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# 1 Distinct early transcriptional regulations by turgor and osmotic

# 2 potential in the root of Arabidopsis

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### 28 Abstract

29 In a context of climate change, deciphering signaling pathways driving plant adaptation to drought, 30 changes in water availability, and salt is key. A crossing point of these plant stresses is their impact on plant water potential ( $\Psi$ ), a composite physico-chemical variable reflecting the availability of water 31 32 for biological processes such as plant growth and stomatal aperture. The  $\Psi$  of plant cells is mainly 33 driven by their turgor and osmotic pressures. Here we investigated the effect of a variety of osmotic 34 treatments in the root of Arabidopsis plants grown in hydroponics. We used, among others, a 35 permeating solute as a way to differentiate variations on turgor from variations in osmotic pressure. 36 Measurement of cortical cell turgor pressure with a cell pressure probe allowed to monitor the 37 intensity of the treatments and thereby preserve the cortex from plasmolysis. Transcriptome analyses 38 at an early time point (15min) showed specific and quantitative transcriptomic responses to both 39 osmotic and turgor pressure variations. Our results highlight how water-related biophysical 40 parameters can shape the transcriptome of roots under stress and provide putative candidates to 41 explore further the early perception of water stress in plants.

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- 43

44 **Keywords:** ethylene glycol, NaCl, osmotic pressure, sorbitol, transcriptional response, turgor

45 pressure, PEG, water potential

# 46 Abbreviations:

EG	Ethylene glycol
PEG	Poly ethylene glycol
Р	Turgor pressure (MPa)
П	Osmotic pressure (MPa)
Ψ	Water potential (MPa)
DEPs	Differentially expressed probes
DEGs	Differentially expressed genes

#### 48 Introduction

49 How the environment is perceived by plants is of major importance for their life cycle. This is particularly true for water deficit (Maurel and Nacry, 2020; Verslues et al., 2023) which can be 50 51 summarized as an imbalance between the plant's requirement and loss of water and its uptake 52 capacity. Water deficit directly impacts the plant water status. One of the ways plant water status is assessed is via plant water potential ( $\Psi$ ), a composite variable which, in plant cells, integrates the 53 54 turgor potential (or turgor pressure, P) and the osmotic potential ( $\Pi$ ) (Haswell and Verslues, 2015). 55 When considering the soil/plant/atmosphere continuum,  $\Psi$  can also be influenced by gravity and 56 matric potentials.  $\Psi$  gradients allow evaluating the motive forces that generate net flows of water 57 between different compartments of this continuum. Together with the viscoelastic properties of the 58 cell wall, P is responsible for the elongation of cells and organs, and for the rigidity of stems and leaves, 59 allowing them to act against gravity and optimize light interception, among others.  $\Pi$  is related to the 60 concentration of solutes in a compartment. The presence of a  $\Pi$  gradient across a semi-permeable 61 barrier causes osmosis: a net directional flow of water, even in the absence of any hydrostatic pressure difference (Bowler, 2017). IT influences biochemical reactions, and can be directly regulated by the cell 62 63 through osmoticum accumulation, synthesis, and transport (Beauzamy et al., 2014).

A critical issue in plant biology is to understand which physico-chemical parameters are 64 65 perceived by plants. Terms such as osmosensing and mechanosensing are employed to describe 66 phenomena related to perceiving the plant water status (Beauzamy et al., 2014; Haswell and Verslues, 2015; Hamant and Haswell, 2017; Scharwies and Dinneny, 2019). Many molecular actors, such as 67 mechanosensitive channels and protein kinases from multiple families (detailed in the reviews cited 68 above) are thought to be involved in this perception or are contributing to associated phenomena. 69 70 However, we lack a clear picture of the early perception of water deficit. One difficulty is that water 71 deficit translates into multiple variations in the cell status. It is still discussed, for example, whether  $\Pi$ 72 or P changes are directly sensed by plants or whether it is rather their impact on cell processes, cell 73 wall status or cell volume (Sack et al., 2018; Verslues et al., 2023).

Another difficulty is that the links between the intensity of the stress causing the water deficit and cells parameters is hard to establish. Measuring plant physico-chemical parameters under physiological conditions is indeed difficult at cell-scale resolution.  $\Pi$  or P can be measured using a combination of challenging, low-throughput, and/or indirect techniques such as pressure chambers and pico-osmometers, cell pressure probes, picogauges or indenters (Beauzamy *et al.*, 2014; Knoblauch *et al.*, 2014; Boursiac *et al.*, 2022). Thus, there is a real need for improved tools and nondestructive techniques using, for example, chemical probes, protein reporters, or marker genes.

Here, we addressed the early stages of water deficit perception by considering that the drop in external  $\Psi$  would primarily provoke a change in either  $\Pi$  or P. Using hydroponically grown Arabidopsis plants that were osmotically challenged with permeating and non-permeating solutes, we first evaluated the impact of a drop in external  $\Psi$  on the P of root cortical cells. We then investigated root transcriptional regulations as a readout, to test whether  $\Pi$  or P can trigger specific and quantitative responses, a first step into the question whether  $\Pi$  or P can be genuinely perceived by plant cells.

#### 88 Material and Methods

#### 89 Plant material and culture conditions

All experiments were performed using Arabidopsis thaliana ecotype Col-0. Seeds were surface 90 91 sterilized and kept at 4°C in dark until sowing on 1/2 Murashige and Skoog basal salt medium agar 92 plates [2.2 g.l<sup>-1</sup> MS (Sigma), 1% sucrose (Euromedex), 0.05% MES (Euromedex), and 0.7% agar (Sigma), pH 5.7 adjusted using KOH]. For pre-germination, plates were incubated vertically in growth chamber 93 94 under long-day conditions (16h/8h, 21°C, 60% humidity). After 10 days, seedlings were transferred to 95 a hydroponic medium [1.25 mM KNO<sub>3</sub>, 0.75 mM MgSO<sub>4</sub>, 1.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 50  $\mu$ M Fe-EDTA, 50 μM H<sub>3</sub>BO<sub>3</sub>, 12 μM MnSO<sub>4</sub>, 0.70 μM CuSO<sub>4</sub>, 1 μM ZnSO<sub>4</sub>, 0.24 μM MoO<sub>4</sub>Na<sub>2</sub>, 100 μM 96 Na<sub>2</sub>SiO<sub>3</sub>] and further grown under the same culture conditions. Cell pressure probe measurements, 97 98 transcriptomic analyses, and treatments for qPCR analysis were performed at 4-8 days, 6 days, or 6-99 11 days after transfer, respectively.

#### 100 **Osmotic treatments**

Osmotic stress treatments were performed using a hydroponic solution containing either 25mM 50mM-75mM-100mM NaCl (Sigma); 50mM-100mM-150mM sorbitol (Sigma); 75g.l<sup>-1</sup>-100 g.l<sup>-1</sup>-125 g.l<sup>-1</sup>
 <sup>1</sup>-150 g.l<sup>-1</sup> polyethylene glycol 8000 (Sigma); or 50mM-100mM-150mM-200mM ethylene glycol
 (Sigma). Table 1 recapitulates the solutions and their respective osmotic potential.

#### 105 Cell Pressure Probe Measurements

Cell pressure probe measurements were performed as described previously (Javot *et al.*, 2003). Our
device uses a pulled and beveled glass microcapillary (tip external diameter: 4 to 8μm), filled with
mineral oil and mounted onto a pressure probe. Primary root tip segments of ~2-3cm were excised
from Arabidopsis seedlings and laid on a filter paper perfused with hydroponic or treatment solution.
Measurements were performed within a distance of 1cm from the elongation of the first root hairs.
Data were recorded using an especially designed software (Pfloek; Department of Plant Ecology,

University of Bayreuth, Germany). Due to the dead volume of the system and the maximal speed of the peristaltic pump, it took approximatively 2min to fully change the perfusion solution around the root.

#### 115 Transcriptomic analyses

116 Osmotic treatments were performed by transferring plants for 15 min into a hydroponic or treatment 117 solution. The whole roots were harvested after 15 min of treatment and immediately frozen in liquid 118 nitrogen. Each sample was a pool of three plants and two sets of plants were treated independently. 119 Frozen samples were ground using a MM 400 mixer mill (Retsch). Total RNA was extracted using TRI 120 Reagent (Molecular Research Center, Inc), DNA contamination was removed by digestion with DNase 121 I (Promega), and further purification of the RNAs was performed using the MinElute Cleanup Kit 122 (Qiagen), all according to the manufacturer's instructions. Concentration and purity of the RNAs was 123 assessed by spectrophotometry and integrity was confirmed using RNA 6000 Nanochips with a 2100 124 Bioanalyzer (Agilent). Gene expression measurements were performed using Arabidopsis Affymetrix 125 Gene1.1 ST array strips (Affymetrix). For each sample, 100 ng of total RNA was processed using the 126 GeneChip WT PLUS Reagent Kit (Affymetrix) following the manufacturer's instructions. Hybridization 127 on array strips was performed for 16h at 48°C. The arrays were washed, and stained, using GeneAtlas 128 Hybridization, Wash and Stain Kit for WT Array Strips following the manufacturer's instructions. Array 129 strips were scanned on the GeneAtlas system.

Microarrays raw data were processed with GCRMA available in the Expression Console Software package developed by Affymetrix. The Affymetrix Microarrays data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus in compliance with Minimum Information About a Microarray Experiment standards (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Gene Expression Omnibus Series accession no. GSE223207.

#### 135 Transcriptomic data analyses

The multi-way type II ANOVA model and one-way ANOVA analyses for genes expression correlation to II and P were run in R (v.4.2.0). Thresholds for the selection of differentially expressed probes (DEPs) were adjusted by comparison of the p-values versus FDR corrected p-values and their frequency. A general cut-off of FDR < 0.2 was ruled out, which yielded non-adjusted p-values thresholds of 0.001 for the solutes (NaCl, Sorbitol, PEG and EG), and 0.0004 and 0.0012 for  $\Pi$  and P, respectively. A few genes were removed from the  $\Pi$ - and P-specific lists since they were associated to at least 2 probes and gave inconsistent ANOVA test results:

- Removed from "Π-specific genes": At1g72850, At1g78270, At2g24540, At2g33810,
   At4g13920, At4g24410, At4g25880, At4g26490,
- Removed from "P-specific genes": At1g07130, At1g07725, At1g08590, At1g51640, At1g56240,
   At1g72850, At2g11851, At2g22960, At3g22070, At3g54630, At3g56770, At4g24410,
   At4g28650, At4g36030, At4g38210, At4g38550, At5g59730.

Treatments clustering was obtained in RStudio (RStudio 2022.07.1+554) by calculating the Euclidean distance between treatments using the function *dist()*, then the clusters obtained by *hclust()* were plotted using *plot()*, with default values. Venn diagrams were elaborated with the nVennR package (Pérez-Silva *et al.*, 2018).

Semantic analysis of the clusters was performed using Genecloud (Krouk *et al.*, 2015) from m2sb.org webpage with an FDR threshold set to 1%. Gene ontology enrichment was performed in R using ClusterProfiler v.4.4.2 (Wu *et al.*, 2021) and org.At.tair.db (v3.15.1) for the Arabidopsis genome wide annotation database (Carlson, 2017). Lists overlaps scores were obtained using the Genesect algorithm from the Virtual Plant platform (Katari *et al.*, 2010).

#### 157 RT-qPCR

158 RNA extraction was performed by using the Direct-zol RNA Miniprep Plus Kits from Zymo Research 159 (NO.2072). cDNA solution was synthesized from 1  $\mu$ g RNA and oligo-DT<sub>15</sub>, dNTPs and M-MLV (Promega) according the manufacturer protocol. At1g13320 (PDF2) and At4g34270 (TIP41-like) were 160 161 selected as internal normalizing genes, because of their stability in roots under abiotic stresses (Czechowski et al., 2005). RT-qPCR primers were designed by using the primer3 online website (version 162 163 4.1.0, Table S1). RT-qPCR reactions were performed according to the procedure recommended by the manufacturer (Takara)(0.5 µL H2O, 0.25 µL F/R, 4 µL cDNA and 5 µL TAKARA SYBR premix Ex Taq. 95°C 164 165 for 30 s; 95°C for 5 s, 60°C for 30 s (40 cycles)). RStudio software was used to calculate gene expression 166 according to Vandesompele's method (Vandesompele et al., 2002).

#### 167 mRNA decay analysis

The half-life time of mRNAs ( $T_{1/2}$ ) was calculated from data available in Sorenson et al. 2018. It is based on the decay rate ( $\alpha$ ) modeled from RNAseq data upon cordycepin treatment on *sov* mutant seedlings (i.e. Col 0), and is calculated as  $T_{1/2} = \ln(2)/\alpha$  (Sorenson *et al.*, 2018).  $T_{1/2}$  of each mRNA from genes in the clusters are presented individually and as boxplots. The values above the boxplots correspond to a non-parametric estimation of the p-value of the  $T_{1/2}$  of a given cluster being smaller than that of the whole genome. In this boostrap-based approach, the median  $T_{1/2}$  of the cluster is compared to the median  $T_{1/2}$  of a sample (of the same size) from the whole genome data. The number of occurrences

- 175 where the genome sample median  $T_{1/2}$  is smaller than the cluster median  $T_{1/2}$  divided by the number
- 176 of tests realized ( $10^4$  tests), aka the frequency, is reported.

#### 177 **Results**

#### 178 Turgor response of root cortical cells to osmotic challenges

179 We determined the P of root cortical cells with a cell pressure probe (Boursiac et al., 2022) upon root 180 perfusion with a standard hydroponic solution, or the same solution supplemented with various 181 concentrations of distinct solutes: sodium chloride (NaCl), sorbitol, poly ethylene glycol 8000 (PEG), or 182 ethylene glycol (EG) (Table 1). In contrast to others, the latter solute can significantly diffuse through 183 cell membranes. Thus, EG is expected to concomitantly reduce the  $\Psi$  of the solution and cells, without significantly changing the  $\Psi$  gradients between compartments (Creelman and Zeevaart, 1985). Figures 184 1A-D show cortical cell pressure measurements over the course of approximately 30 min of a perfusion 185 with various concentrations of NaCl, sorbitol, PEG and EG. For all treatments, we observed a 186 187 progressive reduction in P, which reached a minimal value within 10 min. P remained stable for at least 188 10 additional minutes for most conditions except EG treatments, where a partial restoration of P was 189 eventually observed. We averaged P within 10-20 min of treatment and represented it as a function of 190  $\Pi$  of the bathing solution (Figure 1E). For all solutes except EG, we observed a linear and relatively 191 similar relationship between  $\Pi$  of the solution and cortical cell P. In the 10-20 min time range, EG provoked a reduction in P of ~0.1 MPa, independently of its concentration, and thereby of  $\Pi$ . Note 192 193 that treatments were designed so that P remained positive in cortical cells, and hence cortical cells 194 were not plasmolysed. These results suggest that root cortical cells behave as osmometers with the 195 solutes except EG, and show no major osmotic regulation within the time frame of the experiment. 196 The 15 min time-point, which corresponds to a mostly stable P, seems to be well-adapted to studying 197 the early molecular events triggered by osmotic challenges.

#### 198 Transcriptional response of roots osmotically challenged for 15min.

199 We treated Arabidopsis plants for 15 min using the various conditions tested above, and performed 200 transcriptomic analyses on RNA extracted from their roots. This genome-wide investigation of gene 201 expression in response to 15 distinct osmotic challenges (plus a control condition in which plants were 202 transferred into an identical hydroponic solution, table 1) was recorded using Affymetrix A. thaliana 203 genome arrays (Gene1.1 ST array strip, 2 independent biological experiments). We used the probes 204 data from the genome array to perform a hierarchical classification of the osmotic challenges, and 205 explore their convergence in transcriptional control (Figure 2A). A general feature is that most of the 206 challenges were grouped by the nature of the solute (NaCl, Sorbitol, PEG or EG), suggesting that it represents a main determinant of whole genome transcriptional status. EG treatments were clustered
 next to the hydroponic condition, which echoes to the limited effect of this solute on P. Treatments
 with the two highest PEG concentrations were also apart from the other challenges, which suggests
 that these conditions trigger responses of yet another type.

211

The data sets were then modeled through ANOVA with the following linear model:

212  $Y_i = \alpha.NaCl_{factor} + \beta.Sorbitol_{factor} + \gamma.PEG_{factor} + \delta.EG_{factor}$ 

where Y<sub>i</sub> is the signal intensity of an ATH1 probe,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  are coefficients representing the effect of 213 214 each of the factors, respectively, and NaCl<sub>factor</sub>, Sorbitol<sub>factor</sub>, PEG<sub>factor</sub> and EG<sub>factor</sub> are factors indicating 215 the concentration of each treatment (table 1, full results provided in supplemental data S1) (Ristova et 216 al., 2016). Note that this model uses partial regressions against factor that are derived from the 217 concentration of the solutes. Importantly, the factors are set to 0 when another solute is used as a 218 treatment and, as a consequence, are negatively impacting the score of genes which could be 219 regulated by common underlying processes (such regulations are addressed in the next section). Note 220 also that all solutes are included in the model, despite no co-treatment was performed and thus no 221 interaction is investigated, in a bid to increase the statistical power. We then considered a probe as 222 differentially expressed if its ANOVA p-value was significantly different at p<0.001 (FDR<0.2) for any of 223 the 4 factors. 526 differentially expressed probes (DEPs), corresponding to 436 differentially expressed 224 genes (DEGs) were retrieved with this analysis. In order to estimate the amplitude of the transcriptomic 225 regulation, we first separated and sorted the DEPs according to the conditions in which they were 226 regulated (Figure 2B). Probes regulated specifically by one solute only were the most represented. PEG 227 was the solute with most specific impact, with 182 DEPs (159 DEGs). NaCl, sorbitol and EG treatments 228 resulted in 127 (92), 68 (60) and 14 (12) specific DEPs (DEGs), respectively. The remaining 135 DEPs 229 were regulated significantly in 2 or more solutes treatments (Figure 2B). Because DEGs could be either 230 up or downregulated by the treatments, we separated the genes regulated by each specific solute in 231 two clusters based on their averaged, centered, expression signal. Figure 2C visually confirms that the 232 DEGs identified by this approach indeed exhibit a quantitative transcriptional regulation for a particular 233 solute mostly.

#### 234 **Do** $\Pi$ **or P trigger specific gene regulations?**

Because all treatments share a common osmotic component (table 1), the transcriptional response can also be observed with the prism of a dose-dependent response to osmotic pressure. We therefore performed a one-way ANOVA on our transcriptomic data, using  $\Pi$  as the explanatory, continuous, variable (supplemental data S1). This analysis retrieved 72 DEGs. EG was also used forits capacity to 239 reduce the  $\Psi$  of the solution but, at variance of the other treatments, provoking only a limited 240 reduction in P (Figure 1 D, E). With the aim of differentiating the effect of an osmotic treatment on the 241 transcriptome through either the osmotic potential or its impact on P, we performed a similar one-242 way ANOVA analysis of the 15 min transcriptomic response to the treatments, but with P as the 243 explanatory variable (supplemental data S1). This analysis resulted in 179 DEGs. While 53 DEGs were 244 identified in both  $\Pi$  and P response (see discussion), 19 and 126 DEGs were specific of  $\Pi$  and P, 245 respectively (Figure 3A). Each group of  $\Pi$  or P DEGs was split in 2 clusters, in order to account for 246 potential up- and down-regulations. For the  $\Pi$ -specific genes, the mRNA abundance of the DEGs 247 appeared to be regulated quantitatively for all solutes employed (Figure 3B, upper panels), while a 248 similar regulation for NaCl, sorbitol and PEG, but not EG, was observed for the P-specific genes (Figure 249 3B, lower panels). Most importantly, a clear quantitative correlation to  $\Pi$  or P was confirmed for the II-specific (Figure 3C) and P-specific (Figure 3D) DEGs, respectively. Altogether, our transcriptomic 250 251 approach suggests that while cells remain turgid, at least two components of the osmotic treatment, P and the  $\Pi$  of the bathing solution, are able to provoke specific quantitative responses of the 252 253 transcriptome, resulting in both up- and down-regulations.

#### 254 Are promoter activity and mRNA decay pathways involved in the Π or P transcriptional regulation?

The 1Kb promoter regions of the DEGs were analyzed using the MEME suite (Bailey *et al.*, 2015; Grant and Bailey, 2021, Preprint). Both new and already known (O'Malley *et al.*, 2016) enriched motives were considered for  $\Pi$ -cluster 1 or the P-specific clusters (with only 4 genes, the  $\Pi$ -cluster 2 was not analyzed, supplemental data S2, S3A-C). All clusters showed an enrichment in motives (or similar motives) known to bind ABI3VP1 transcription factors (TFs). All other motives were found enriched in 1 cluster only: REM, C2C2dof and BBRBPC binding elements for P-cluster 2, C2H2 and ZFHD binding motives for P-cluster 1, and an ARID binding motif for  $\Pi$ -cluster 1.

262 We also considered whether the regulation of the mRNA abundance of the DEGs could be post-263 transcriptional, and in particular due to their degradation. Using the transcription inhibitor cordycepin 264 and a model-assisted RNAseq approach, Sorenson et al. (2018) performed a global evaluation of mRNA 265 decay rates in Arabidopsis and evaluated the implication of the three main decay pathways (Sorenson 266 et al., 2018). As a first hint into this type of regulation for the DEGs identified herein, we used the 267 mRNA decay rates obtained in the above-mentioned study, for the sov Col genotype, to calculate the 268 mRNA half-life of our genes of interest in their growth conditions ( $T_{1/2}$ ). The median  $T_{1/2}$  of all mRNAs 269 detected in this study was around 101 min. The median  $T_{1/2}$  calculated for genes of the  $\Pi$ -specific 270 clusters and P-specific cluster 2 were significantly lower, with mean values of 56 min, 23 min and 69 271 min, respectively (Figure 4), and was not different for genes of P-specific cluster 1.

#### 272 A short list of $\Pi$ or P correlated genes

273 In a bid to confirm the robustness of the microarray approach, and select potentially good candidates 274 that could serve as markers of P or  $\Pi$ , we ranked genes of the 4 categories ( $\Pi$ - or P-specific, up or 275 downregulated) according to 3 parameters: the adjusted R square of a linear fit of their averaged 276 centered expression as a function of  $\Pi$  or P, the slope of the linear fit, and the average expression level 277 in control conditions (Supplemental data S4). We randomly selected a few genes, among the best 278 ranked of each list, to confirm their regulation by RT-qPCR, upon 15min NaCl, sorbitol and EG 279 treatments, and in three new, independent, biological replicates. Figure 5 shows plots of the 280 comparison between the means of the microarray signals and of the signals obtained by RT-qPCR. 10 281 genes out of 12 displayed a significant correlation between both signals, thus globally confirming the results obtained by the microarray approach. The  $\Pi$ -specific cluster 2 showed poor reliability, with 282 283 only 1 gene out of 3 having a similar behavior upon confirmation by RT-qPCR and in new biological 284 replicates. The P-specific cluster 1 exhibited the highest rate of confirmation, in both the p-value of 285 the correlation and the R<sup>2</sup> of the relationship. Among them, At1g64640 stood out with a correlation p-286 value below 1e-3 and a  $R^2 > 0.9$ . The expression of this gene is therefore robustly and quantitatively 287 correlated to P, at 15 min after an osmotic challenge.

#### 288 What are the gene functions altered by $\Pi$ or P?

A semantic analysis of the gene annotation present in the 4 clusters ( $\Pi$ - or P-specific, up or downregulated) using Genecloud (Krouk *et al.*, 2015) revealed that the  $\Pi$ -specific clusters do not show any particular semantic enrichment. Arabinogalactan, Cys/His rich proteins, "protein kinase C" and TFs related terms were detected in the cluster 1 of P-specific genes (Figure 6A, left). Cluster 2 of P-specific genes showed enrichments in terms related to ethylene-dependent and other transcriptional regulations as well as defense responses (Figure 6A, right).

295 A Gene Ontology (GO) enrichment analysis was also performed.  $\Pi$ -specific cluster 1 was found 296 specifically enriched in genes associated with defense responses and the cell wall (Figure 6B, left). 297 Results for P-specific clusters were quite consistent with the semantic analysis. P-specific cluster 1 was 298 enriched in "anchored components" which echoes to the arabinogalactan term above (not shown). P-299 specific cluster 2 was enriched in terms associated to ethylene and defense responses. In a bid to 300 sharpen the above mentioned GO analysis, we also evaluated the overlap between our gene lists and 301 the Gene Ontology list "response to NaCl" (GO:0009651) as well as two of the upstream terms: 302 "response to abiotic stress" (GO:0009628) and "response to stimulus" (GO:0050896) (Figure 6C). The two upregulated clusters,  $\Pi$ -specific cluster 1 and P-specific cluster 2, showed a significant overlap 303 with the genes in the categories "response to stimulus" and "response to abiotic stress", but not with 304

305 "response to salt stress", indicating the convergence of our approach to the existing knowledge. 306 Because the specificity of the response might not prevail under short term treatment, and a general 307 stress response (GSR) may rather be activated (Bjornson et al., 2021), we also compared our lists to 308 the genes identified as GSR from the work of Ma and Bohnert (2007). In this study, the authors 309 analyzed a collection of transcriptome profiles of plants under various treatments, and highlighted a 310 stress-dependent cluster that could represent cell-level stress responses (Ma and Bohnert, 2007). П-311 specific cluster 1 and P-specific cluster 2 showed a significant overlap with the GSR genes, with a 312 greater Z-score than for the previous comparisons (Figure 6C). This analysis suggests that some of the 313 upregulated genes that are quantitatively (and inversely) correlated to  $\Pi$  or P belong to a common and 314 early response to stresses.

Finally, our candidates were also compared to a list of genes encoding transcription factors (Pruneda-Paz *et al.*, 2014), and showed no significant enrichment in this category of genes (Figure 6C).

#### 317 **Discussion**

This study was designed to improve our comprehension of which component of a water deficit can be perceived by plant roots. For this, we considered both the II of the bathing solution or the P of root cells as possible input signals (Figure 1) and used whole genome transcriptional responses as a readout. The approach was meant to unravel any quantitative relationship between the input signals and responses.

#### 323 Parameters at the origin of the transcriptional responses

324 In addition to solute specific transcriptional responses (Figure 2D), our study unraveled 72 genes which 325 expression correlated to  $\Pi$ , independently of the solute used ( $\Pi$ -specific clusters, Figure 3, A and C). 326 These results suggest that plant cells have the capacity to sense and transduce the external osmotic 327 potential. Measurement with a cell pressure probe allowed us to also look for correlations between 328 gene expression and the P of root cortical cells. In this approach, the use of EG as a permeating 329 osmoticum was critical to make a distinction between the effects of the solutes on P and  $\Pi$  (Figure 1E). 330 241 genes were found to be truly correlated to P (P cluster 1 and 2, Figure 3, A and D) and suggest that 331 plant cells also have the capacity to specifically sense and respond to the internal pressure. Due to the 332 experimental design and variability, 53 genes could not be assigned to a  $\Pi$ - or P-specific response (Figure 3A) and would deserve more investigation, in particular with the use of other permeating 333 334 solutes. Nevertheless, the identification of genes which expression is quantitatively correlated to all 335 possible combinations ( $\Pi$  or P, up- or down-regulation) highlights the multiplicity of water deficit 336 responses in plant cells. Since we uncovered potentially distinct regulatory mechanisms, our results

will help clarifying studies on mechano- and osmo-sensing as well as our understanding of plant response to water deficit. For example, turgor recovery upon plant adaptation to low external water potential by solutes synthesis/accumulation necessarily implies an uncoupling between P and  $\Pi$ .

340  $\Pi$  or P may not be the exact physico-chemical parameters that are genuinely perceived by 341 plants. It has been suggested that, in leaves, accumulation of ABA is triggered by a drop in relative 342 water content (RWC) rather than variations in P or  $\Pi$  (Jia *et al.*, 2001; Sack *et al.*, 2018). This distinction was made possible by experiments of leaf dehydration beyond the turgor point loss. In our 343 344 experiments, turgor was preserved because we anticipated that plasmolysis could trigger distinct 345 responses and, on a crude assumption, the relative change in cell volume ( $\Delta V/V$ ) is linearly correlated 346 to the variation in P ( $\Delta$ P) according to  $\Delta$ P =  $\varepsilon$ .  $\Delta$ V/V where  $\varepsilon$  stands for the cell wall elastic modulus 347 (Hüsken et al., 1978). Thus, we cannot differentiate P or RWC and may use them interchangeably in 348 our interpretations. It would also be interesting to establish whether the internal (intracellular) 349 osmotic potential ( $\Pi_{int}$ ) can be sensed and trigger specific transcriptional responses. Because cells 350 behave as osmometers in the presence of NaCl, sorbitol and PEG,  $\Pi_{int}$  can be expressed, at equilibrium, as  $\Pi_{int}$  =  $\Pi$  + P. Since the effects on  $\Pi$  and P were close (Figure 1E), those solutes do not allow to 351 distinguish  $\Pi_{int}$  from  $\Pi$ . EG flux was not completely equilibrated after a 15min treatment (Figure 1D), 352 and  $\Pi_{int}$  could be calculated based on equations that describe P variations in cells perfused with a 353 354 permeating solute (Steudle, 1989). However, experimental variations did not allow us to reach a 355 sufficient resolution of the hydraulic and solute relaxation phases in cells under EG treatment. Thus, 356 our current study cannot conclude on the ability of root cells to respond to changes in  $\Pi_{int}$ .

357 Finally, we would like to point other kinds of avenues for interpreting our data. Firstly,  $\Pi$ 358 changes are isotropic in the hydroponic solution, so that all root parts were somewhat homogeneously 359 challenged. In contrast, P, which was only measured in resting cortical cells, close to root tip, may be 360 different in other cell types. For example, epidermal cells of Arabidopsis roots usually show a P that is 361 about 0.1MPa lower than that of cortical cells (Javot et al., 2003). This difference translates into a shift in the response curve of P to  $\Pi$  and could eventually lead to plasmolysis, in a limited number of cell 362 363 types, and under the most severe osmotic challenges. Secondly, gene expression data were obtained 364 from a whole root mRNA extraction, and could mask cell-type specific regulations which are known to exist (Ma and Bohnert, 2007; Dinneny et al., 2008). Finally, the transcriptome status at 15min is the 365 366 consequence of regulatory mechanisms that were activated within the first 10 min, where turgor 367 pressure was in a transient status. A more detailed kinetic of the early events would shed light on the 368 gene regulatory networks (Krouk *et al.*, 2010) activated very early by  $\Pi$  or P variations. All these aspects

deserve more investigations at the cell, gene and genome levels, for which our current work provide awell-defined framework.

#### 371 Mechanisms of mRNA abundance regulation by $\Pi$ or P

In this study, mRNA abundance, as monitored by microarrays or RT-qPCR, was employed as a readout
of water deficit signaling. We realize that changes in a mRNA abundance can be due to many molecular
aspects acting on their synthesis or decay. We gathered informations for two of them: the
corresponding promoter activity and its RNA degradation.

376 We first analyzed the promoters of genes in the  $\Pi$ - or P-specific clusters for the presence of 377 binding sites for putative TFs that could regulate their expression at the transcriptional level (Sup data 378 S3 and S4). We mostly identified binding sites for TFs belonging to the C2C2dof, ABI3VP1, BBR/BPC, 379 C2H2, ZFHD, and REM families. Members of these families have been involved in a broad range of 380 processes but are not specific of water deficit (Yamaguchi-Shinozaki and Shinozaki, 2006; Coutand et 381 al., 2009; Noguero et al., 2013; Mantegazza et al., 2014; Taylor-Teeples et al., 2015; Perrella et al., 382 2018; Lai et al., 2021; Yan et al., 2021; Wang et al., 2022). This result corroborates the idea that a 383 multiplicity of TFs can regulate each gene. It also indicates that the short-term responses to both  $\Pi$ and P likely occur through transcriptional regulation, for which we could not identify representative 384 385 motives nor the critical role of specific TFs.

386 To address a possible role of mRNA degradation, we referred to a previous work that studied mRNA decay, but under control conditions (Sorenson et al., 2018). With a median T<sub>1/2</sub> of >100 min at 387 388 the whole genome level, we hypothesized that a short T<sub>1/2</sub> in resting conditions might be a prerequisite 389 for genes we identified as down-regulated within 15 min by the osmotic treatments ( $\Pi$  cluster 2 and 390 P cluster 1, Figures 3 and 4). Indeed, the relatively short  $T_{1/2}$  calculated for genes of  $\Pi$ -specific cluster 391 2, together with a promoter activity arrest, is compatible with the regulation we observed. Conversely, 392 this may not be the case for genes of P cluster 1 which showed a median  $T_{1/2}$  similar to that of the 393 whole genome. Here, we speculate that on top of a down-regulation of their promoter activity, a 394 reduction in their  $T_{1/2}$  should be induced by the osmotic challenges, thereby leading to their rapid 395 downregulation. Indeed, phosphorylation of proteins of the mRNA decapping complex is regulated by 396 osmotic stresses (Sieburth and Vincent, 2018). The multiplicity of  $\Pi$  or P responses that we identified 397 therefore seems to translate into a similar complexity of mRNA regulation mechanisms, and provides 398 an interesting avenue for further investigation.

#### 399 Functions regulated by $\Pi$ or P

400 Our approach could possibly identify genes which function in  $\Pi$  or P signaling. Semantic and gene 401 ontology enrichments were performed on the gene lists and identified complementary terms. Generic 402 terms retrieved by this approach were mainly associated to transcriptional regulation, responses to 403 abiotic or biotic stimuli, and the cell wall (Figure 6A and B). It is difficult to extract any precise signaling 404 pathway here since many annotations of these genes are inferred, and some of the terms define 405 diverse functions. For example, arabinogalactan proteins are involved in many processes in roots 406 including biotic and abiotic responses (Hromadová et al., 2021), and genes of the C1-like domain 407 superfamily have been associated to various biological/developmental processes, including root 408 epidermal cell differentiation (Bruex et al., 2012). It is also somehow surprising to extract terms related 409 to biotic stresses and defense responses. However, this may result from genes whose annotation originates from, but not necessarily restricts to, "biotic" conditions, or whose function was only 410 411 indirectly inferred. Indeed, our approach uncovers genes associated to the "short-term", less specific, 412 general stress response (Figure 6C)(Bjornson et al., 2021). Importantly, we introduce here the notion 413 that there is a quantitative relationship between the mRNA abundance of these genes and physico-414 chemical parameters (Figure 3C).

#### 415 Quantitative responses to physico-chemical parameters

416 Our study integrates into earlier works focused on the perception of the physico-chemical conditions 417 of cells. We investigated here the dose dependent effects of physico-chemical parameters on the root 418 transcriptome. Such an approach has been successfully applied in poplar, where it was shown that the 419 abundance of ZFP2 mRNA is correlated to the sum of strains upon stem bending (Coutand et al., 2009), 420 and which initiated great advances on the understanding of thigmomorphogenesis. With respect to 421 water, a study was performed in sunflower where a generalized linear model fed by the expression 422 level of 3 genes was developed in order to compute integrated parameters such as the pre-dawn water 423 potential or the soil water content (Marchand et al., 2013). There is a gap between obtaining 424 correlations between physico-chemical parameters and gene expression -such as what we present 425 here, and creating biomarkers or biosensors (Jones, 2014). Nevertheless, the genes identified in the 4 426 clusters could serve as molecular reporters to investigate the perception and signaling of  $\Pi$  or P. 427 Indeed, this has been successfully achieved for temperature sensing, where the promoter of HSP70 428 was used as a quantitative reporter of ambient temperature, and allowed to discover the role of H2A.Z 429 proteins in the temperature-dependent modulation of transcription (Kumar and Wigge, 2010).

#### 430 Conclusion

Thanks to a combination of physiological techniques and a transcriptome approach, we showed theexistence of rapid, specific transcriptional responses to water-related physico-chemical parameters.

- We propose herein a list of early responsive genes whose mRNA abundance in quantitatively
  correlated to external Π or to cell P. This list provides potential reporter genes that could serve to
  elaborate biomarkers of the plant cells water status. This study also paves the way for future dissection
- 436 of the molecular perception of water deficit in plants, through the identification of how their mRNA
- 437 abundance is regulated.

### 438 Supplementary data

- 439 Table S1: primers sequences for the RT-qPCR analysis
- 440 Supplemental data S1: p-values of the ANOVA analyses and probe/AGI correspondence for the441 Gene1.1 ST array
- 442 Supplemental data S2: summary of TFs binding sites enrichment in the Π and P clusters
- 443 Supplemental data S3 A, B, C: output of the promoter analysis for each cluster
- Supplemental data S4: gene list of each cluster, highlighting the genes that were tested further byRT-qPCR

446

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### 451 Authors contributions

YB and GK conceptualized the research. AC and YH performed the research with contributions from TB
for physiological measurements. YH, GK and YB analyzed the data with contributions from SR and CM.
YB wrote the article with insights from CM. All authors contributed to reviewing the manuscript and
agreed to its content.

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### 462 **Conflict of interest**

463 None declared

### 464 **Data availability**

The transcriptomic data that support the findings of this study are openly available in Gene Expression
Omnibus Series (http://www.ncbi.nlm.nih.gov/geo/) GSE223207.

The code and source files (besides transcriptomic data) used to analyze the data and/or to generate 467 468 the figures 1 to 5 of this study can be downloaded from 469 https://github.com/ybinrae/Watermarker paper1.git .

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## 569 Tables

## 570 Table 1: Summary of the osmotic treatments applied to the roots, and the factors of the ANOVA 571 model.

- 572 Solutes were dissolved in the hydroponic solution. The osmotic potential of the solution was measured
- 573 at 20°C with an osmometer (Wescor), 3 digits after the decimal point are shown. The osmotic potential
- 574 is the opposite of the osmotic pressure.

Solute	Concentration	П	P <sub>cort</sub>	NaCl <sub>factor</sub>	Sorbitol <sub>fac</sub>	PEG <sub>factor</sub>	EG <sub>f7650</sub> r
	(mM, g/l for				tor		
	PEG)						576
None	0	0.021	0.41	0	0	0	0
NaCl	25	0.117	0.32	25	0	0	0
	50	0.238	0.25	50	0	0	0
	75	0.355	0.14	75	0	0	0
	100	0.425	0.05	100	0	0	0
Sorbitol	50	0.134	0.32	0	50	0	0
	100	0.255	0.18	0	100	0	0
	150	0.370	0.1	0	150	0	0
Polyethylene	75	0.073	0.31	0	0	75	0
glycol 8000	100	0.131	0.26	0	0	100	0
	125	0.209	0.2	0	0	125	0
	150	0.280	0.14	0	0	150	0
Ethylene Glycol	50	0.151	0.3	0	0	0	50
	100	0.268	0.29	0	0	0	100
	150	0.394	0.29	0	0	0	150
	200	0.516	0.26	0	0	0	200

### 577 Figure legends

### 578 Figure 1: Osmotic treatments reduce the P of root cortical cells in Arabidopsis.

579 A portion of ~3cm of root of 21 day-old plants, laid on a perfused Whatman paper, was treated with 580 various solutes at different concentrations. Cortical cell P was measured with a cell pressure probe. A to D: measurement kinetics performed on plants treated with NaCl at concentrations of 0, 25, 50, 75, 581 100mM (the darker the color, the more concentrated, n>2 for each treatment), sorbitol at 50, 100 and 582 150mM, PEG8000 at 75, 100, 125 and 150 g.l<sup>-1</sup>, and EG at 50, 100, 150 and 200mM, respectively. Zero 583 584 in the time axis indicates the change in perfusion from hydroponic solution to the same solution 585 complemented with treatments. A lowess smoothing was added in order to highlight the general 586 behavior of P after each treatment E: plot recapitulating the measurements of P within the 10-20min 587 time frame as a function of the osmotic pressure of the solution (average value  $\pm$  SEM, n  $\geq$  2, blue: 588 sorbitol, pink: NaCl, green: PEG, red: EG).

589

### 590 Figure 2: Features of the early transcriptomic response to osmotic treatments.

A: Dendrogram illustrating the effects of the solute nature and concentration on the regulation of gene expression. B: number of DEPs classified according to the solute used for the treatment. The matrix below indicates if those DEPs were found for a single or for various solute(s). C: Gene expression signals in the different treatments. Genes regulated by one solute only were selected from B and split in 2 clusters. The average, centered, expression value for each gene is plotted against the combination of solutes and concentrations used in the transcriptomic approach.

597

### 598 Figure 3: Correlations between gene expression and osmotic or turgor pressure.

599 A: Venn diagram showing the number of genes, in the same transcriptomic approach as in figure 2, 600 which expression is significantly correlated to  $\Pi$ , P, or both. **B**: The 137 " $\Pi$ -specific" genes and 401 "P-601 specific" genes were split in 2 clusters each, and their average centered expression is expressed as a 602 function of the solute/treatment combination corresponding to the biological assays of the 603 transcriptome approach. C: The 137 " $\Pi$ -specific" genes were separated into 2 clusters and their 604 average centered expression is expressed as a function of the osmotic potential of the solution of 605 treatment, or the cortical cell turgor pressure. D: same representation as in C but for the 401 "P-606 specific" genes.

607

### **Figure 4: mRNA half-life time is reduced in 3 clusters in control conditions.**

The half-life time of mRNAs ( $T_{1/2}$ ) was calculated from Sorenson et al. 2018, based on the decay rate ( $\alpha$ ) modeled upon cordycepin treatment on *sov* mutant seedlings (i.e. Col 0,  $T_{1/2} = \ln(2)/\alpha$ ) (Sorenson *et al.*, 2018).  $T_{1/2}$  of each mRNA from genes in the clusters are presented individually and as boxplots. The numbers above the boxplots correspond the p-value of a bootstrap based test of  $T_{1/2}$  of the cluster

being smaller than the whole genome median  $T_{1/2}$  (see M&M).

614

### **Figure 5: RT-qPCR validation of osmotic or turgor pressure clusters.**

- The expression of three candidates per list:  $\Pi$  or P, clusters 1 and 2 were investigated by RT-qPCR in 3 independent biological replicates. The plants were harvested 15min after transfer into a hydroponic solution, or the solution complemented with 25, 50,75, 100mM NaCl, or 50, 100, 150mM Sorbitol, or 50, 100, 150, 200g.l<sup>-1</sup> EG. For each gene, normalized RT-qPCR signal (± SEM) is expressed as a function of the averaged signal (± SEM) obtained from the transcriptome analysis. The p-value of a correlation test (Pearson) as well as the linear fit (with its R<sup>2</sup> value) between the average values are indicated for
- 622 each gene.
- 623

### Figure 6: classification of the $\Pi$ and P correlated genes.

A: output of the Genecloud semantic analysis for the 2 clusters showing a significant enrichment. B:

626 Output of a gene ontology analysis for the 2 clusters showing significant enrichments in GO terms. The

627 GO terms, gene counts for each GO term and adjusted p-value of the enrichment are presented. C:

- Degree of overlap (Z-score) between our genes lists and public genes lists related to osmotic stress,
   the general stress response, and a list of transcription factors, using the Genesect algorithm.
- 630



A portion of ~3cm of root of 21 day-old plants, laid on a perfused Whatman paper, was treated with various solutes at different concentrations. Cortical cell P was measured with a cell pressure probe. A to D: measurement kinetics performed on plants treated with NaCl at concentrations of 0, 25, 50, 75, 100mM (the darker the color, the more concentrated, n>2 for each treatment), sorbitol at 50, 100 and 150mM, PEG8000 at 75, 100, 125 and 150 g.l-1, and EG at 50, 100, 150 and 200mM, respectively. Zero in the time axis indicates the change in perfusion from hydroponic solution to the same solution complemented with treatments. A lowess smoothing was added in order to highlight the general behavior of P after each treatment E: plot recapitulating the measurements of P within the 10-20min time frame as a function of the osmotic pressure of the solution (average value  $\pm$  SEM, n  $\ge$  2, blue: sorbitol, pink: NaCl, green: PEG, red: EG).





A: Dendrogram illustrating the effects of the solute nature and concentration on the regulation of gene expression. B: number of DEPs classified according to the solute used for the treatment. The matrix below indicates if those DEPs were found for a single or for various solute(s). C: Gene expression signals in the different treatments. Genes regulated by one solute only were selected from B and split in 2 clusters. The average, centered, expression value for each gene is plotted against the combination of solutes and concentrations used in the transcriptomic approach.





A: Venn diagram showing the number of genes, in the same transcriptomic approach as in figure 2, which expression is significantly correlated to Pi, P, or both. B: The 137 "Pi-specific" genes and 401 "P-specific" genes were split in 2 clusters each, and their average centered expression is expressed as a function of the solute/treatment combination corresponding to the biological assays of the transcriptome approach. C: The 137 "Pi-specific" genes were separated into 2 clusters and their average centered expression is expressed as a function of the solution of the osmotic potential of the solution of treatment, or the cortical cell turgor pressure. D: same representation as in C but for the 401 "P-specific" genes.



# Figure 4

The half-life time of mRNAs (T1/2) was calculated from Sorenson et al. 2018, based on the decay rate ( ) modeled upon cordycepin treatment on sov mutant seedlings (i.e. Col 0, T1/2=  $\ln(2)/$ ) (Sorenson et al., 2018). T1/2 of each mRNA from genes in the clusters are presented individually and as boxplots. The numbers above the boxplots correspond the p-value of a bootstrap based test of T1/2 of the cluster being smaller than the whole genome median T1/2 (see M&M).



Figure 5

The expression of three candidates per list: or P, clusters 1 and 2 were investigated by RT-qPCR in 3 independent biological replicates. The plants were harvested 15min after transfer into a hydroponic solution, or the solution complemented with 25, 50,75, 100mM NaCl, or 50, 100, 150mM Sorbitol, or 50, 100, 150, 200g.l-1 EG. For each gene, normalized RT-qPCR signal ( $\pm$  SEM) is expressed as a function of the averaged signal ( $\pm$  SEM) obtained from the transcriptome analysis. The p-value of a correlation test (Pearson) as well as the linear fit (with its R<sup>2</sup> value) between the average values are indicated for each gene.



## Figure 6

A: output of the Genecloud semantic analysis for the 2 clusters showing a significant enrichment. B: Output of a gene ontology analysis for the 2 clusters showing significant enrichments in GO terms. The GO terms, gene counts for each GO term and adjusted p-value of the enrichment are presented. C: Degree of overlap (Z-score) between our genes lists and public genes lists related to osmotic stress, the general stress response, and a list of transcription factors, using the Genesect algorithm.