid accumulation of

81   the ethylene precursor ACC (1-aminocyclopropane-1-carboxylate) and presumably also

82   ethylene itself, instead of the classic drought-related hormone abscisic acid (ABA) (Skirycz

83   *et al.*, 2011*a*). Unravelling the growth regulation upon stress is thus preferably studied in

84   growing tissue instead of mature leaves or whole seedlings, as growing tissues are more

85   subdued by growth inhibitory mechanisms. In growing leaf tissue exposed to mannitol-

86   induced stress, a highly-interconnected gene regulatory network (GRN) is induced. The

87   transcription factors that are part of this network regulate each other's expression and have

88   been shown to regulate leaf growth upon osmotic stress (Van den Broeck *et al.*, 2017). Some

89   members of this GRN, such as ETHYLENE RESPONSE FACTOR 6 (ERF6), ERF9 and

90   WRKY15, can activate the expression of *GIBBERELLIN2-OXIDASE6* (*GA2-OX6*), a gene

91   encoding a gibberellin degradation enzyme (Rieu *et al.*, 2008; Dubois *et al.*, 2013; Van den

92   Broeck *et al.*, 2017). This results in decreased levels of gibberellin and DELLA protein

93   stabilisation, which pushes the cells to permanently exit the cell division phase and into the

94   cell differentiation phase (Claeys *et al.*, 2012). The first transcriptional changes occur very

95   rapidly, after 40 minutes of stress. However, early signalling and associated phosphorylation

96   events that precede part of these transcriptional changes are largely unknown, likely because

97   most phosphoproteomic studies focused on severe, lethal stress or on whole seedlings,

98   masking the growth-specific phosphorylation events (Bhaskara *et al.*, 2017*a*).

99   While the transcriptional events orchestrating leaf growth upon mild stress have been

100   studied extensively, the early proteome and phosphoproteome changes are not yet fully

101   understood. In this study, we performed full proteome and phosphoproteome analyses on

102   growing leaf tissue exposed to mannitol-induced stress and captured the rapid (within the first

103   half hour) events associated with this stress. We demonstrate differences in proteome changes

104   in the early (30 min) and later (4 h) mannitol-triggered proteome, such as the translational

105   machinery and oxidation-reduction processes. Next, we evaluated the phoshoproteome and

106   found several connections with the GA–DELLA pathway, which form interesting candidates

107   for follow-up studies. We compared four phosphoproteome datasets after mild and severe

108   stress highlighting their distinct signalling pathways. Finally, through validation of some

109   candidates for a growth phenotype upon mild mannitol treatment, we identified H(+)-

110   ATPASE 2 (AHA2) and CYSTEINE-RICH REPEAT SECRETORY PROTEIN 38

111   (CRRP38) as novel regulators of osmotic stress signalling and response.

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113   **MATERIAL AND METHODS**

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115   **Plant material and growth conditions**

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117   Wild-type plants (Col-0) were grown *in vitro* at 21°C under a 16-h-day (110 mmol/(m^{2}s)) and

118   8-h-night regime. 64 wild-type seeds were sown on a 14-cm-diameter Petri dish with solid ½

119   MS medium (Murashige and Skoog, 1962; 6.5 g/L agar, Sigma), overlaid with a nylon mesh

120   (Prosep) of 20-μm pore size. During growth, plates were randomized. For the short-term (30

121   min mannitol stress) proteome and phosphoproteome analyses, half of the plants were

122   transferred to ½ MS medium containing 25 mM mannitol (Sigma) at 15 days after

123   stratification (DAS). The third leaf was harvested after 30 min. The other half of the plants

124 was not transferred, and the third leaf was harvested before transfer (time point 0). In total, 4

125 biological repeats were performed. For the proteomic experiment after 4 h of mannitol stress

126 and the expression analysis, half of the plants were transferred to control ½ MS medium, the

127 other half to ½ MS medium containing 25 mM mannitol at 15 DAS. The third leaf was

128 harvested 20 min and 40 min (for expression analysis) or 4 h (for proteomics) after transfer.

129 In total 3 biological replicates were performed, and approximately 100 mg leaf material was

130 harvested per sample. All experiments were performed independently.

131

132 **qPCR analyses**

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134 Samples were immediately frozen in liquid nitrogen and ground with a Retsch machine and

135 3-mm metal beads. Subsequently, RNA was extracted with TriZol (Invitrogen) and further

136 purified with the RNeasy plant mini kit (Qiagen). For cDNA synthesis, the iScript

137 cDNASynthesis Kit (Bio-Rad) was used with 1 µg of RNA as starting material. qRT-PCR

138 was performed with the LightCycler 480 Real-Time SYBR Green PCR System (Roche). The

139 data were normalised against the average of housekeeping genes AT1G13320 and

140 AT2G28390 (Czechowski, 2005), as follows: dCt = Ct (gene) – Ct (average [housekeeping

141 genes]) and ddCt = dCt (Control) – dCt (Treatment). Ct represents the number of cycles at

142 which the SYBR Green fluorescence reached a threshold during the exponential phase of

143 amplification. Primers were designed with Primer-BLAST

144 (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (**Supplementary Information**).

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146 **(Phospho)proteome workflow**

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148   Plant material was flash-frozen in liquid nitrogen and ground into a fine powder. Subsequent

149   proteome and phosphoproteome analyses were performed as previously described (Vu *et al.*,

150   2016; Nikonorova *et al.*, 2018). For details, we refer to the **Supplementary Information**.

151   LC-MS/MS analysis was performed as previously described (Vu *et al.*, 2016). Both the

152   proteome and phosphoproteome samples were analysed using 3 h gradients on a quadrupole

153   Orbitrap instrument (Q Exactive).

154   MS/MS spectra were searched against the Arabidopsis proteome database (TAIR10,

155   containing 35,386 entries; http://www.arabidopsis.org/) using the MaxQuant software

156   (version 1.5.4.1). Details on settings can be found in **Supplementary Information**. All MS

157   proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE

158   partner repository with the data set identifier PXD008900. For the quantitative proteome and

159   phosphoproteome analyses, the "ProteinGroups" and 'Phospho(STY)sites' output files,

160   respectively, generated by the MaxQuant search were loaded into Perseus software. For

161   phosphoproteome data only high-confidence hits with phosphorylation localisation

162   probabillity > 0.75 were included in analysis. Data analysis was performed as described

163   previously (Vu *et al.*, 2016), and modifications are added in the main text.

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165   ***In silico analyses, data visualisation and statistics***

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167   To generate networks for known and predicted protein-protein interactions, the datasets were

168   loaded to the STRING database (https://string-db.org; version 10.5) using high confidence

169   interaction score (> 0.7). As active interaction sources text mining, experiments, databases,

170   co-expression, neighbourhood, gene fusion and co-occurrence were selected.

171   (Phospho)proteins that did not have any interaction were removed from the network.

172   Biological Process terms were retrieved from the TAIR portal (www.arabidopsis.org, Bulk

173   Data Retrieval). Further data visualisation was performed in Cytoscape (version 3.5.1) on the

174   extracted interaction network and GO annotations. GO-enrichment analysis was performed in

175   PLAZA 4.0 workbench using entire species as background model and 0.05 as a p-value cut-

176   off. The HMMER web server was used for the prediction of kinase domains. For Motif-X

177   analyses (Chou and Schwartz, 2011), the sequences (limited to 13 amino acids) of up- and

178   downregulated phosphosites were pre-aligned with the phosphosite centered. The IPI

179   Arabidopsis Proteome was used as the background database. The occurrence threshold was

180   set at the minimum of 5 peptides, and the p-value threshold was set at $<10^{-6}$. Venn diagrams

181   were created using the Venny 2.1 online tool (http://bioinfogp.cnb.csic.es/tools/venny).

182   Barcharts, boxplots and statistical analyses for qPCR and phenotyping data were performed

183   using R software (https://www.R-project.org) and all figures were generated with Inkscape or

184   Photoshop. Details on ANOVA results can be found in **Supplementary Information**.

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186   **Genotyping**

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188   The *crrsp38-1* (SALK_151902) and *aha2-4* (SALK_082786) mutant *Arabidopsis* plants were

189   obtained from Nottingham Arabidopsis Stock Centre (NASC). The homozygous *Arabidopsis*

190   mutants were identified by PCR using primers specific to the insertion T-DNA

191   (**Supplementary Information**) and the LB primer (ATTTTGCCGATTTCGGAAC). The

192   SALK lines used are in Col-0 background.

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194   **Leaf growth phenotyping**

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196   Both wild-type (Col-0) and mutant plants were grown *in vitro* on 14-cm-diameter Petri dishes

197   at 21°C under a 16-h-day (110 mmol/(m^{2}s)) and 8-h-night regime. The mutant line was

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198   grown together with the appropriate control on one plate to correct for plate effects. For each

199   condition (MS or mannitol), 4-6 plates with 8 wild-type and 8 mutant seeds per plate were

200   sown. Half of the plants were grown on solid (9 g/L agar, Sigma) ½ MS control medium and

201   the other half on solid ½ MS medium with the addition of 25 mM D-mannitol (Sigma). In

202   total, 1 and 2 independent experiments were performed for *aha2-4* and *crrsp38*, respectively.

203   The plates were photographed at 22 DAS and all images were analysed using ImageJ

204   (Schindelin *et al.*, 2015) to measure the projected rosette area.

205

206   **RESULTS AND DISCUSSION**

207

208   *Proteome and phosphoproteome profiling to unravel the early mannitol response*

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210   To gain insight in the early molecular changes associated with mannitol-triggered osmotic

211   response, we focused on changes in the proteome and phosphoproteome in expanding leaf

212   tissue of *Arabidopsis thaliana*. We opted for low concentrations of the osmoticum mannitol

213   (25 mM), which enabled a mild stress that does not affect plant survival but solely represses

214   growth (Skirycz *et al.*, 2011*a*; Claeys *et al.*, 2014). Specifically, *A. thaliana* seedlings at 15

215   days after stratification (DAS) were transferred to ½ MS medium containing 25 mM mannitol

216   and after 30 min, the third expanding leaf was harvested in 4 biological repeats. We chose the

217   30 min time point because this time point coincides with the earliest changes observed in the

218   transcriptome data on mild osmotic stress in growing leaves (Van den Broeck *et al.*, 2017).

219   Next, proteins were extracted and used for two parallel analyses: (i) the total proteome,

220   enabling us to identify key proteins responding to mannitol, and (ii) the phosphoproteome,

221   allowing us to gain insights into the early phosphorylation events. The proteome analysis of

222   control and mannitol-treated samples resulted in total in the identification of 2932 protein

223   groups (a protein group includes proteins that cannot be unambiguously identified by unique

224   peptides but have only shared peptides) (**Figure 1 and Supplementary Table S1**). The

225   phosphoproteome analysis led to the identification of 3698 phosphorylated peptides that

226   could be mapped on 1466 proteins (**Figure 1 and Supplementary Table S2**). In this list of

227   identified phosphopeptides, the contributions of pS, pT, and pY were 82.2%, 17.0%, and

228   0.8%, respectively (**Figure 1**). To address if the early mannitol-triggered leaf proteome is

229   significantly different from a more long-term exposure, we also performed a 4 h mannitol

230   treatment. This time point was chosen as it is 2 h later than the maximum in expression of

231   several key transcription factors that were previously identified (Van den Broeck *et al.*,

232   2017). The third expanding leaf was harvested in 3 biological replicates and led to

233   identification of 2633 proteins (**Figure 2 and Supplementary Table S3**).

234

235   ***Data filtering approach to identify relevant candidates for further studies***

236

237   One of the challenges in quantitative proteomics is dealing with missing data (Karpievitch *et*

238   *al.*, 2012; Lazar *et al.*, 2016), which cannot be processed using standard regression methods

239   (e.g., t-test and ANOVA). To overcome this, missing values can be imputed or ignored

240   (Karpievitch *et al.*, 2012). However, this can lead to a misinterpretation of results and, more

241   importantly, to the loss of potentially interesting proteins or phosphosites for further studies

242   (Lazar *et al.*, 2016). A protein or a phosphorylated peptide that is present in a treated sample

243   and absent in the control conditions ("one-state" or "unique" proteins or phosphopeptides,

244   hereafter referred to as unique) and vice versa could be of great biological interest.

245   To date, post-Mass Spectrometry (MS) data analysis remains a highly debated field

246   with numerous imputation techniques and sophisticated statistical approaches that aim to deal

247   with a complete dataset with missing values (Koopmans *et al.*, 2014; Lazar *et al.*, 2016;

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248 Wang *et al.*, 2017*b*). Here, to overcome the problem of missing values without imputation or

249 complex statistical analysis, we applied a hybrid approach for data analysis that treats

250 intensity-based and presence/absence data separately (**Figures 1 and 2 and Supplementary**

251 **Figure 1**). The original, complete dataset containing *log2*-transformed intensities was split in

252 three subsets (**Supplementary Figure 1; see Supplementary Information for details**).

253 Dividing the dataset in three subsets allowed us to minimise the number of missing values in

254 the input for regression analysis (subset 1), eliminate unreliable detections or quantifications

255 (subset 2) and include unique proteins or phosphopeptides (subset 3) (**Figures 1 and 2,**

256 **Supplementary Figure 1 and Supplementary Tables S1-3**). The follow-up statistical

257 workflow was performed as described previously (Vu *et al.*, 2016). In brief, the dataset was

258 centred around zero by a subtraction of the medium within each replicate and subjected to a

259 two-sample t-test ($p < 0.05$) (**Figures 1 and 2**).

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261 *Early (30 min) mannitol-triggered effects on the leaf proteome*

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263 For the proteome analysis, the above-described approach resulted in 18 and 21 unique

264 proteins detected only in the control and mannitol-treated sample, respectively (**Figure 1 and**

265 **Supplementary Table S1**). In addition, statistical analysis determined 128 differentially

266 abundant proteins: 27 higher and 101 that were found lower in abundance upon mannitol

267 treatment compared to the control (**Figure 1 and Supplementary Table S1**). To simplify

268 data characterisation further, we arranged proteins in two groups: upregulated (proteins

269 detected only in mannitol-treated samples and significantly more abundant upon mannitol

270 treatment) and downregulated (proteins detected only in control samples and significantly

271 more abundant in control conditions).

272   Using the PLAZA 4.0 platform, GO enrichment analysis was conducted on the dataset

273   in the context of biological process (**Supplementary Table S4**). GO enrichment on

274   biological processes showed that differentially regulated proteins (including unique ones)

275   were involved in various processes, such as response to stress and abiotic stimulus.

276   To better understand the relationships between the 167 up- and down-regulated

277   proteins, we constructed a protein-protein interaction network consisting of 105 interacting

278   proteins with 242 (potential) interactions (see Material and Methods for details). To get

279   insight into the early biological processes affected by mild osmotic stress, GO annotations of

280   up- and down-regulated proteins were superimposed on the network and nodes were grouped

281   accordingly (**Figure 3**). This approach revealed that most interacting proteins were involved

282   in protein metabolism, specifically in amino acid biosynthesis, translation and ribosome

283   biogenesis, protein folding, intracellular protein transport and ubiquitin-dependent protein

284   degradation.

285   In a first large group of differentially abundant interacting proteins, we observed that

286   ribosomal proteins were highly regulated upon mannitol exposure, suggesting an altered

287   capacity for protein translation. Six ribosomal proteins were unique for the mannitol

288   treatment and two were upregulated. This is in line with the observation of long-term

289   proteome changes in growing Arabidopsis leaves subjected to mannitol, where the levels of

290   ribosomal and translational proteins were also found highly regulated, both up and

291   downregulated (Skirycz *et al.*, 2011*b*).

292   A second large group of proteins in our network was involved in oxidation-reduction

293   processes and biotic and abiotic stress responses. CAT1 was detected as a unique protein for

294   the mannitol-treated samples, most likely protecting plant cells against toxic effects of ROS

295   produced upon osmotic stress. Surprisingly, other reduction-oxidation protein family

296   members (2 glutathion peroxidases, 2 ascorbate peroxidases and 9 thioredoxines, 1

297   periredoxin, 1 glutaredoxin and 1 dehydro-ascorbate reductase) appeared to be

298   downregulated. This is in contrast with the long-term proteome changes in growing leaf

299   tissue (Skirycz *et al.*, 2011*b*) and not expected because peroxidases have been widely

300   described as ROS scavengers and are involved in the ROS damage repair (Kapoor and

301   Sveenivasan, 1988; Caverzan *et al.*, 2012; Bela *et al.*, 2015). ROS are known to play a dual

302   role: being toxic and destructive molecules, but, they can also serve as signalling molecules

303   regulating stress responses, growth and development (Kovtun *et al.*, 2000; Foyer and Noctor,

304   2005; Vanderauwera *et al.*, 2005; Gadjev *et al.*, 2006; Pitzschke and Hirt, 2006; Brown and

305   Griendling, 2009; Hossain *et al.*, 2015). The downregulation of these scavenger proteins

306   might be a result of the lack of ROS production during the very early stages of mild osmotic

307   stress or because these enzymes are a source of hydrogen peroxide (H_2O_2) (Blokhina *et al.*,

308   2003). The first hypothesis is in line with the observation that H_2O_2 levels do not accumulate

309   within the first 10 min upon a hyperosmotic treatment and even decrease to levels lower than

310   the control at later time points in Arabidopsis cell cultures (Beffagna *et al.*, 2005). On a

311   transcript level, glutathione peroxidases have already been shown to be downregulated in rice

312   upon drought stress (Passaia *et al.*, 2013). Deficiency in some ROS scavengers led to an

313   increase in the plant's sensitivity to oxidative stress while exposure to osmotic and salt

314   stresses resulted in an increased tolerance (Miller *et al.*, 2007).

315   The third largest cluster in the network consisted of proteins related to photosynthesis

316   and carbohydrate metabolism. Members of this group were mainly downregulated under

317   mannitol stress except for a phosphoenolpyruvate carboxylase family protein (PEPC,

318   AT1G77060). This is in agreement with previous studies where it was shown that abiotic

319   stresses can induce *PEPC* gene expression in wheat, Arabidopsis and sorghum (Echevarría *et*

320   *al.*, 2001; González *et al.*, 2003; García-Mauriño *et al.*, 2003; Sánchez *et al.*, 2006).

321   Moreover, recent studies demonstrated that the maize *PEPC* gene was able to confer drought

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322   tolerance and increase grain yield in transgenic wheat plants (Qin *et al.*, 2016).

323   Photosynthesis-related proteins, such as PHOTOSYSTEM II SUBUNIT Q and

324   PHOTOSYSTEM II SUBUNIT P, were downregulated upon mannitol-induced stress, as

325   were many chloroplast-located proteins (18 of the 121) (Lawlor and Tezara, 2009). This was

326   expected as the photosynthetic electron transfer chain can produce ROS species

327   (Ramachandra Reddy *et al.*, 2004).

328   Another interesting protein identified as unique for mannitol-treated samples was

329   SUPER SENSITIVE TO ABA AND DROUGHT 2 (SAD2)/ ENHANCED MIRNA

330   ACTIVITY 1 (EMA1) (**Supplementary Table S1**), an importin protein that regulates nuclear

331   transport and is involved in the regulation of miRNA's (Verslues *et al.*, 2006*b*; Wang *et al.*,

332   2011). *SAD2/EMA1* is transcriptionally not induced by ABA and stress treatments, but

333   mutant lines show a hypersensitivity to ABA treatment and salt stress (Verslues *et al.*,

334   2006*b*).

335   To conclude, our results point towards a downregulation of photosynthesis and

336   upregulation of CAT1 probably for ROS scavenging, both systems potentially reduce ROS

337   production. In addition, our results suggest an unexpected new role for peroxidases in the

338   early mannitol response as these were downregulated.

339

340   ***Lack of correlation between early mannitol-triggered transcript and protein fold changes***

341

342   Because some proteins are mainly regulated at post-transcriptional level, such as the above-

343   mentioned EMA1, we evaluated to what extent changes in transcript abundance for a

344   selection of 14 genes correlated with protein levels. For this, expanding leaf tissue (leaf 3, 15

345   days after sowing) was harvested 20 and 40 min after mild mannitol treatment. We included

346   the genes with the highest change in protein abundance and some of the unique proteins.

347   Surprisingly, we found almost no obvious changes after 20 and 40 min of mannitol treatment

348   at the transcriptional level for the genes (**Figure 4**). This suggested that changes in protein

349   levels at 30 min were not caused by changes in transcript abundance, but more likely due to

350   differences in protein degradation or stabilisation. We concluded that even though the

351   transcript level was tested for only a subset of proteins, the transcriptome poorly reflects the

352   proteome, which is in agreement with other studies (Jogaiah *et al.*, 2013; Bai *et al.*, 2015;

353   Walley *et al.*, 2016).

354

355   ***Effects of four hours mannitol treatment on the leaf proteome***

356

357   To get an idea on changes in protein abundance upon more prolonged mild osmotic stress, we

358   also performed a proteome analysis of growing leaf tissue after 4 hours of mannitol

359   treatment. The above described data analysis workflow led to the identification of 15 unique

360   proteins (based on absence or presence in 3 out of 3 biological replicates), 14 for the control

361   and one for the mannitol-treated sample. Together with the statistical analysis, this resulted in

362   83 differentially abundant proteins (49 more abundant and 34 less abundant upon stress)

363   (**Figure 2 and Supplementary Table S3**). GO enrichment on biological processes showed

364   that differentially regulated proteins (including unique ones) were, among several others,

365   involved in response to stress and to abiotic stimulus (**Supplementary Table S4**). A protein-

366   protein interaction network was built for up- and downregulated proteins (see Material and

367   Methods for details), and similar to 30 min mannitol treatment, the vast majority of

368   interacting proteins was involved in translation and ribosome biogenesis (**Figure 5**).

369   To unravel dynamic changes of proteins that were up- and downregulated at 30 min,

370   we traced these proteins in the 4 h mannitol dataset. Despite that we cannot compare the

371   exact values from both experiments, as they were processed separately, we can make some

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372   assumptions based on the protein fold changes. Thus, 167 up- and downregulated proteins of

373   the 30 min dataset were mapped on the total 4 h proteome data and 120 proteins were

374   retained (**Figure 6 and Supplementary Table S5**). However, only 6 of these proteins were

375   significantly differentially abundant after 4 h (**Supplementary Table S3**). Of the 120

376   mapped proteins 59 proteins showed the same trend at 30 min and 4 h after mannitol

377   treatment of which 16 proteins remained upregulated and 43 proteins downregulated. This

378   group of "stable" proteins contained various reduction-oxidation proteins, such as 5

379   thioredoxins, 2 peroxiredoxins, 1 ascorbate peroxidase, 1 glutathione peroxidase, 1

380   superoxide dismutase (SOD1) and 1 catalase (CAT1). Another group of proteins, consisting

381   of 11 proteins, was upregulated at 30 minutes and downregulated after 4 h of mannitol

382   treatment. The group of proteins that were downregulated at 30 minutes and became

383   upregulated after 4 h of mannitol treatment included 50 proteins with diverse functions.

384   Overall, these observations suggested a dynamic control of protein levels during mild

385   osmotic stress response.

386

387   *Early (30 min) mannitol-triggered effects on the leaf phosphoproteome*

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389   In the phosphoproteome analysis, 96 unique phosphorylated peptides were detected – 56 and

390   40 for control and mannitol-treated samples, respectively (**Figure 1 and Supplementary**

391   **Table S2**). The statistical workflow resulted in a list of 76 differentially abundant

392   phosphopeptides: 40 higher and 36 lower in abundance upon mannitol treatment compared to

393   the control (**Figure 1 and Supplementary Table S2**). Similar as for the proteome data, we

394   combined unique and differentially abundant phosphopeptides in two sets of upregulated and

395   downregulated proteins and subjected them to further analyses.

396 GO analysis of biological process terms revealed that proteins with differentially

397 regulated phosphosites under mild osmotic stress (including unique phosphosites) were

398 involved in several processes, including (protein) phosphorylation, regulation of cellular

399 response to stress, response to abscisic acid and mannitol metabolic process (**Supplementary**

400 **Table S4.**).

401 To unravel possible interactions between the proteins that were differentially

402 phosphorylated and unique, we constructed, as for the proteome datasets, a protein-protein

403 interaction network consisting of 44 interacting phosphoproteins with 42 (potential)

404 interactions combined with GO categorisation for biological processes (see Material and

405 Methods for details). This approach revealed that interacting phosphoproteins were mainly

406 involved in photosynthesis, splicing, chromatin remodelling and transport (**Figure 7**). In

407 general, this network analysis showed that 27% of the detected phosphoproteins are part of a

408 subnetwork, suggesting that upon a stimulus, more than one protein of the subnetwork is

409 differentially phosphorylated, potentially affecting its activity. However, it should be noted

410 that we cannot pinpoint a role for the identified change in phospho-status in relation to the

411 protein function or activity.

412 Upon mild osmotic stress, the DELLA proteins play an important role in the growth

413 inhibition in leaves upon mild osmotic stress (Claeys *et al.*, 2012). GA binds to its receptor

414 GA INSENSITIVE DWARF1 (GID1) (Ueguchi-Tanaka *et al.*, 2005) and forms a complex

415 with DELLA proteins, leading to its degradation (Nakajima *et al.*, 2006). Under mild stress,

416 the gene encoding a GA-degradation enzyme, *GA2-OX6*, is upregulated by several mannitol-

417 responsive transcription factors (Van den Broeck *et al.*, 2017), which leads to a decrease in

418 GA levels and subsequently to the stabilisation of DELLA proteins. Such stabilisation of

419 DELLA proteins leads to changes in the activity of transcription factors and expression of

420 GA-regulated genes. In our phosphoproteomic dataset, we found a few differentially

17

421   phosphorylated proteins that are linked to DELLAs. For example, bZIP16 (AT2G35530;

422   Ser[152], >1.5-fold downregulated) (**Table 1**), a transcriptional repressor, has been shown to

423   directly repress *REPRESSOR OF GA-LIKE 2* (*RGL2*), encoding a DELLA protein (Hsieh *et*

424   *al.*, 2012). We thus hypothesize that a decrease in phosphorylation of bZIP16 might decrease

425   its activity and its repression on *RGL2*. Another example is the CALCIUM-DEPENDENT

426   PROTEIN KINASE (CDPK/CPK)-RELATED PROTEIN KINASE 2 (TAGK2/CRK2)

427   (AT3G19100), for which a phospho-site at Ser[57] was upregulated 1.6-fold upon mannitol

428   treatment (**Table 1**). Recently it was shown that TAGK2/CRK2 phosphorylates the GA

429   RECEPTOR RING E3 UBIQUITIN LIGASE (GARU) at Tyr[321]which results in the

430   disruption of the interaction between GARU and the GA RECEPTOR GA INSENSTIVE

431   DWARF1 (GID1). Because this interaction is disrupted, GARU is not able to induce the

432   degradation of GID1. *TAGK2*-overexpressing plants thus show an increased GID1

433   stabilisation and DELLA degradation (Nemoto *et al.*, 2017). However, the role of

434   phosphorylation associated with TAGK2 has not yet been studied.

435   Considering the interest in early signalling cascades underlying responses to mild

436   osmotic stress, we focused on proteins with up- and downregulated phosphosites possessing

437   protein kinase activity. Using the HMMER online tool (Finn *et al.*, 2015), we found 7 and 10

438   (potential) protein kinases of which phosphosites were up- and downregulated, respectively

439   (**Supplementary Table S6**). Upregulation of phosphorylation sites of a kinase can indicate

440   an activation of the kinase itself and, as a consequence, its downstream signaling cascades;

441   and dephosphorylation can imply the opposite (Wang *et al.*, 2007; Tarrant and Cole, 2009;

442   Day *et al.*, 2016). Next, we looked into predicted overrepresented kinase motifs as the

443   sequence consensus of phosphopeptide motifs reflects the kinase-specific regulation of

444   substrate phosphorylation and the identity of the corresponding kinases (**Supplementary**

445   **Figure S2**). This revealed that for the upregulated phosphopetides, the protein motifs [SP]

18

446   and [TP] were the most enriched motifs. Peptides containing the proline (P)-directed [SP] and

447   [TP] motifs are suggested to be targets of MAP-kinases, SnRK2, RLKs, CDPKs, CDKs,

448   AGC family protein kinases and STE20-like kinases (van Wijk *et al.*, 2014). For the

449   downregulated phosphopeptides, [SP] and [RxxS] motifs were overrepresented, of which the

450   [RxxS] motif is recognized by MAP kinases.

451   Some examples of the identified kinases that have a changed phospho-status upon

452   mild mannitol stress are CALCINEURIN B-LIKE PROTEIN (CBL) - INTERACTING

453   PROTEIN KINASE 8 (CIPK8, AT4G24400), MAP kinase kinase kinase 7 (MKKK7,

454   AT3G13530), brassinosteroid-signalling kinase 1 (BSK1, AT4G35230) and SnRK2.4

455   (AT1G10940) (**Table 1**). CIPKs are involved in calcium signalling cascades and are found to

456   be induced in response to stress (Hu *et al.*, 2009; Pandey *et al.*, 2014). Mitogen activated

457   protein kinase (MAPK) cascades are known to be involved in drought stress (Ichimura *et al.*,

458   2000) and in PAMP signalling (Asai *et al.*, 2002; Pitzschke *et al.*, 2009). The detected

459   MKKK7 was demonstrated as a negative regulator of flg22-triggered signalling and basal

460   immunity (Mithoe *et al.*, 2016), but could have other functions as well. BSK1 is a target of

461   the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) and plays a role

462   in the brassinosteroid signalling during plant immunity (Shi *et al.*, 2013). SnRK2.4 is a well-

463   known regulator of the salt stress response in plants. According to recent findings, SnRK2.4

464   belongs to the SnRK2 group 1 of which its members are not activated by abscisic acid (ABA)

465   (Kulik *et al.*, 2011). The importance of phosphorylation for SnRK2.4 activity was previously

466   demonstrated upon salt stress and the abolishment of SnRK2.4 activity in mutant lines led to

467   an increased sensitivity to salt (Krzywińska *et al.*, 2016). The first phase of salt stress is

468   osmotic stress, strengthening a role for SnRK2.4 in this process (Shavrukov, 2013). However,

469   our analysis showed a different mannitol-regulated phosphorylation site than those previously

470   described (Kline *et al.*, 2010), namely Ser[357]. The Ser[357] residue was dephosphorylated upon

471   mannitol treatment, and is located in a protein-protein interaction motif outside the activation

472   loop of the kinase that has not been linked to osmotic stress responses (Kulik *et al.*, 2011).

473

474   *A normalised early mannitol-triggered differential leaf phosphoproteome*

475

476   A common question related to quantitative phosphoproteomics is whether the measured

477   phosphorylation changes result from changes in kinase or phosphatase activity or from

478   changes in phosphoprotein abundance. In the context of quantitative phosphoproteomics it is

479   important to correct – to the extent possible – the measured phosphorylation changes with

480   respect to changes in protein abundance (Vu *et al.*, 2016). Taking into account that

481   phosphopeptide abundance is directly depending on protein abundance, we normalised

482   phosphosite intensities. It should be noted that this analysis is only possible for a subset of

483   identified phosphosites because as a result of the enrichment for phosphorylated proteins

484   during the phosphoproteome analysis, most of them are not detected in the whole proteome

485   where no enrichment was performed. Furthermore, it should be mentioned that this

486   normalised phosphoproteome data set does not imply that the non-normalised

487   phosphopeptides are not interesting to investigate; merely that these changes could not be

488   corrected for protein abundance. 172 up- and downregulated phosphorylated peptides derived

489   from 158 phosphoproteins were mapped on the total proteome data and 32 proteins were

490   found to be overlapping. Next, the log_2 fold change of the protein was subtracted from the

491   log_2 fold change of the phosphosite. This defined a set of phosphorylation events that are

492   fully due to changes in kinase and phosphatase activity and not due to changes in protein

493   abundance (to the extent these changes in phosphorylation status do not impact on protein

494   abundance) (**Figure 8 and Supplementary Table S7**). Sixteen phosphosites could not be

495   normalised as they belonged to a group of unique phosphosites and did not have a fold

20

496 change value. Within the normalised phosphopeptides, two phosphosites of a remorin family

497 member REM1.3 (AT2G45820), Thr[58] and Ser[64], were down regulated upon mannitol

498 treatment (**Figure 8 and Table 1**). REM1.3 was already reported to be phosphorylated upon

499 oligo-galacturonide treatment which elucidates a plant stress response (Kohorn *et al.*, 2016).

500 REM1.3 is proposed as a scaffolding protein for signalling at the plasma membrane and is

501 thus an interesting candidate for mannitol-induced signalling (Marín *et al.*, 2012).

502

503 ***Comparative phosphoproteome analysis identifies bZIP30 and RBB1 as general players in***

504 ***osmotic stress response***

505

506 To assess whether the mannitol-regulated phosphorylated proteins in growing leaf tissue are

507 part of a more general stress response or are specific for the stress-induced growth-regulating

508 response, we compared our data set (30 min 25 mM mannitol) with 3 previously published

509 osmotic stress-related phosphoproteome datasets (Xue *et al.*, 2013; Stecker *et al.*, 2014;

510 Bhaskara *et al.*, 2017*b*). Given the differences in experimental set-up (long-term versus short-

511 term or mild versus severe stress) (see **Table 2** for details), we found little overlap between

512 all four datasets; only two proteins, BASIC LEUCINE-ZIPPER 30 (BZIP30)/DRINK ME

513 (DKM) and REGULATOR OF BULB BIOGENESIS1 (RBB1) (Han *et al.*, 2015), were

514 detected in all datasets, possibly indicating a general role in osmotic stress responses (**Figure**

515 **9 and Supplementary Table S8**). Interestingly, the same phosphorylation site, Ser[176], of

516 bZIP30 was found to be upregulated in the 3 datasets where mannitol was used. It should be

517 noted that bZIP30 (Ser[176], >1.5-fold upregulated, **Table 1**) was also identified in our network

518 as a central phosphoprotein in the longest interaction chain (**Figure 7**). This transcription

519 factor influences the expression of cell cycle and cell expansion genes, two processes that are

520 affected by mild mannitol treatment (Skirycz *et al.*, 2011*b*; Lozano-Sotomayor *et al.*, 2016).

21

521   In addition, we found 4 common mannitol-responsive phosphoproteins (based on overlap

522   between (Xue *et al.*, 2013), (Stecker *et al.*, 2014) and this study). The phosphorylation site

523   Ser^{680} of VARICOSE-RELATED PROTEIN (VCR, AT3G13290) was identified as

524   upregulated in all studies where mannitol was used. In addition, six phosphoproteins were

525   exclusively found in the two mild osmotic stress studies (Bhaskara *et al.*, 2017*a* and this

526   study), including a mitogen activated protein kinase kinase kinase-like protein (AT3G58640)

527   and MPK3/6-TARGETED VQ MOTIF-CONTAINING PROTEIN 1 (MVQ1, AT1G28280).

528   Apart from the different methodologies, the limited overlap points out the large

529   difference between short and long-term phosphorylation response upon stress, indicating the

530   transient and dynamic behaviour of phosphorylation events. Additionally, the severity of the

531   stress determines the phosphorylation response (Bhaskara *et al.*, 2017*a*).

532

533   *(Phospho) proteome profiling identifies AHA2 and CRRSP38 as growth regulators under*

534   *mannitol stress*

535

536   To assess the involvement of proteins with a differential abundance or phosphorylation status

537   in shoot growth and/or mannitol response, and thus the quality of our data set, we selected

538   two candidates for phenotypic analysis. From the mannitol-regulated proteome data set

539   (**Supplementary Table S1**), we selected the uncharacterized CYSTEINE-RICH REPEAT

540   SECRETORY PROTEIN 38 (CRRSP38, AT3G22060). Interestingly this protein was

541   identified as down-regulated after 30 min of mannitol application and was not detected after 4

542   h of mannitol stress treatment. This is in contrast to the absence of any mannitol-induced

543   transcriptional change at 20 and 40 min, and the increasing expression from 2 h on

544   (**Supplementary Figure S4**). Possibly, this is due to a transcript - protein level feedback

545   mechanism.We obtained a knock-out allele of CRRSP38 (SALK_151902), referred to as

546   *crrsp38-1* (**Figure 10 and Supplementary Figure S3**). Phenotypic analyses of *crrsp38-1* at

547   22 days after sowing revealed a larger rosette area both under control and mild mannitol

548   conditions (**Figure 10**), suggesting that this protein is potentially a general growth regulator

549   instead of specifically involved in the growth regulation upon mild stress. It will be

550   interesting to explore the molecular function of CRRSP38 in leaf growth regulation, and its

551   precise role upon mild mannitol-induced stress.

552   From the normalised phosphoproteome, we focused on AHA2 (AT4G30190), an

553   H(+)-ATPASE 2 of which phosphosite Thr^{881} showed an 1.9-fold increase in phosphorylation

554   after mannitol treatment and had not yet been connected to mannitol stress, and does not

555   show any obvious mannitol-induced transcriptional change in a time course experiment

556   (**Figure 8, Supplementary Figure S4 and Supplementary Table S7**). Thr^{881} is situated in

557   the conserved autoinhibitory region I of the C-terminal domain of AHA2 (Rudashevskaya *et*

558   *al.*, 2012; Falhof *et al.*, 2016), and rapid PLANT PEPTIDE CONTAINING SULFATED

559   TYROSINE 1 (PSY1)-induced *in planta* phosphorylation of AHA2 at Thr^{881} increases proton

560   efflux (Fuglsang *et al.*, 2014). We hypothesized AHA2 might play a role in growth inhibition

561   upon mild osmotic stress. Therefore, we characterized the strong knock-down *aha2-4* mutant

562   with only about 10% *AHA2* expression compared to wild type (Haruta *et al.*, 2010). As for

563   *crrsp38-1*, the rosette area of the *aha2-4* mutant was measured at 22 DAS under normal and

564   mild mannitol conditions. The *aha2-4* plants have a slightly larger rosette area in control

565   conditions (8%) and were significantly more sensitive to mannitol compared to wild-type

566   (**Figure 10**). Specifically, the *aha2-4* mutant showed a significant reduction of 59% under

567   mannitol conditions compared to a reduction of 38% in the wild-type plants. This suggested

568   that AHA2 can indeed regulate the mannitol-induced growth inhibition.

569   In summary, our (phospho)proteome-centred approach allowed the identification of

570   novel mannitol stress-related players.

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CONCLUSIONS

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In this study, label-free proteomic and phosphoproteomic analyses were performed on expanding Arabidopsis leaves exposed to mild osmotic stress. By performing the proteome analysis at two different time points, 30 min and 4 h, dynamic patterns in protein abundance could be observed. In general, after 30 min of stress ribosomal proteins were upregulated upon mannitol treatment, and photosynthesis and reduction-oxidation-related proteins were downregulated. While after 4 h, ribosomal proteins were downregulated. Furthermore, the lack of correlation between transcriptional changes prior to changes in protein abundance points towards an important role of protein degradation/stabilisation upon stress. In addition, we identified several proteins that had an altered phosphorylation status upon mild osmotic stress, suggesting an important role for kinase and phosphatase-mediated signalling. We also identified several important regulators, such as the transcription factor bZIP30, which is likely a central component of both mild and severe osmotic stress. However, previously and in this study, no transcriptional changes were observed for bZIP30, indicating that by solely studying transcriptomics, central proteins involved in stress response are likely missed. On the other hand, several transcription factors from the recently described mild mannitol stress-associated gene regulatory network were not identified in our (phospho)proteomes. In addition, we identified proteins that were specifically phosphorylated under short-term mild osmotic stress, such as AHA2. Phenotypic analysis of an *aha2* knock-out mutant indeed confirmed a role for AHA2 in the regulation of growth upon mild osmotic stress.

594   Taken together, our datasets further stress the importance of proteome- and

595   phosphoproteome-based approaches, in addition to transcriptomics, for unravelling the

596   molecular mechanisms underlying growth regulation under stress.

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600   **SUPPLEMENTARY DATA**

601

602   **Supplementary Information**

603   **Supplementary Figure S1.** Visual explanation for the 3 subsets described in the main text.

604   **Supplementary Figure S2.** Visual depiction of predicted overrepresented kinase motifs

605   based on Motif X analysis.

606   **Supplementary Figure S3.** Details of *crrsp38-1* T-DNA line.

607   **Supplementary Table S1.** Proteins identified at 30 min after mannitol treatment, including

608   raw data, differentially abundant and unique proteins.

609   **Supplementary Table S2.** Phosphosites identified at 30 min after mannitol treatment,

610   including raw data, differentially abundant and unique phosphosites.

611   **Supplementary Table S3.** Proteins identified at 4 h after mannitol treatment, including raw

612   data, differentially abundant and unique proteins.

613   **Supplementary Table S4.** GO enrichment on biological processes.

614   **Supplementary Table S5.** Comparative analysis of proteome datasets from 30 min

615   (significantly different and unique proteins) and 4 h (all dataset) mannitol treatment.

616   **Supplementary Table S6.** Protein kinases with differentially phosphorelated and unique

617   phosphosites predicted by HMMER.

618   **Supplementary Table S7.** Phosphorylated proteins normalised for protein abundance.

619 **Supplementary Table S8.** Comparative analysis of outputs from 4 phosphoproteomic

620 studies on osmotic stress.

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885

886 **TABLES**

887 **Table 1.** Phosphorylated sites mentioned in text.

Group	Protein	Name (TAIR)	Position	*Log2* fold change	Localisation probability	Peptide with localisation probabilities
Upregulated	AT3G19100	CALCIUM-DEPENDENT PROTEIN KINASE (CDPK/CPK)-RELATED PROTEIN KINASE 2 (TAGK2/CRK2)	Ser^{57}	0.683	0.95	ASPFFPFY(0.02)T(0.03)PS(0.95)PAR
	AT2G21230	BASIC LEUCINE-ZIPPER 30 (BZIP30)	Ser^{176}	0.665	1.00	SIS(1)GEDTSDWSNLVK
	AT3G13290	VARICOSE-RELATED PROTEIN (VCR)	Ser^{680}	0.552	0.99	T(0.005)S(0.005)S(0.99)ADY(0.001)FYVR
	AT1G28280	MPK3/6-TARGETED VQ MOTIF-CONTAINING PROTEIN 1 (MVQ1)	Ser^{194}	0.618	0.95	S(0.018)GS(0.018)S(0.018)NQS(0.945)PNELAAEEK
Downregulated	AT3G13530	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 7 (MAPKKK7)	Ser^{776}	-0.274	0.95	LAS(0.051)IS(0.95)GGLDGQAPR
	AT2G45820	REMORIN 1.3 (REM1.3)	Thr^{58}	-0.460	1.00	ALAVVEKPIEEHT(1)PK
			Ser^{64}	-0.450	0.95	AS(0.01)S(0.95)GS(0.04)ADRDVILADLEK
	AT2G35530	BASIC REGION/LEUCINE ZIPPER TRANSCRIPTION FACTOR 16 (bZIP16)	Ser^{152}	-0.708	1.00	GS(1)LGSLNMITGK
Unique for mannitol	AT4G24400	CBL-INTERACTING PROTEIN KINASE 8 (CIPK8); SNF1-RELATED PROTEIN KINASE 3.13 (SnRK3.13)	Thr^{166}	-	0.85	T(0.85)T(0.15)CGTPNYVAPEVLSHK
Unique for control	AT5G40450	REGULATOR OF BULB BIOGENESIS (RBB1)	Ser^{2802}	-	1.00	S(0.997)LS(0.003)DHIQK
	AT4G35230	BRASSINOSTEROID-SIGNALING KINASE 1 (BSK1)	Thr^{353}	-	0.80	KQEEAPS(0.201)T(0.798)PQRPLS(0.001)PLGEACSR
	AT1G10940	SNF1-RELATED PROTEIN KINASE 2.4 (SNRK2.4)	Ser^{357}	-	1.00	EVHAS(1)GEVR

37

888 **Table 2.** Details on phosphoproteomic studies for comparative analysis

Study	Material	Growth medium	Compound	Concentration	Time	Setup	Method
Current study	Leaf #3 of 15-days-old seedlings	Agar plates	Mannitol	25 mM	30 min	Transfer	Label-free
Bhaskara et al., 2017	7-day-old seedlings	Agar plates	PEG	a low water potential (21.2 MPa)	96 h	Transfer	ITRAQ
Stecker et al., 2014	10-day-old seedlings	Liquid culture	Mannitol	300mM	5 min	Medium replacement	^{15}N-labelling
Xue et al., 2013	12-day-old seedlings	Liquid culture	Mannitol	800mM	30 min	Transfer	Label-free

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FIGURE LEGENDS

Figure 1. Mannitol-triggered protein and phosphoprotein changes upon short exposure (30 min). This workflow illustrates the steps to obtain a reliable set of proteins or phosphosites following LC-MS/MS. The portions for proteins and phosphosites (including the % serine (S), threonine (T) and tyrosine (Y) sites) are indicated.

Figure 2. Mannitol-triggered protein changes upon long exposure (4 h). This workflow illustrates the steps to obtain a reliable set of proteins following LC-MS/MS. The numbers for detected and selected proteins are indicated.

Figure 3. Protein-protein interaction network of significant mannitol-regulated proteins (30 min treatment). GO annotations for biological process of up- and down-regulated proteins were superimposed on the network and nodes were grouped accordingly. Colored backgrounds indicate functions related to protein metabolism (yellow), photosynthesis and carbohydrate metabolism (green) and oxidation-reduction processes (orange). Unique proteins were indicated with dashed lines while differentially abundant proteins were coloured from dark green ranging to red depending on the log_2 fold change. Thickness of connecting lines indicates a combined score of interaction.

Figure 4. Comparison of protein abundance with transcript level of corresponding genes. The differential expression of significant differentially up- or down-regulated proteins was analysed. The expression and protein levels were measured in expanding leaf tissue upon mannitol treatment and compared to control conditions. Dashed lines indicate proteins unique for control (green) or mannitol-treated (red) samples. RAD23C - RADIATION

924   SENSITIVE23C; 4CL1 - 4-COUMARATE:COA LIGASE 1; PRXQ - PEROXIREDOXIN

925   Q; CDSP32 - CHLOROPLASTIC DROUGHT-INDUCED STRESS PROTEIN OF 32 KD;

926   LEA26 - LATE EMBRYOGENESIS ABUNDANT 26; SAL1 – SAL1 phosphatase;

927   EIF(ISO)4E - EUKARYOTIC TRANSLATION INITIATION FACTOR ISOFORM 4E;

928   FAD7 - FATTY ACID DESATURASE 7; CAT1 – CATALASE 1.

929

930   **Figure 5.** Protein-protein interaction network of significant mannitol-regulated proteins (4 h

931   treatment). GO annotations for biological process of up- and down-regulated proteins were

932   superimposed on the network and nodes were grouped accordingly. Unique proteins were

933   indicated with dashed lines while differentially abundant proteins were coloured from dark

934   green ranging to red depending on the *log₂* fold change. Thickness of connecting lines

935   indicates a combined score of interaction.

936

937   **Figure 6**. Venn diagram showing the overlap between the significant up- and down-regulated

938   proteins from the 30 min proteome data set and all quantifiable proteins from 4 h proteome

939   data set. In the overlap, three subsets of proteins are identified based on the changes in their

940   abundances from 30 min to 4 h of mannitol stress; stable proteins are up- or downregulated at

941   both 30 min and 4 h, "UP to DOWN" indicate proteins that are upregulated after 30 min but

942   downregulated at 4 h and the "DOWN to UP" indicates the opposite.

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944   **Figure 7.** Protein-protein interaction network of significant mannitol-regulated

945   phosphopeptides mapped on the corresponding proteins (30 min treatment). GO annotations

946   for biological process of up- and down-regulated proteins were superimposed on the network

947   and nodes were grouped accordingly. Unique phosphosites were indicated with dashed lines

948   while differentially abundant phosphosites were coloured from dark green ranging to red

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949   depending on the *log₂* fold change. Thickness of connecting lines indicates a combined score

950   of interaction.

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952   **Figure 8.** A normalised mannitol-triggered phosphoproteome. Significantly up- and

953   downregulated phosphosites were normalised by subtracting the *log₂* fold change of the

954   protein abundance from the *log₂* fold change of the phosphosite, with the exception of the

955   unique phosphosites. In total, 32 differentially phosphorylated proteins could be mapped on

956   total proteome data.   RBB1- REGULATOR OF BULB BIOGENESIS1; PHOS34 -

957   Phosphorylated protein of 34 kDa; NDK1 - NUCLEOSIDE DIPHOSPHATE KINASE 1;

958   ADSS - ADENYLOSUCCINATE SYNTHASE; CPN20 - CHAPERONIN 20; REM1.3 –

959   Remorin 1.3; PSAE1 - Photosystem I reaction center subunit IV A; H1.2 – HISTONE 1.2;

960   REC2 - REDUCED CHLOROPLAST COVERAGE 2; PHOT1 - PHOTOTROPIN 1; TOC86

961   - TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLASTS 86;

962   AMPD - ADENOSINE 5'-MONOPHOSPHATE DEAMINASE; ELF5A-3 - EUKARYOTIC

963   ELONGATION FACTOR 5A-3; AHA2 - H(+)-ATPASE 2; PSBA - PHOTOSYSTEM II

964   REACTION CENTER PROTEIN A; REC3 - REDUCED CHLOROPLAST COVERAGE 3;

965   PIP3 - PLASMA MEMBRANE INTRINSIC PROTEIN 3; RBCS1A - RIBULOSE

966   BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A; DAYSLEEPER - Zinc finger

967   BED domain-containing protein; CRWN1 - CROWDED NUCLEI 1.

968

969   **Figure 9.** Venn diagram showing the overlapping phosphoproteins from four recent

970   phosphoproteomic studies of osmotic stress responses, including our current study. Details on

971   selected studies, concerning the osmoticum and concentration that was used, are indicated in

972   Table 2. bZIP30 - BASIC LEUCINE-ZIPPER 30; RBB1- REGULATOR OF BULB

973   BIOGENESIS1; VCR - VARICOSE-RELATED PROTEIN; MVQ1 - MPK3/6-TARGETED

974   VQ MOTIF-CONTAINING PROTEIN 1.

975

976   **Figure 10. Phenotypes of T-DNA insertion lines for selected candidates. (A, C)**

977   Representative pictures of *crrsp38*-1 and *aha2-4* lines compared to Col-0 at 22 days after

978   stratification grown on control (MS) or mannitol (25 mM). Scale bar, 5 mm. **(B, D)**

979   Quantification of the rosette area of *crrsp38-1* (B) and *aha2-4* (D) at 22 days after

980   stratification grown on control (MS) or 25 mM mannitol (MA). Boxplots are combined

981   values of at least 30 seedlings from 4-6 different plates and 2 or 1 independent experiments

982   for *crrsp38-1* or *aha2-4*, respectively. Asterisk indicates a significant difference with $p < 0.05$

983   based on ANOVA. In addition, the ANOVA analysis indicated that the genotype:treatment

984   interaction for *aha2-4* was significant ($p < 0.05$).

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

Translation and ribosome biogenesis

Photosynthesis and carbohydrate metabolism

Protein folding

Methionine metabolic process
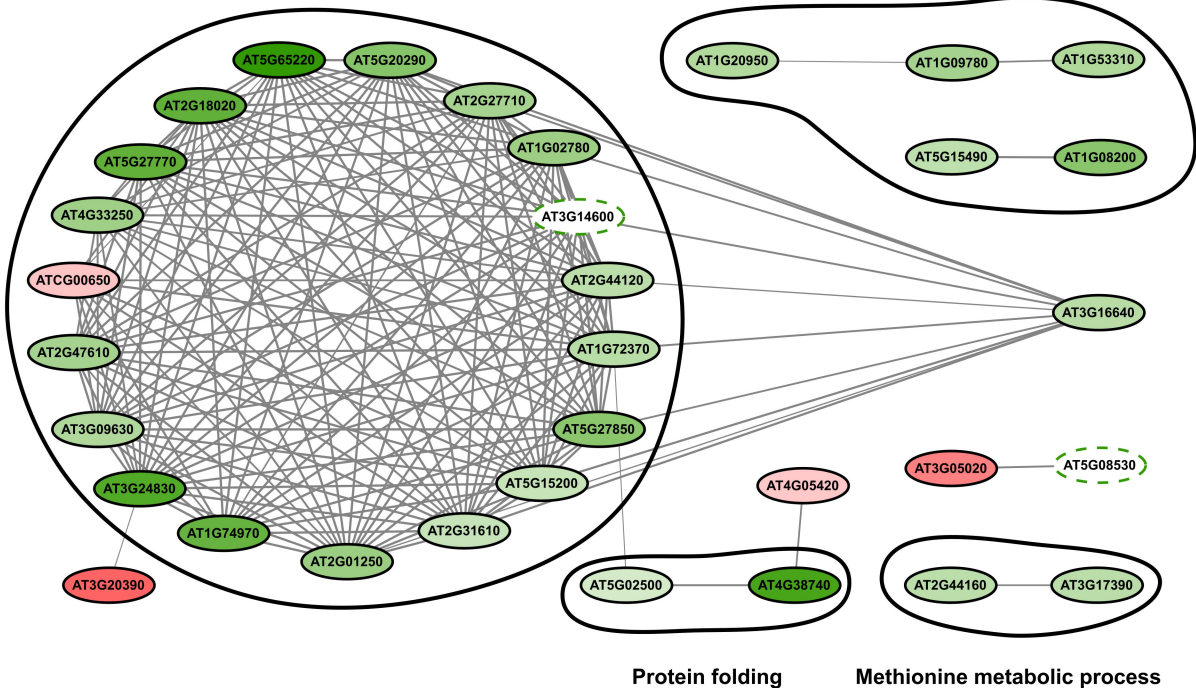
Unique for control

Log2 fold change

-0.71   0.0   1.17

FIGURE 5

30 min proteome (up- and downregulated proteins)

4 h proteome (all quantifiable proteins)

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UP ➜ DOWN (11 proteins)

STABLE (59 proteins)

DOWN ➜ UP (50 proteins)

FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 9

FIGURE 10