Mimicking drought transcriptional responses using a high throughput plate assay

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Abstract

Soil-free assays that induce water stress are routinely used for investigating plant responses to drought conditions. Due to their ease of use, the research community often relies on polyethylene glycol (PEG), mannitol and salt (NaCl) treatments to reduce the water potential of growth media, thus mimicking drought conditions in the laboratory. However, while these types of stress can create phenotypes that resemble those of drought, it remains unclear how they compare at the molecular signaling level. Here, using transcriptomics, we demonstrate that while these assays elicit differential expression of root genes involved in drought signaling, many drought-induced genes were repressed by such treatments (and vice versa). In light of this, we designed a new method for simulating drought. By simply adding less water to agar, we found our 'low-water' agar (LW) assay elicits root gene expression responses that hold greater directional agreement with drought signaling responses. Furthermore, we show our approach can be leveraged as a high-throughput assay to investigate natural variation in water stress responses.

Introduction

As climate change advances, improving crop drought tolerance will be key for ensuring food security (*1, 2*). This has led to intense research at the molecular level to find novel loci and alleles that drive plant responses to drought conditions. Such investigations demand simple assays that can reproduce drought phenotypes at both the physiological and molecular levels. While some researchers use soil-based assays, these are cumbersome. For example, extracting intact root systems from the soil is difficult, and reproducing the rate at which water evaporates from the soil can be challenging (*3*). In light of this, chemical agents such as polyethylene glycol (PEG), mannitol, or salt (NaCl) are often employed to simulate drought stress. When present in aqueous or agar media, they allow precise and dose-dependent control of water potential (*4, 5*). When exposed to these media types, plants exhibit the hallmarks of drought physiology, such as reduced growth rate, reduced stomatal conductance, and increased leaf senescence (*4, 6, 7*). Notably, each type of stress can exert distinct effects (*8*). For example NaCl not only induces osmotic stress, but can cause salt toxicity (*6*). Since it is not metabolized by most plants mannitol is considered less toxic (*3*), however evidence suggests it may act as a signaling molecule (*5, 9*). Since both NaCl and mannitol can enter the pores of plant cell walls (*8, 10*), they can induce plasmolysis, a process that does not typically occur under mild water deficit (*8*). Due to its higher molecular weight, PEG treatment avoids this, and instead causes cytorrhysis (*8*), a physiology more common under drought settings (*10, 11*).

At the molecular signaling level, it remains unclear whether the stress that PEG, mannitol or NaCl elicit is comparable to drought signaling, where we define drought signaling as the transcriptional responses in plant organs in response to water deficit. While some experimental data suggests that they are comparable (*4, 12, 13*), to our knowledge a side-by-side comparison has not been performed. By taking a transcriptomic approach, we show that genes dose-responsive to PEG, mannitol and NaCl treatment in the root display 27 %, 48 % and 57 % directional agreement respectively with genes induced or repressed by drought. Inspired to develop a new method to simulate drought, we present a ‘low-water’ agar (LW) assay, which exhibited a 87 % directional agreement with drought signaling genes.

PEG, mannitol and NaCl treatments repress drought inducible genes in roots

To benchmark PEG, mannitol and NaCl treatments to one another, we first tested their physiological effect across a range of doses. To this end, we grew *Arabidopsis* seedlings on agar plates supplemented with Linsmaier & Skoog (LS) nutrients for 14 days on three different doses of each stress type. Dose ranges were chosen based on published literature, and ranged from mild to severe stress levels (*3, 4, 11*). As the dose of each stress type increased, the media's water potential significantly decreased in a dose-dependent manner (Spearman, $p < 0.05$). Across the doses tested, we found that each stress type's impact on water potential was not statistically different from one other (ANCOVA post-hoc, $p > 0.17$). In response to these treatments, we found shoot biomass significantly decreased in a dose-dependent manner ($p < 2 \times 10^{-6}$) (**Figure 1A - C**, **Figure 1-figure supplement 1**, **Supplementary File 1**).

Genes that change their expression in response to an environmental signal often do so in a dose-responsive manner (*4, 14*). In light of this, we sought to discover genes whose expression was dose-responsive to the amount of PEG, mannitol, or NaCl applied. Crucially, by identifying genes that were stress responsive across a range of doses, we ensured such genes responded to and were directional with the stress as a whole, and not simply induced or repressed at an individual dose. Taking this approach, we sequenced root and shoot bulk transcriptomes by RNA-seq, and associated each gene's expression with the dose of stress with a linear model. To ensure we captured steady-state differences in gene expression, and avoided those that were transient, we sequenced root and shoot transcriptome profiles after 14 days of stress exposure (*3, 6, 15*). By these means, we found hundreds of genes that were dose-responsive to each treatment within root and shoot tissue (**Figure 1E – 1H, Figure 1-figure supplement 2, Supplementary File 2**) (adj. $p < 0.05$). We note many of these dose-responsive genes were shared across treatments (**Figure 1D**, **Figure 1-supplement Figure 3**).

Next, we wanted to compare these different methods of lowering water potential to an assay that more closely resembled drought. To create this benchmark, we subjected mature *Arabidopsis* plants grown in pots on vermiculite supplemented with LS media to a mild drought stress by withholding water for 5 days. During this period, field capacity reduced from 100 % to 41 %. This treatment led to a reduction in plant biomass ($p = 1.8 \times 10^{-3}$), as well as seed yield ($p = 1.2 \times 10^{-4}$), but did not induce visible signs of senescence or wilting (**Figure 1-figure supplement 4**, **Supplementary File 3**). We assayed root and shoot gene expression responses each day during

137 water loss by RNA-seq. We observed a dose-dependent relationship between a decrease in field
138 capacity and gene expression responses in both roots and shoots, identifying 1,949 drought-
139 responsive genes in roots and 1,792 in shoots (adj. $p < 0.01$) (**Figure 1I, Figure 1-figure**
140 **supplement 2, Supplementary File 2**). We ensured these genes' expression patterns recovered
141 upon rewatering (**Figure 1I**). We note the genes we found differentially expressed agreed with a
142 previous report detailing transcriptional responses to water deficit in soil (*16*) (**Figure 2-figure**
143 **supplement 1**), as well as agreed with genes responsive to transient treatment with Abscisic Acid
144 (ABA), a stress hormone whose levels rise in response to water deficit (*4*) (**Figure 2-figure**
145 **supplement 2**). Thus, we considered the differentially expressed genes we find through our
146 vermiculite drying approach to be involved in drought signaling responses, but acknowledge that
147 this definition excludes other climatic effects associated with drought such as heat stress or light
148 intensity.
149
150 To assess how PEG, mannitol and NaCl treatments compared to the drought signaling response
151 described above, we overlapped genes found differentially expressed in each experiment. For
152 shoot tissue, we found genes that were differentially expressed under drought stress overlapped
153 significantly with genes that were differentially expressed by either PEG, mannitol and NaCl
154 treatments (Fisher test, adj. $p < 0.05$). Additionally, there was 88 – 99 % directional agreement
155 within these overlaps, indicating that genes induced or repressed by drought signaling were
156 similarly induced or repressed by agar stress treatments (**Figure 2A** & **2B**). Along these lines,
157 across all conditions we saw differential expression of stress markers *RESPONSE TO*
158 *DESSICATION 20;29B (RD20;29B) (17, 18)*, the osmo-protectant gene *DELTA1-PYRROLINE-*
159 *5-CARBOXYLATE SYNTHASE 1* (*P5CS1*), and ABA signaling and biosynthesis genes
160 *HOMEOBOX 7* (HB7) (*19*) and *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED3)* (*20*)
161 (**Figure 2-figure supplement 3**).
162
163 In contrast, we did not find a similar result in the root. Here, we found a reduced directional
164 agreement; a greater number of genes that were upregulated during drought signaling were
165 downregulated by PEG, mannitol and NaCl treatments (and vice versa) (**Figure 2A**). This trend
166 persisted when we assessed genes found differentially expressed at each discrete dose of stress
167 (**Figure 2B**). For example, 27 % of PEG dose-responsive genes shared the same direction of
168 expression seen in drought signaling responses. We note that previously published PEG
169 transcriptome datasets agreed with our own (**Figure 2-figure supplement 1**). Such mis-
170 regulation is exemplified by the expression of stress markers *HOMEOBOX 12* (*HB12*) (*19*)

171 (**Figure 2C**), *GRC2-LIKE 1* (*GCL1*) (*21*), and *RESPONSE TO DEHYDRATION* 21 (*RD21*) (*22*)
172 (**Figure 2-figure supplement 3**). Mannitol and NaCl held a 48 % and 57 % agreement in gene
173 expression direction respectively. Examples of stress genes that followed a pattern of mis-
174 regulation in mannitol and NaCl treatments were *DROUGHT HYPERSENSITIVE 2* (*DRY2*) (*23*)
175 (**Figure 2D**) and *ROOT HAIR SPECIFIC 18* (RHS18) (*24*).
176
177 Why does PEG down-regulate drought-induced genes in the root? We hypothesize that the genes
178 that were repressed by PEG may be the result of hypoxia. We base this on PEG's ability to impede
179 oxygen solubility (*25, 26*). This appears to be reflected at the molecular level, where genes
180 downregulated by PEG are over-represented in the 'monooxygenase activity', and 'oxygen
181 binding' Gene Ontology (GO) Terms ($p < 1 \times 10^{-15}$, **Supplementary File 4**). In contrast, NaCl-
182 responsive GO Terms included a specific downregulation of 'phosphorous metabolic processes'
183 ($p = 5.2 \times 10^{-6}$), suggesting that the roots were changing phosphate levels in response to NaCl, a
184 process known to help maintain ion homeostasis (*27*). For mannitol, we observed a specific
185 downregulation of 'cell wall organization or biogenesis' and 'microtubule-based processes' ($p <$
186 7.8×10^{-3}), suggesting a unique root developmental response to mannitol, possibly mediated by
187 mannitol acting as a signaling molecule (*9*). For both mannitol and NaCl stress, we saw
188 photosynthesis-related GO Terms enriched among genes upregulated in the roots, which may be
189 due to an interaction between the osmotic stress and exposing the roots to light (*28*)
190 (**Supplementary File 4**).
191

192 **The 'low-water agar' assay recapitulates drought signaling responses in the root**

193
194 In addition to examining PEG, mannitol and NaCl transcriptional responses, we were also
195 motivated to design a new way of simulating drought on an agar plate. We hypothesized that
196 instead of adding a compound to reduce water potential, we could mimic drought by adding less
197 water to agar media (leading to both higher agar and nutrient concentrations). We called this
198 media 'low-water' (LW) agar, and by testing three different doses (80 %, 60 % and 40 % water
199 content), found that it limited plant shoot dry weight and media water potential in a similar way to
200 PEG, mannitol and NaCl (**Figure 1A - C**). Additionally, we found that LW treatment limited primary
201 root growth rate, shoot water potential, and photosynthesis efficiency (**Figure 2-figure**
202 **supplement 4**). At the molecular level, RNA-seq revealed 1,376 and 1,921 genes that were dose-
203 responsive to the level of LW stress in roots and shoots respectively (**Figure 1E & Figure1-figure**

204 **supplement 2**). We found that these gene expression responses overlapped significantly with
205 those found involved in drought signaling (Fisher test, $p < 1 \times 10^{-32}$) (**Figure 2A** & **2B**). Compared
206 to PEG, mannitol and NaCl, we found genes differentially expressed in response to LW agar held
207 a greater directional agreement with drought signaling responses (87 % directional agreement).
208 Such directional agreement can be seen in stress marker expression of genes such as *HB12*
209 (**Figure 2C**), *GCL1* and *RD21* (**Figure2-figure supplement 3**).
210
211 Adding less water to nutrient agar solution increases both nutrient and agar concentrations. An
212 increase in nutrient concentration can induce salt-like stress while increasing agar concentration
213 will increase tensile stress (*29*). We tested each of these variables separately to understand the
214 role each played in eliciting the gene expression responses found in the LW assay. To do this,
215 we repeated our LW dose experiment, but now increasing only the concentration of LS nutrients
216 (1, 1.25, 1.67 and 2.5 X) or the concentration of agar (2, 2.5, 3.3 and 5 %) (**Figure 2E**). We found
217 a significant decrease in shoot area size in response to an increase in nutrient concentration
218 (Pearson $p = 1.7 \times 10^{-7}$) and agar concentration ($p = 3.9 \times 10^{-13}$), where the latter more closely
219 phenocopied the effect of LW (**Figure 2E**, **Figure 2-figure supplement 5, Supplementary File**
220 **5**). Since the increase in nutrient concentration alone was responsible for changing media water
221 potential, the phenotypic response to increased agar concentration was not in response to a lower
222 water potential (**Figure 2-figure supplement 5**). Next, we examined the transcriptional responses
223 underlying nutrient and agar responses by sequencing root tissue across each dose tested.
224 Through linear modeling, we found 1,043 genes and 938 genes that were dose-responsive to
225 nutrient or agar concentration, respectively. Next, we investigated whether these genes agreed
226 with those found in drought signaling responses. We found that genes differentially expressed in
227 response to an increase in agar or nutrient concentration overlapped 12 % and 17 % of drought
228 responsive gene expression respectively (permutation test, $p < 0.05$) (**Figure 2F, Figure 2-figure**
229 **supplement 5, Supplementary File 6**). However, we found genes differentially expressed in
230 response to LW treatment led to a higher overlap (26 %), suggesting that both nutrient and agar
231 concentration contribute to LW performance.
232
233 Finally, we tested if our LW assay was sensitive enough to detect phenotypic variability. To
234 achieve this, we grew 20 different *Arabidopsis* ecotypes on 50 % LW agar, where ecotypes were
235 selected from a previous drought study that assessed fitness in a common garden experiment
236 (*30*). By comparing the total shoot area after three weeks of growth, we found that our assay
237 revealed variability in shoot growth responses (**Figure 2G** & **2H**, **Supplementary File 7**).

238 Similarly, we found that the relative impact LW agar had on an accession's shoot size was
239 associated with the relative impact drought had on its fitness, as measured under field conditions
240 (Spearman p = 0.04, **Figure 2-figure supplement 6**) (*30*). This suggests that our assay may be
241 useful for screening for novel drought-associated loci among a wider group of accessions or
242 mutants.
243
244 In summary, the LW agar assay presents a new approach for simulating root drought signaling
245 responses on an agar plate. We note that our assay cannot mimic many additional effects drought
246 stress can have on gene expression in real environments – such as those that arise from changes
247 in soil structure or the root microbiome (*31*). However, by inducing growth arrest and gene
248 expression responses comparable to drought signaling, LW agar offers a high-throughput method
249 to screen phenotypes and probe gene regulatory responses associated with drought signaling.
250 We describe how to make LW media in the Materials and Methods.
251
252
253 Supplementary
254
255 **Supplementary File 1** – Plant physiological measurements
256 **Supplementary File 2** – Differentially expressed genes and normalized counts in LW, PEG,
257 Mannitol, NaCl or Vermiculite drought stress experiments
258 **Supplementary File 3** – Vermiculite drought stress assay measurements
259 **Supplementary File 4** – GO Term enrichment of differentially expressed genes
260 **Supplementary File 5** – Shoot area of seedlings grown under different agar and nutrient
261 concentrations
262 **Supplementary File 6** – Differentially expressed genes and normalized counts in response to
263 changes in nutrient or agar concentration
264 **Supplementary File 7** – Shoot area of different *Arabidopsis* accessions grown on LW media
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Data Availability

Raw sequencing data is available at the National Center for Biotechnology Information Sequence Read Archive (accession number PRJNA904764). Normalized read counts and raw phenotypic datasets can be found in the Supplementary Material.

Materials and Methods

Low-Water Agar Assay – *Arabidopsis* seedlings were grown on vertical plates for 8 days under short day conditions (8 h light, 21 C, 150 umoles light) on agar media (1 × Linsmaier & Skoog (LS) (Cassion LSP03) media, 1% sucrose, 2% agar, pH 5.7). After 8 days, plants were transferred to 'low-water' (LW) plates that contained varying water content. The 100% treatment plate, where water is not lacking, contained 75 mL of 2% agar and 1× LS media. Drought was simulated by preparing the same media but reducing the amount of water present. For example, the 80% treatment plate contained 60 mL of 2.5% agar and 1.25× LS media, pH 5.7. Plants were grown upon 3 different treatments (80%, 60%, and 40% water amount) for 14 days. We note that the volume of LW itself does not impact gene expression responses (**Figure 2-figure supplement 7**). On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). To test different *Arabidopsis* accessions on LW agar, plants were sown on either 100% or 50% treatments as described above, however supplemented with 0.5% or 1% sucrose respectively to encourage germination. Seedlings were grown for 3 weeks under short day conditions in before imaging plates in duplicate (n = 2 - 5 plants per plate) (**Supplementary File 7**). Shoot area was calculated from images using Plant Growth Tracker (GitHub - https://github.com/jiayinghsu/plant_growth_tracker).

305 Vermiculite Drought Assay – *Arabidopsis* seedlings were grown on vertical plates for 17 days
306 under short day conditions (8 h light, 21 C, 150 umoles light) on agar media (1×LS, 1% sucrose,
307 2% agar, pH 5.7), before transfer to vermiculite saturated with 0.75 × LS media. Plants were then
308 grown on vermiculite at 100% field capacity (FC) for 12 days (8 h light, 21 C, 150 umoles light).
309 On the 13th day, the first time point was sampled (4.5 hours after subjective dawn) where tissue
310 was flash frozen in liquid nitrogen. After this, excess aqueous solution was drained from each pot,
311 and then each pot was calibrated to 1 × FC. Plant tissue was harvested each day on subsequent
312 days at the same time of day. Each day, pots were weighed to measure extent of evaporation. By
313 these means, FC was measured (**Figure 1-figure supplement 4**). After the 5th day sample was
314 taken, water was re-added to the remaining pots to an excess of 1× FC. ~ 15 plants were sampled
315 per time point. Plants were then left to grow under long day conditions until flowering. Seeds were
316 harvested, dried, and weighed (n = 50 plants per treatment).
317
318 Polyethylene Glycol (PEG) Stress Assay – *Arabidopsis* seedlings were grown on vertical plates
319 for 8 days under short-day conditions (8 h light, 21 C, 150 umoles light) on agar media (1×LS, 1%
320 sucrose, 2% agar, pH 5.7), before transfer to polyethylene glycol (PEG) media of varying
321 concentrations. PEG media plates were prepared by dissolving crystalline 6000 MW PEG into
322 freshly autoclaved 1× LS media pH 5.7 and pouring 50 mL of PEG media solution onto 1× LS, 2%
323 agar, media plates (pH 5.7), letting the PEG solution diffuse into the solid media overnight, then
324 pouring off excess and transferring seedlings to PEG infused media plates as described in (*11*).
325 Plants were grown under 3 different treatments (12%, 20%, and 28% PEG solution w/v) for 14
326 days. On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6
327 plants per replicate).
328
329 Mannitol and NaCl Osmotic Stress Assays – *Arabidopsis* seedlings were grown on vertical plates
330 for 8 days under short-day conditions (8 h light, 21 C, 150 umoles light) on agar media (1× LS,
331 1% sucrose, 2% agar, pH 5.7), before transfer to either mannitol or salt (NaCl) media of varying
332 concentrations. Mannitol and NaCl media plates were prepared by adding respective volume of
333 stock solution to 1× LS, 2% agar, pH 5.7 media before autoclaving. Plants were grown under 3
334 different treatments of mannitol or NaCl (50 mM, 100 mM and 200 mM for mannitol, 30 mM, 75
335 mM, and 150 mM for NaCl) for 14 days. On day 14, 2 hours after subjective dawn, shoot and root
336 samples were flash frozen (6 plants per replicate).
337

338 ABA Exogenous Treatment Assay – *Arabidopsis* seedlings were grown on vertical plates for 8
339 days under short-day conditions (8 h light, 21 C, 150 umoles light) on agar media (1× LS, 1%
340 sucrose, 2% agar, pH 5.7), before transfer to 1× LS, 2% agar, pH 5.7 control media and grown
341 for 14 days. On day 14, abscisic acid (ABA) solutions of 1 uM, 5 uM and 10 uM were prepared
342 from 10 mM ABA dissolved in ethanol stock, as well as a mock treatment solution containing 0.1%
343 ethanol concentration. 30 min after subjective dawn, 15 mL of each solution was dispersed onto
344 the roots of the seedlings. After 1 min of treatment, the ABA solution was removed from the plates,
345 and the plates returned to the growth chamber. 2 hours after subjective dawn, shoot and root
346 samples were flash frozen (6 plants per replicate).
347
348 Osmotic Potential Measurements: The water potential of media was determined considering it
349 equivalent to the osmotic potential (Ψs). Osmotic potential was measured using a vapor pressure
350 osmometer (Model 5600, ELITech Group; Puteaux, France). Readings were taken from melted
351 agar media constituted with the particular stress type. Osmolality readings for each sample
352 obtained were converted to megapascals (MPa) using the equation Ψs = -CRT, where C is the
353 molar concentration, R is the universal gas constant, T is the temperature in Kelvin. Two weeks
354 after transplanting the seedlings to LW agar media, we assessed the osmotic potential of shoot
355 tissue. After immersion in liquid nitrogen 3 shoots were placed into 0.5-ml tubes and centrifuged
356 to extract the tissue sap. The osmotic potential (Ψs) of the extracted sap was determined using a
357 vapor pressure osmometer.
358
359 Chlorophyll fluorescence measurements: Chlorophyll fluorescence was assessed in eight
360 seedlings of each plate using the Walz PAM IMAGING PAM M-series IMAG-K7 (MAXI)
361 fluorometer. For every experiment, leaves were pre-conditioned in the dark for 1 h. The maximum
362 quantum yield of PSII (F_v/F_m) was calculated using the formula:
363 $$F_v/F_m=(F_m-F_0)/F_m$$
364 Where F_v is the variable fluorescence, F_m is the maximal fluorescence following 1 h of dark
365 adaptation and F_0 is the minimal fluorescence level of a dark-adapted leaf when all photosystem
366 II (PSII) reaction centers are open.
367
368 Root Growth Rate Measurements – *Arabidopsis* seedlings were grown on vertical plates for 8
369 days under short day conditions (8 h light, 21 C, 150 umoles light) on agar media (1× LS, 1%
370 sucrose, 2% agar), before transfer to 40% LW, 28% PEG, 150 mM mannitol or 150 mM NaCl
371 treatment plates as described above. Root images were acquired every two days for a total of 8

372 days using scanners. Primary root length, defined as the length (scaled to
373 cm) from hypocotyl base to root tip, was quantified using image J. For each treatment we
374 screened 4 plates, with each plate holding 4 individual plants.
375
376 RNA-extraction and Library Preparation – Plant tissue was crushed using the TissueLyser
377 (Agilent) and RNA extracted using RNeasy Mini Kit (Qiagen). RNA quality was assessed using
378 Tape station High Sensitivity RNA assay (Agilent). 0.5 - 1 ug of total RNA proceeded to library
379 preparation, where libraries were prepared using TruSeq stranded mRNA kit (Illumina). Resulting
380 libraries were sequenced on the NovaSeq 6000 (Illumina) with 2x150 bp paired-end read
381 chemistry. Read sequences were aligned to the *Arabidopsis* TAIR10 genome using HISAT2 (*32*),
382 and gene counts called using HT-seq (*33*), by relying on Araport11 annotation (*34*). Normalized
383 counts can be found in **Supplementary File 2**. For each organ, libraries from all experiments
384 were normalized together before calling differential expression.
385
386 Statistical Analysis – To detect differential expression in our drought assay on vermiculite, we
387 called differential expression using a linear model using the DESeq2 LRT function to associate a
388 change in field capacity with change in gene expression. The same statistical approach was used
389 to associate a change in a gene's expression to changes in dose of LW, PEG, mannitol, and
390 NaCl, as well as changes in agar concentration, nutrient concentration and volume of agar used.
391 Resulting model p-values were adjusted to account for false discovery (p-value < 0.05). The
392 complete list of differentially expressed genes for each experiment can be found in
393 **Supplementary File 2** and **Supplementary File 6**. Pairwise differential gene expression was
394 called using DESeq2 (*35*). Specifically, for plate based assays, we called differential expression
395 by comparing the control treatment to each treatment dose, using an adjusted p-value threshold
396 of 0.05. Overlap analyses were performed using Fisher exact tests, with an adjusted p-value
397 threshold of 0.05. The background for these intersects was all expressed genes within the
398 respective organ. Permutation tests and GO Term enrichment analyses was performed in
399 VirtualPlant (*36*), with all expressed genes within the respective organ used as background.
400
401

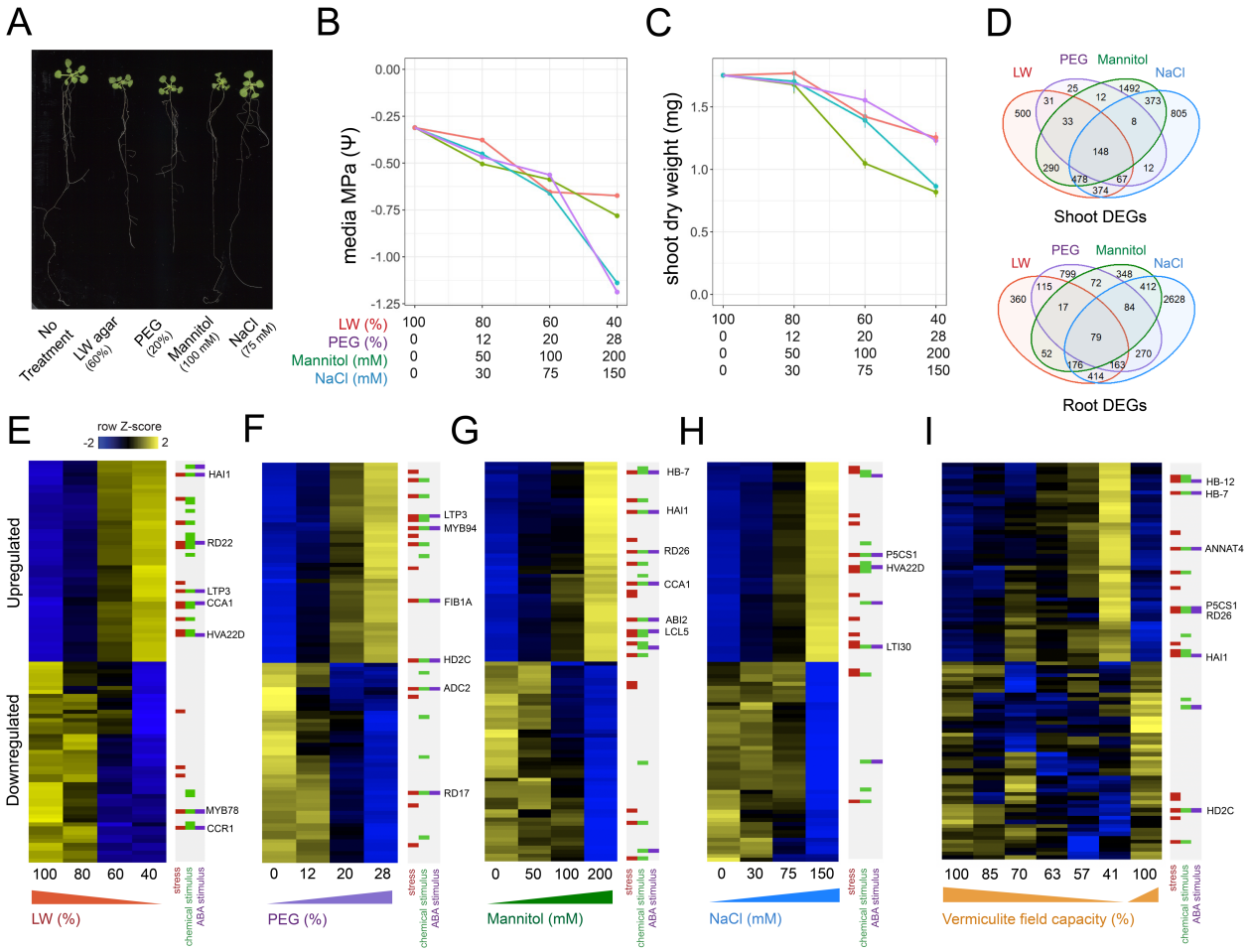

Figure 1 - Benchmarking the impact different stress assays have on *Arabidopsis* physiology and gene expression. A: 22-day-old *Arabidopsis* growth on plates under either 60 % low-water (LW) agar, 20 % PEG, 100 mM mannitol, or 75 mM NaCl treatments. **B**: Water potential measurements of treatment media. **C:** Dry weight of 22-day-old *Arabidopsis* seedlings under different doses of each stress treatment (n = 11 - 12). **D:** Number and intersect of differentially expressed genes (DEGs) that are dose-responsive to each stress treatment within root and shoot tissue. **E - I:** Heatmaps displaying the top 50 most significant upregulated or downregulated genes in response to **(E)** LW, **(F)** PEG, **(G)** mannitol, **(H)** NaCl and **(I)** vermiculite-induced drought stress in the *Arabidopsis* root. Membership of Gene Ontology (GO) Terms for 'response to stress', 'response to chemical stimulus' or 'response to ABA stimulus' are indicated.

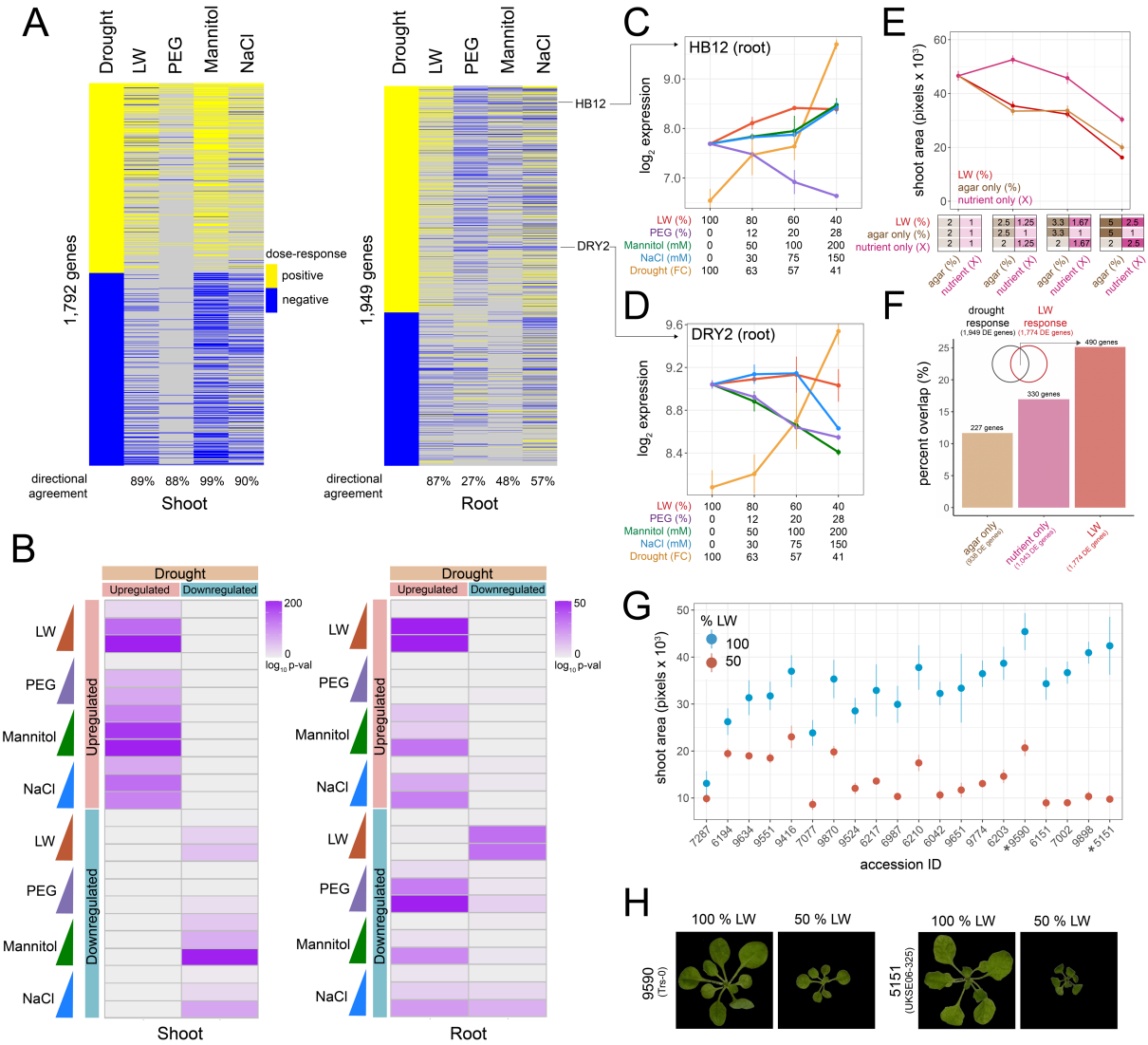

Figure 2 - Comparative transcriptomic analysis reveals PEG, mannitol, and NaCl downregulate drought-inducible genes in the root. A: Heatmap displaying genes differentially expressed under drought stress in shoot or root tissue compared to their dose-responsive expression within each agar stress assay. Level of 'directional agreement' (i.e., differentially expressed in the same direction) found within each assay reported. **B:** Overlap analysis of genes found differentially expressed under drought stress, compared to those found differentially expressed within each dose of PEG, mannitol, NaCl or LW agar assays in both shoot and root. **C - D:** Expression patterns of stress marker genes *HOMEOBOX12* (*HB12)* and *DROUGHT HYPERSENSITIVE 2* (*DRY2)* across each assay in root tissue. **E:** Shoot area of seedlings grown under increasing doses of LW, agar or nutrient concentrations (n = 19). **F:** Number and percent overlap of genes found differentially expressed in response to increasing doses of LW, agar or nutrient concentrations with those differentially expressed in response to drought stress. **G:** Total shoot area of *Arabidopsis* accessions grown under either 100 % or 50 % LW agar treatment (n = 5 - 12). **H:** Images of *Arabidopsis Trs-0* or *UKSE06-325* accessions grown on either 100 % or 50 % LW treatment.

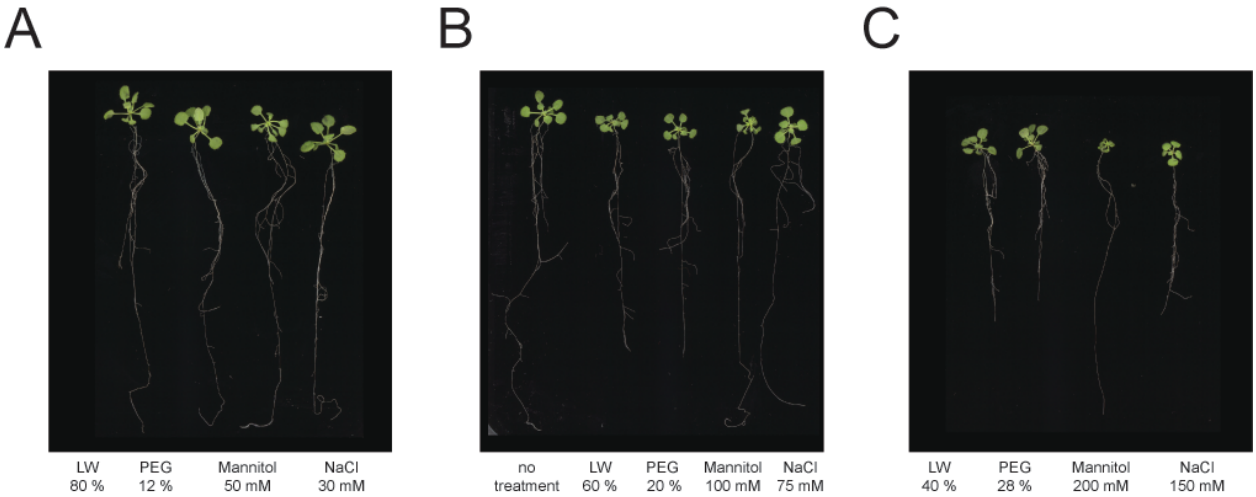

Figure 1 – figure supplement 1

Plant growth responses to stress assays. A – C: Images of 22-day-old *Arabidopsis* seedlings grown under different doses of each agar stress assay.

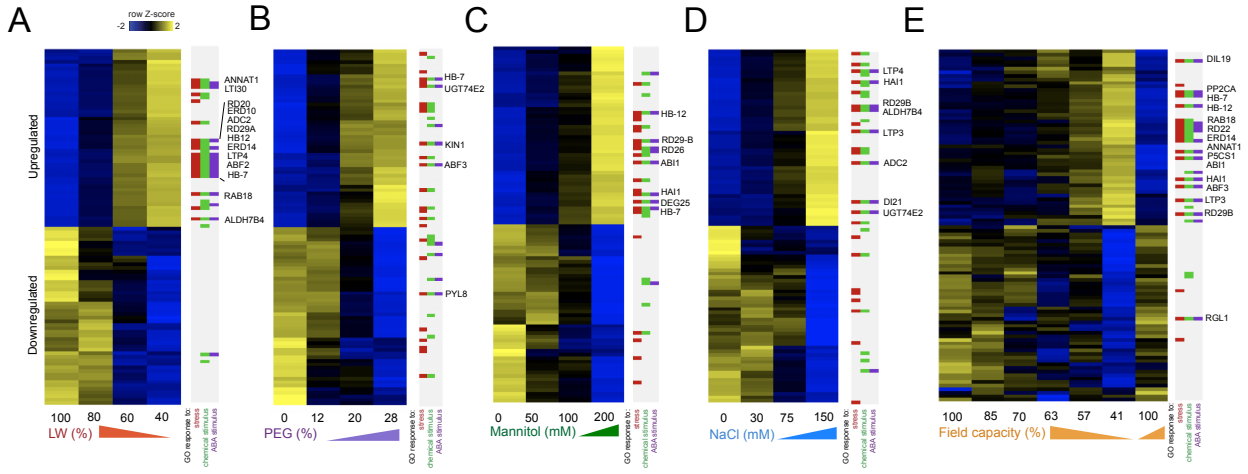

Figure 1 – figure supplement 2

Shoot gene expression responses to each stress assay are dose responsive. Heatmap displaying the top 50 most significant upregulated or downregulated genes in shoots in response to **(A)** LW, **(B)** PEG, **(C)** mannitol, **(D)** NaCl and **(E)** vermiculite induced drought stress. Membership of Gene Ontology (GO) Terms for 'response to stress', 'response to chemical stimulus' or 'response to ABA stimulus' indicated.

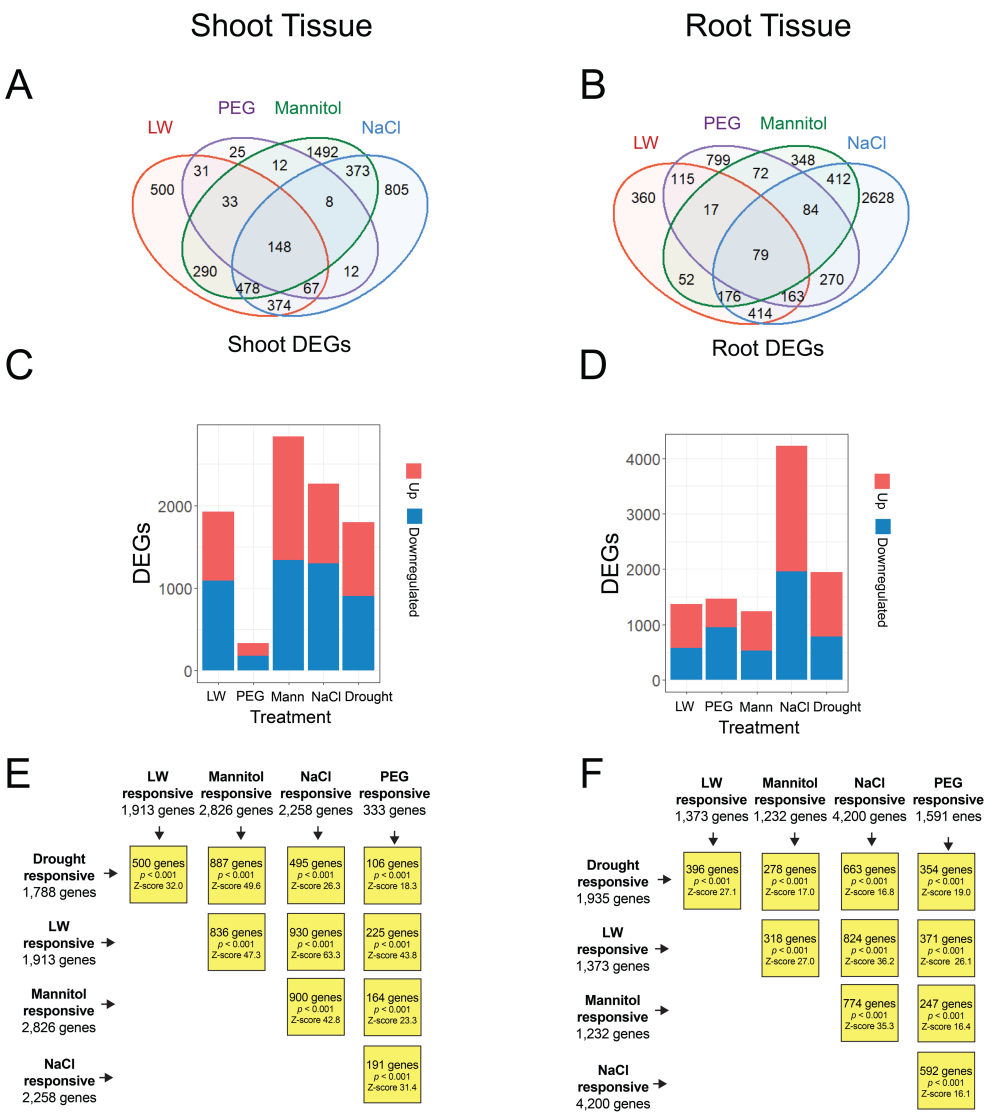

Figure 1 - figure supplement 3

Overlapping differentially expressed genes (DEGs) in response to different assay types. Overlap of dose-responsive differentially expressed genes in shoot **(A)** and root **(B)** in response to either LW, PEG, mannitol or NaCl (replicated from Figure 1). Number of upregulated or downregulated dose-responsive genes in response to each treatment type in shoot **(C)** and root **(D)**. Overlapping gene sets in **(E)** shoot or **(F)** root tissue (permutation test, $p < 0.001$).

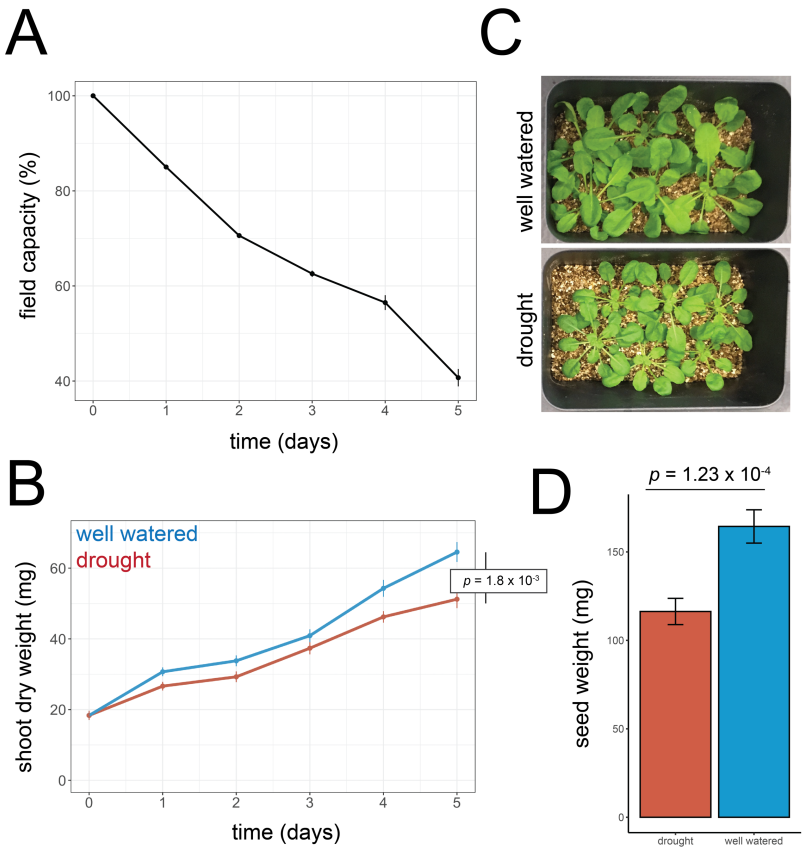

Figure 1 - figure supplement 4

Treating vermiculite-grown *Arabidopsis* plants to mild drought stress. A: Field capacity measurements of vermiculite as water evaporated over a 5-day period (n = 6 - 12). **B:** Shoot dry weight of *Arabidopsis* rosettes as they grew either under well-watered conditions or drought conditions over a 5-day period (t-test, n = 12). **C:** Images of plants after 5 days of water stress. **D:** Seed yield resulting from *Arabidopsis* plants after drought recovery (t-test, n = 50).

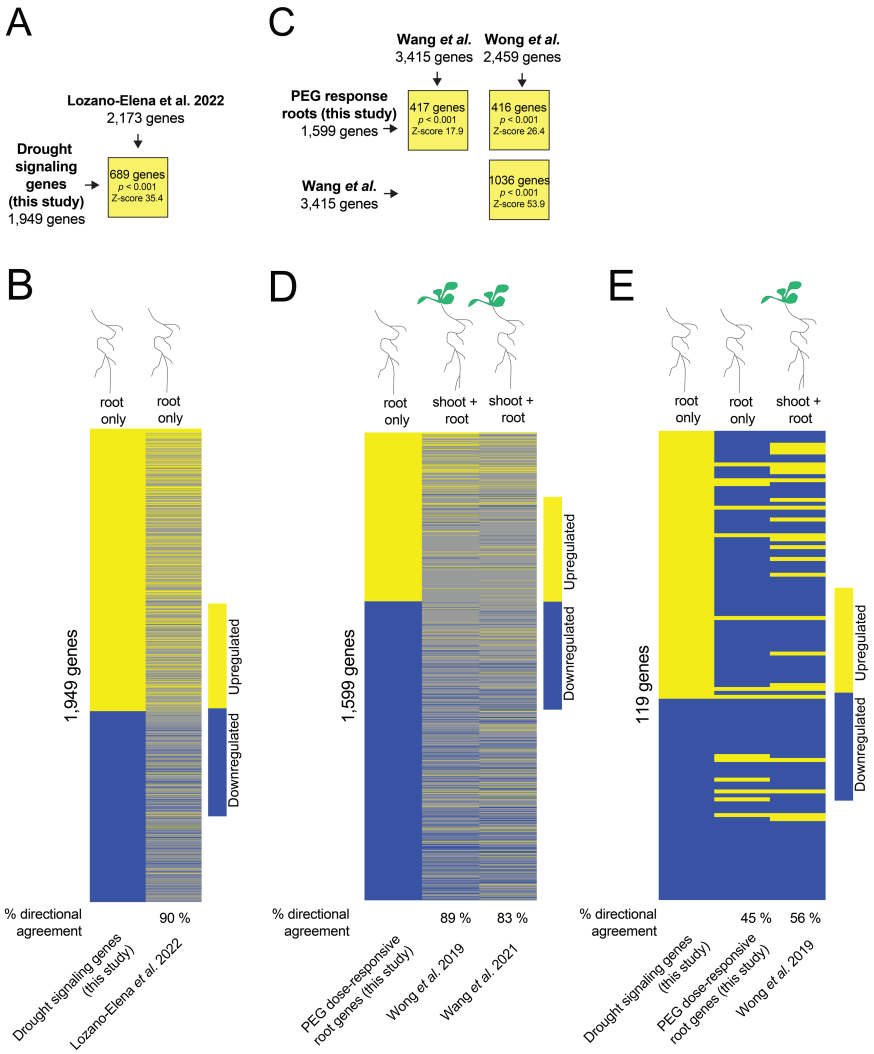

Figure 2-figure supplement 1

Comparative transcriptomic analysis indicates drought signaling genes and PEG dose-responsive genes agree with previous studies. A: Intersect analysis of root genes found differentially expressed in response to vermiculite drying within this study, and genes found differentially expressed in response to soil drying from Lozano-Elena *et al.* (*16*) (permutation test, $p < 0.001$). **B:** Heatmap displaying direction of root genes differentially expressed in response to either vermiculite drying (this study) or soil drying (*16*). Directional agreement with this study's drought response indicated. **C:** Intersect analysis of genes found differentially expressed in response to PEG treatment in this study, Wong *et al.* (*37*) and Wang *et al.* (*38*) (permutation test, $p < 0.001$). **D:** Heatmap displaying direction of genes differentially expressed in response to PEG treatment across each study. Directional agreement with this study's PEG response indicated. We note that both Wong *et al.* and Wang *et al.* assess transcriptomic responses of whole seedlings (both root and shoot), while our study assesses root tissue only. **E:** Examining the 119 genes that were differentially expressed in response to drought (this study), PEG treatment (this study) and PEG treatment reported in Wong *et al.* Directional agreement with this study's drought signaling response and each PEG treatment experiment indicated.

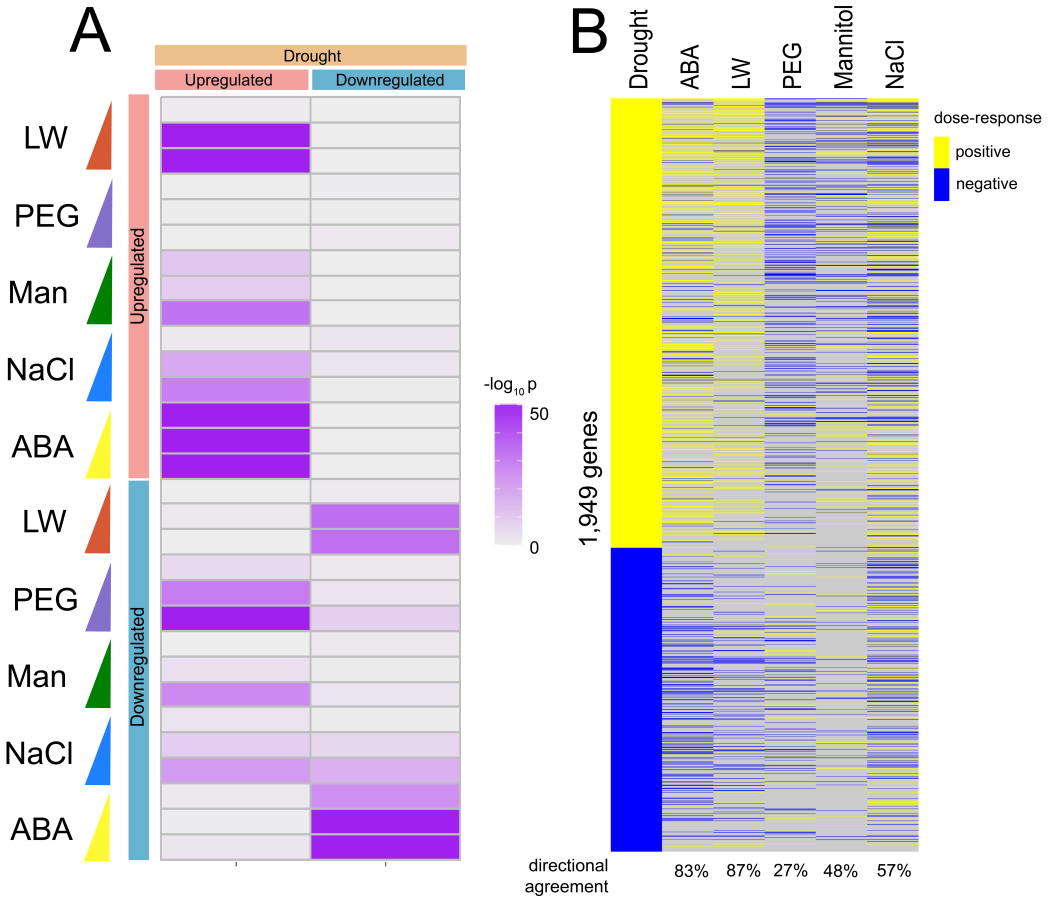

Figure 2-figure supplement 2

Comparative transcriptomic analysis reveals ABA-induced differential expression is comparable to drought and low-water (LW) agar signaling. A: Overlap analysis of genes found differentially expressed under drought treatment, compared to those within each dose of either PEG, mannitol, NaCl, ABA or LW agar assays in both root and shoot (Fisher exact test, adj. $p < 0.05$). **B:** Heatmap displaying genes differentially expressed under drought stress in root tissue compared to their dose-responsive expression within each stress assay. Direction of gene expression agreement with drought-responsive gene expression (i.e., 'directional agreement') indicated.

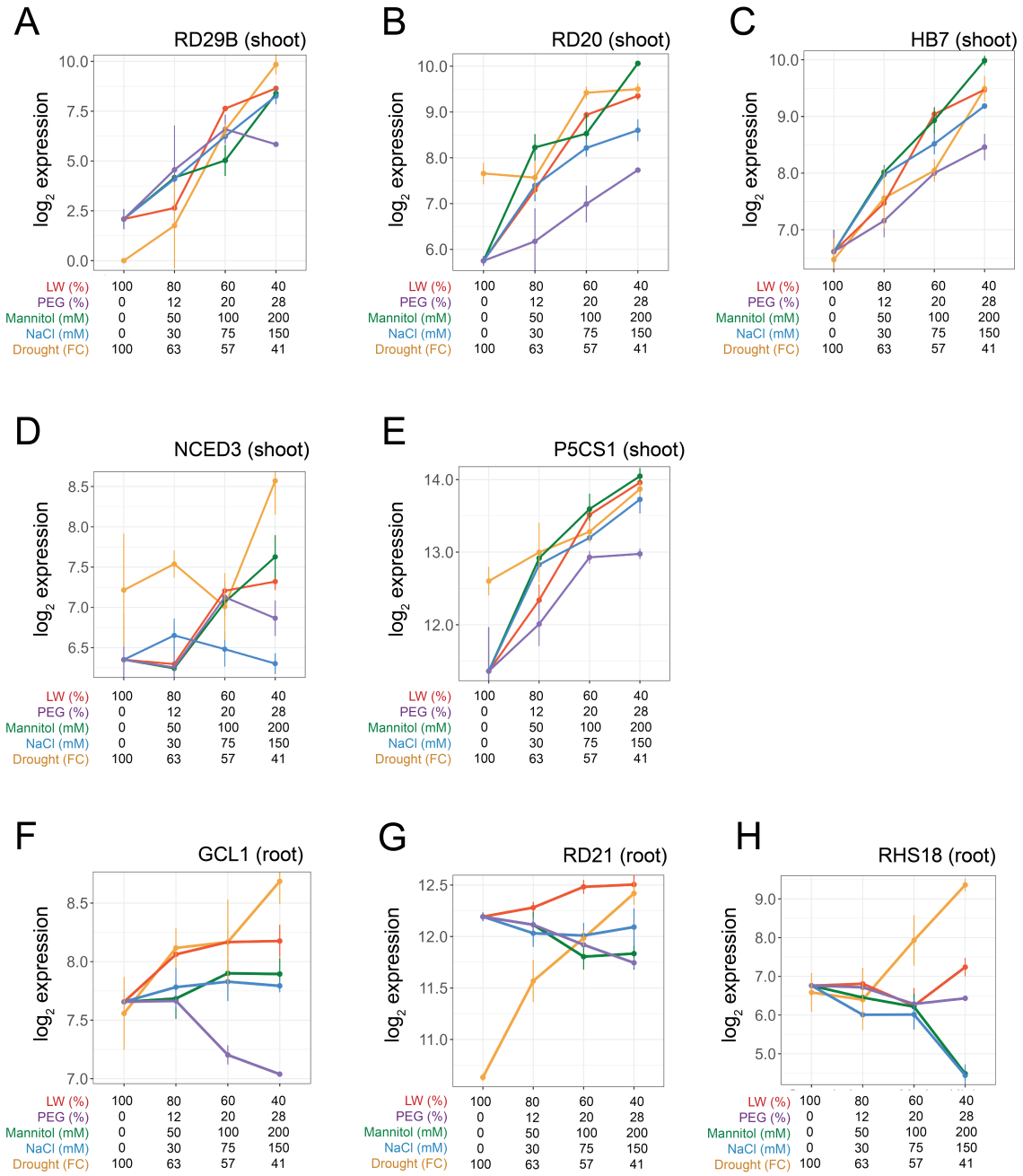

Figure 2-figure supplement 3

Gene expression profiles of stress markers. A - E: Expression patterns of drought marker genes under doses of each assay in shoot tissue **(A)** RD29B, **(B)** RD20, **(C)** HB7, **(D)** NCED3 and **(E)** P5CS1. **F - H:** Expression patterns of drought marker genes under doses of each assay in root tissue **(F)** GCL1, **(G)** RD21 and **(H)** RHS18.

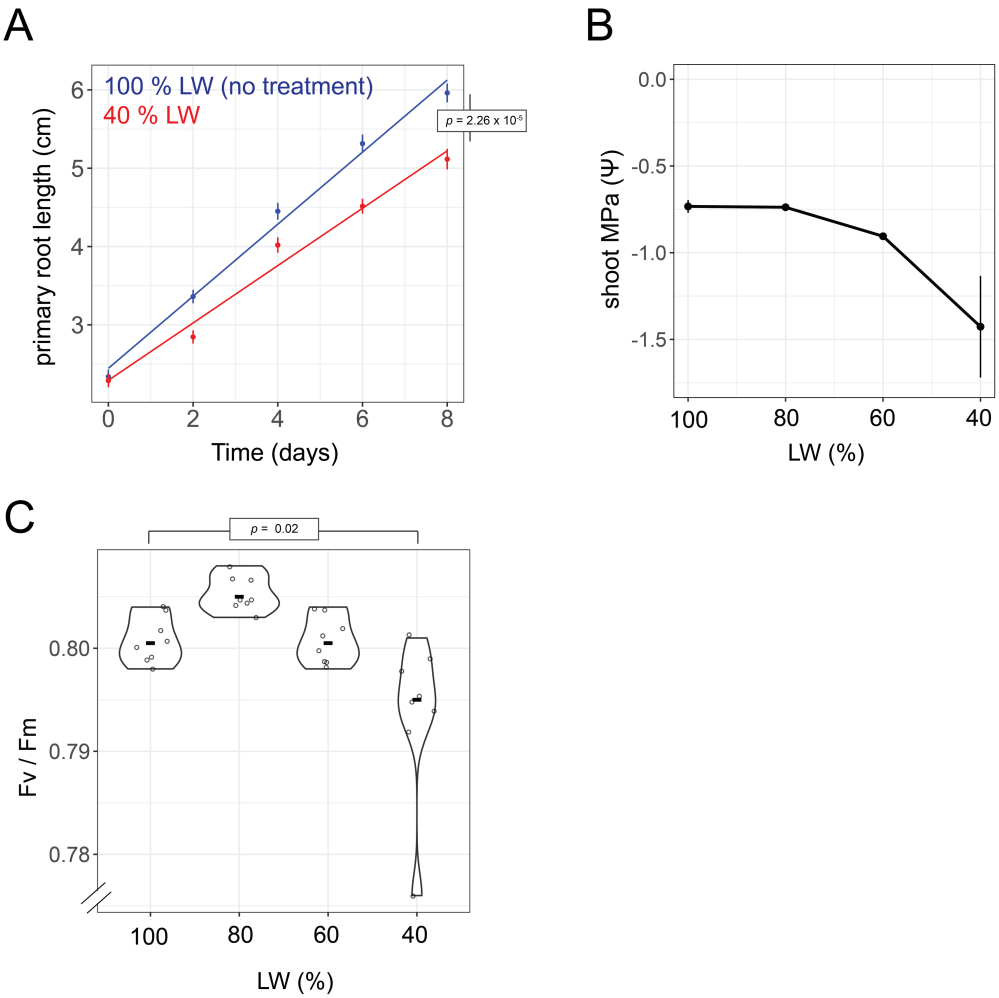

Figure 2-figure supplement 4

Physiological measurements of *Arabidopsis* seedlings in response to LW treatment. A: Measurement of primary root growth rate across 8 days of growth under no treatment and 40 % LW conditions (n= 16, t-test *p*). **B**: Shoot water potential measurements of seedlings grown under different LW media doses (n = 3, Pearson *p* = 0.009). **C**: Measurement of maximum quantum yield of PSII (Fv / Fm) under different LW media doses (n=4, t-test *p*).

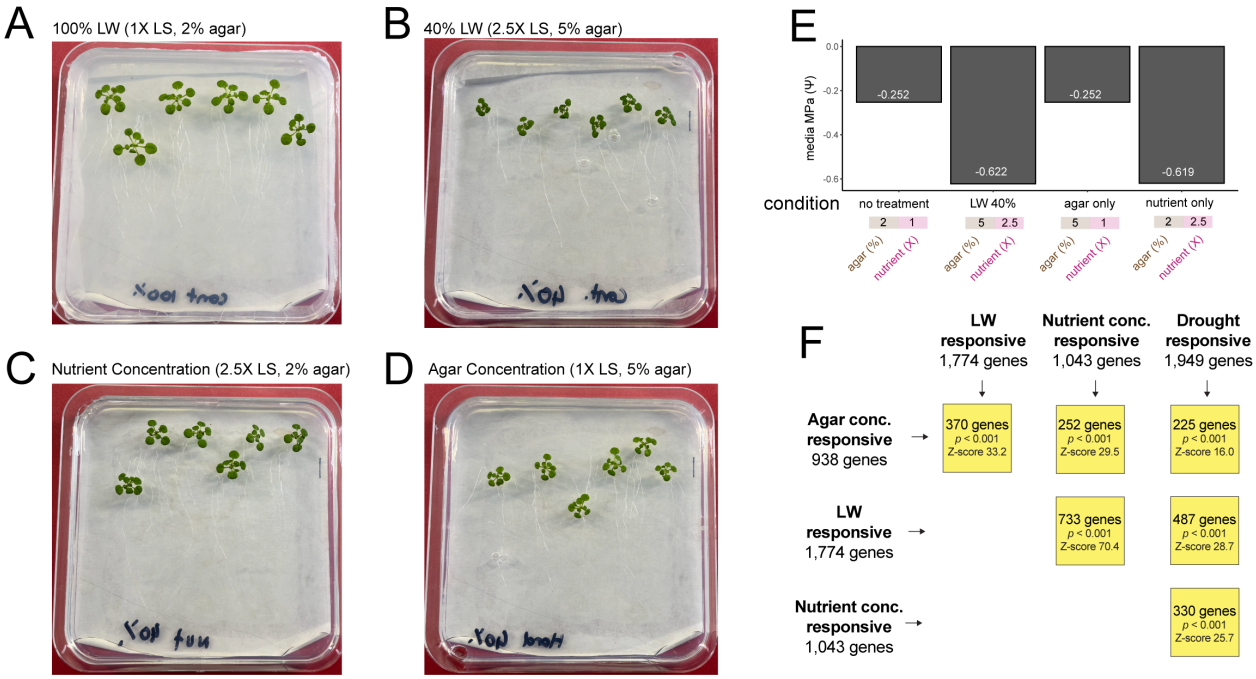

Figure 2-figure supplement 5

Comparing the separate effects of nutrient concentration and agar concentration on seedling growth. Image of *Arabidopsis* seedlings grown on either **(A)** 100% LW agar (i.e. 1X LS, 2% agar) or **(B)** 40% LW, which increased both nutrient and agar concentrations to 2.5X and 5%, respectively. **C:** Image of seedlings grown on an increased 2.5X nutrient concentration (without a change in agar concentration). **D:** Image of seedlings grown on an increased 5% agar concentration (without a change in nutrient concentration). **E:** Water potential measurements of media presented in **A – D**. **F:** Intersection of differentially expressed genes responsive to either agar concentration, nutrient concentration, LW treatment or drought stress (permutation test, $p < 0.001$).

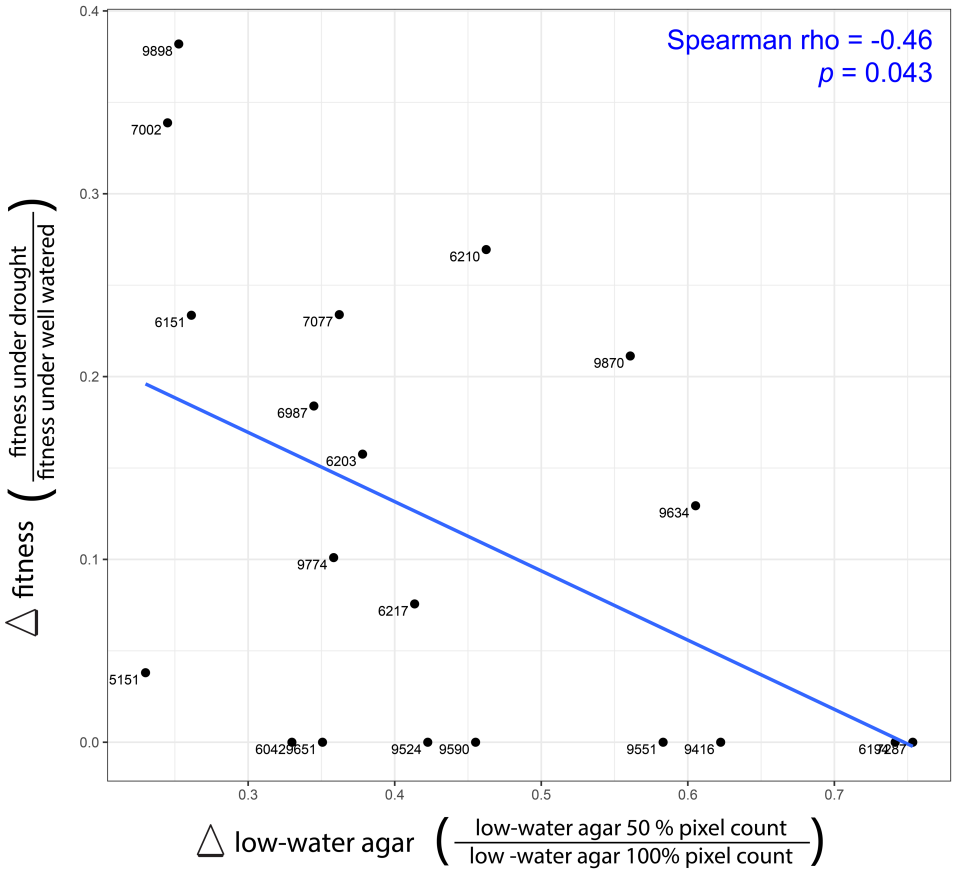

Figure 2-figure supplement 6

Associating low water (LW) agar's impact on shoot size with plant fitness. Comparing the impact low-water agar treatment has on shoot size of 20 different *Arabidopsis* accessions to the change in their fitness found under drought conditions in the field, as reported in (*30*).

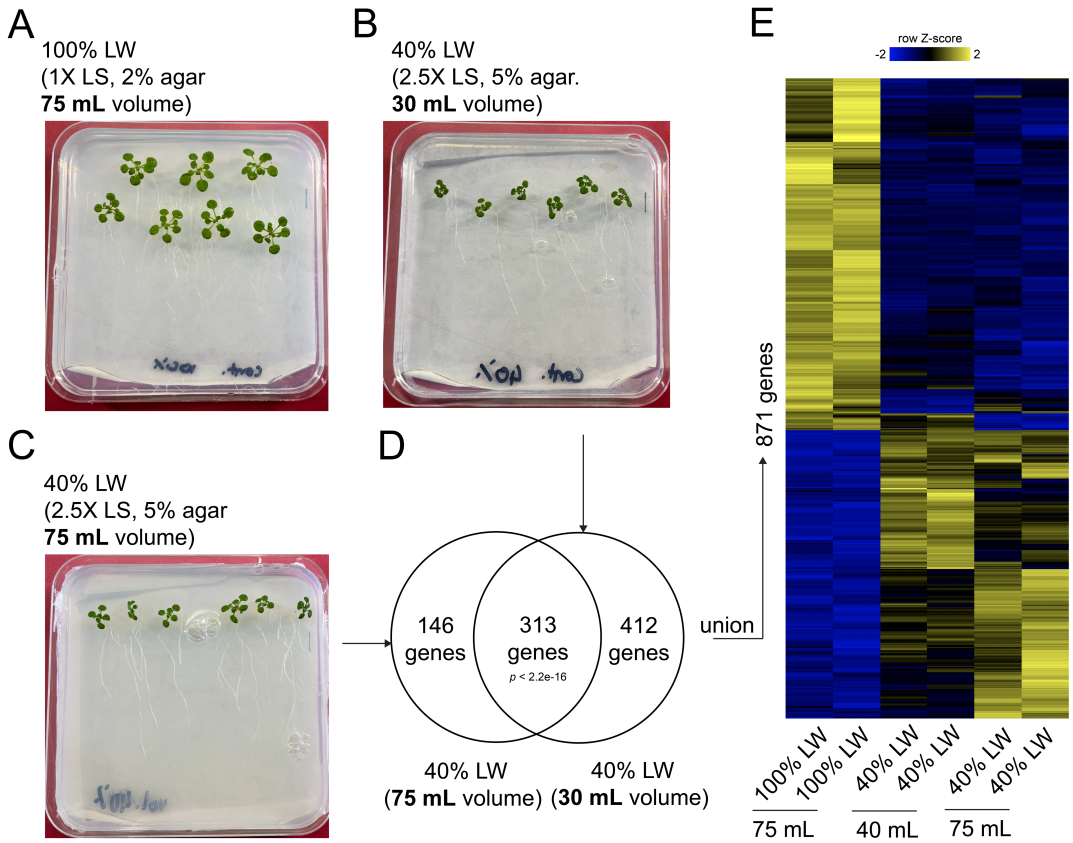

Figure 2-figure supplement 7

The volume of LW agar does not impact gene expression. Image of *Arabidopsis* seedlings grown on either **(A)** 75 mL of 100% LW agar, **(B)** 30 mL of 40% LW agar, or **(C)** 75 mL of 40% LW agar. **(D)** Overlap genes found differentially expressed on either 75 mL or 30 mL of LW agar is statistically significant (Fisher exact test). **(E)** Heatmap of the union of genes found either differentially expressed on 75 mL or 30 mL of LW agar indicates both volumes elicit similar transcriptional trends.

References

1. H. C. J. Godfray *et al.*, Food Security: The Challenge of Feeding 9 Billion People. *Science* **327**, 812-818 (2010).
2. D. S. Battisti, R. L. Naylor, Historical warnings of future food insecurity with unprecedented seasonal heat. *Science* **323**, 240-244 (2009).
3. M. Dubois, D. Inze, Plant growth under suboptimal water conditions: early responses and methods to study them. *J Exp Bot* **71**, 1706-1722 (2020).
4. H. Claeys, S. Van Landeghem, M. Dubois, K. Maleux, D. Inze, What Is Stress? Dose-Response Effects in Commonly Used in Vitro Stress Assays. *Plant Physiol* **165**, 519-527 (2014).
5. M. Hohl, P. Schopfer, Water Relations of Growing Maize Coleoptiles : Comparison between Mannitol and Polyethylene Glycol 6000 as External Osmotica for Adjusting Turgor Pressure. *Plant Physiol* **95**, 716-722 (1991).
6. R. Munns, Comparative physiology of salt and water stress. *Plant Cell Environ* **25**, 239-250 (2002).
7. K. C. Jisha, J. T. Puthur, Halopriming of seeds imparts tolerance to NaCl and PEG induced stress in Vigna radiata (L.) Wilczek varieties. *Physiol Mol Biol Plants* **20**, 303-312 (2014).
8. P. E. Verslues, M. Agarwal, S. Katiyar-Agarwal, J. Zhu, J. K. Zhu, Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J* **45**, 523-539 (2006).
9. C. Trontin *et al.*, A pair of receptor-like kinases is responsible for natural variation in shoot growth response to mannitol treatment in Arabidopsis thaliana. *Plant J* **78**, 121-133 (2014).
10. T. E. Juenger, P. E. Verslues, Time for a drought experiment: Do you know your plants' water status? *Plant Cell* **35**, 10-23 (2023).
11. C. M. van der Weele, W. G. Spollen, R. E. Sharp, T. I. Baskin, Growth of Arabidopsis thaliana seedlings under water deficit studied by control of water potential in nutrient-agar media. *J Exp Bot* **51**, 1555-1562 (2000).
12. G. Zeller *et al.*, Stress-induced changes in the Arabidopsis thaliana transcriptome analyzed using whole-genome tiling arrays. *Plant J* **58**, 1068-1082 (2009).
13. J. A. Kreps *et al.*, Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. *Plant Physiol* **130**, 2129-2141 (2002).
14. J. Swift, J. M. Alvarez, V. Araus, R. A. Gutierrez, G. M. Coruzzi, Nutrient dose-responsive transcriptome changes driven by Michaelis-Menten kinetics underlie plant growth rates. *Proc Natl Acad Sci U S A* **117**, 12531-12540 (2020).
15. N. Nikonorova *et al.*, Early mannitol-triggered changes in the Arabidopsis leaf (phospho)proteome reveal growth regulators. *J Exp Bot* **69**, 4591-4607 (2018).
16. F. Lozano-Elena, N. Fabregas, V. Coleto-Alcudia, A. I. Cano-Delgado, Analysis of metabolic dynamics during drought stress in Arabidopsis plants. *Sci Data* **9**, 90 (2022).
17. J. Msanne, J. Lin, J. M. Stone, T. Awada, Characterization of abiotic stress-responsive Arabidopsis thaliana RD29A and RD29B genes and evaluation of transgenes. *Planta* **234**, 97-107 (2011).
18. S. Takahashi, T. Katagiri, K. Yamaguchi-Shinozaki, K. Shinozaki, An Arabidopsis gene encoding a Ca2+-binding protein is induced by abscisic acid during dehydration. *Plant Cell Physiol* **41**, 898-903 (2000).
19. A. E. Valdes, E. Overnas, H. Johansson, A. Rada-Iglesias, P. Engstrom, The homeodomain-leucine zipper (HD-Zip) class I transcription factors ATHB7 and ATHB12

736 modulate abscisic acid signalling by regulating protein phosphatase 2C and abscisic
737 acid receptor gene activities. *Plant Mol Biol* **80**, 405-418 (2012).
738 20. B. C. Tan *et al.*, Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid
739 dioxygenase gene family. *Plant J* **35**, 44-56 (2003).
740 21. Y. Gao *et al.*, Genetic characterization reveals no role for the reported ABA receptor,
741 GCR2, in ABA control of seed germination and early seedling development in
742 Arabidopsis. *Plant J* **52**, 1001-1013 (2007).
743 22. M. Koizumi, K. Yamaguchi-Shinozaki, H. Tsuji, K. Shinozaki, Structure and expression of
744 two genes that encode distinct drought-inducible cysteine proteinases in Arabidopsis
745 thaliana. *Gene* **129**, 175-182 (1993).
746 23. D. Pose *et al.*, Identification of the Arabidopsis dry2/sqe1-5 mutant reveals a central role
747 for sterols in drought tolerance and regulation of reactive oxygen species. *Plant J* **59**, 63-
748 76 (2009).
749 24. O. P. Ponce *et al.*, Transcriptome profiling shows a rapid variety-specific response in two
750 Andigenum potato varieties under drought stress. *Front Plant Sci* **13**, 1003907 (2022).
751 25. J. Mexal, J. T. Fisher, J. Osteryoung, C. P. Reid, Oxygen availability in polyethylene
752 glycol solutions and its implications in plant-water relations. *Plant Physiol* **55**, 20-24
753 (1975).
754 26. P. E. Verslues, E. S. Ober, R. E. Sharp, Root growth and oxygen relations at low water
755 potentials. Impact Of oxygen availability in polyethylene glycol solutions. *Plant Physiol*
756 **116**, 1403-1412 (1998).
757 27. K. Miura, A. Sato, M. Ohta, J. Furukawa, Increased tolerance to salt stress in the
758 phosphate-accumulating Arabidopsis mutants siz1 and pho2. *Planta* **234**, 1191-1199
759 (2011).
760 28. K. Kobayashi *et al.*, Shoot Removal Induces Chloroplast Development in Roots via
761 Cytokinin Signaling. *Plant Physiol* **173**, 2340-2355 (2017).
762 29. S. Verger, Y. Long, A. Boudaoud, O. Hamant, A tension-adhesion feedback loop in plant
763 epidermis. *Elife* **7**, (2018).
764 30. M. Exposito-Alonso *et al.*, Natural selection on the Arabidopsis thaliana genome in
765 present and future climates. *Nature* **573**, 126-129 (2019).
766 31. D. Naylor, D. Coleman-Derr, Drought Stress and Root-Associated Bacterial
767 Communities. *Front Plant Sci* **8**, 2223 (2017).
768 32. D. Kim, J. M. Paggi, C. Park, C. Bennett, S. L. Salzberg, Graph-based genome
769 alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* **37**, 907-
770 915 (2019).
771 33. S. Anders, P. T. Pyl, W. Huber, HTSeq-a Python framework to work with high-throughput
772 sequencing data. *Bioinformatics* **31**, 166-169 (2015).
773 34. C. Y. Cheng *et al.*, Araport11: a complete reannotation of the Arabidopsis thaliana
774 reference genome. *Plant J* **89**, 789-804 (2017).
775 35. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for
776 RNA-seq data with DESeq2. *Genome Biology* **15**, (2014).
777 36. M. S. Katari *et al.*, VirtualPlant: a software platform to support systems biology research.
778 *Plant Physiol* **152**, 500-515 (2010).
779 37. M. M. Wong *et al.*, Phosphoproteomics of Arabidopsis Highly ABA-Induced1 identifies
780 AT-Hook-Like10 phosphorylation required for stress growth regulation. *Proc Natl Acad*
781 *Sci U S A* **116**, 2354-2363 (2019).
782 38. Y. Wang, Z. Fang, L. Yang, Z. Chan, Transcriptional variation analysis of Arabidopsis
783 ecotypes in response to drought and salt stresses dissects commonly regulated
784 networks. *Physiol Plant* **172**, 77-90 (2021).
785