Mimicking genuine drought responses using a high throughput plate assay

Stephen Gonzalez [1*], Joseph Swift [1*], Jiaying Xu [1], Natanella Illouz-Eliaz [1], Joseph R. Nery [2] and Joseph R. Ecker [1,2,3†]

1. Plant Biology Laboratory, The Salk Institute for Biological Studies, 10010 N Torrey Pines Rd, La Jolla, CA 92037, United States.
2. Genomic Analysis Laboratory, The Salk Institute for Biological Studies, 10010 N Torrey Pines Rd, La Jolla, CA 92037, United States.
3. Howard Hughes Medical Institute, The Salk Institute for Biological Studies, 10010 N Torrey Pines Rd, La Jolla, CA 92037, United States.

* These authors contributed equally to this work

† corresponding author

Abstract

Simple, soil-free assays that can mimic drought conditions are incredibly useful for investigating plant stress responses. Due to their ease of use, the research community often relies on polyethylene glycol (PEG), mannitol and salt treatments to simulate drought conditions in the laboratory. However, while these types of osmotic stress can create phenotypes that resemble those of drought, it remains unclear how they compare at the molecular level. Here, using transcriptomics, we demonstrate that these assays are unable to replicate drought signaling responses in the *Arabidopsis* root. Indeed, we found a significant number of genes that were induced by drought were in fact repressed by such treatments. Since our results question the utility of PEG, mannitol and salt, we designed a new method for simulating drought. By simply adding less water to agar, our 'low-water agar' assay elicits gene expression responses that compare more favorably to drought stress. Furthermore, we show our approach can be leveraged as a high-throughput assay to investigate natural variation in drought 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 drought responses. Such investigations demand simple assays that can reproduce drought phenotypes at both the physiological as well as 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. In light of this, many molecular biologists prefer to simulate osmotic stress using chemical agents, such as polyethylene glycol (PEG), mannitol, and salt (NaCl) (*3*). When dissolved in agar or aqueous solution, these chemicals work by lowering water potential, in effect making it harder for plants to absorb water (*4*). Given their ability to produce phenotypes similar to drought stress, PEG, mannitol and NaCl have been used to simulate drought for decades (*5, 6*).

However, at the molecular level, it remains unclear whether the osmotic stress that PEG, mannitol or NaCl elicit are comparable to that of bona-fide drought. While some experimental data suggests that they are (*4, 7, 8*), to our knowledge a rigorous side-by-side comparison has not been performed. By taking a comparative transcriptomic approach, we show that PEG, mannitol and NaCl stress are unable to induce the root gene expression responses that occur under drought stress. Inspired to develop a better method to simulate drought, we present our own 'low-water agar' assay, which proved superior in recapitulating the molecular signaling responses seen under true drought stress.

PEG, mannitol and NaCl treatments repress drought inducible genes

We sought to detect PEG, mannitol and NaCl's effect on the *Arabidopsis* transcriptome. To this end, we grew *Arabidopsis* seedlings on vertical agar plates for 14 days on three different doses of either PEG, mannitol or NaCl. As expected, we saw a reduction in plant biomass as the dose of each treatment became more severe (**Figure 1A & B, Supplementary Figure 1**). For each treatment, we sequenced root and shoot transcriptomes by RNA-seq. By these means, we found over 800 differentially expressed genes for each treatment (adj. p-value < 0.05) (**Figure**

1D & 1E), where expression responses were largely dose responsive to the severity of stress (**Figure 1F**).

Next, we wanted to compare these different ways of simulating drought stress to the transcriptomic responses seen under bona-fide drought conditions. To create this benchmark, we subjected mature *Arabidopsis* pot-grown plants to drought stress by withholding water for 5 days, which led to a reduction in plant biomass (**Figure 1C**). We assayed root and shoot gene expression responses each day of water loss, and using a linear model, found 1,900 and 1,793 drought responsive genes in the root and shoot respectively (adj. p-value < 0.01) (**Figure 1D & 1E**). To ensure these were truly drought responsive genes, we required their expression to recover upon rewatering (**Figure 1G**).

To assess how PEG, mannitol and NaCl treatments compared to the drought stress described above, we overlapped genes found differentially expressed in each experiment. For shoot tissue, we found genes that were differentially expressed under drought stress overlapped significantly with genes that were differentially expressed by either PEG, mannitol and NaCl treatments (adj. p-val < 0.05), indicating that these treatments compared favorably to drought stress (**Figure 2A & 2B**). Along these lines, across all conditions we saw expected differential expression of the canonical drought markers RD29B (*9*) and RD20 (*10*), the osmo-protectant genes P5CS1 (*11*) and ALDH10A8 (*12*), and ABA signaling and biosynthesis genes HB7 (*13*) and NCED3 (*14*) (**Supplementary Figure 2**).

In contrast, we did not find a similar result in the root. To our surprise, we found a significant number of genes that were upregulated by drought stress were in fact downregulated by PEG, mannitol and NaCl treatments, a trend which increased as the stress became more severe (**Figure 2A & 2B**). Furthermore, it appeared that PEG performed the worst; only 27 % of PEG responsive genes were concordantly regulated in the same direction seen under true drought stress. Such mis-regulation is exemplified by the expression of drought markers HB12 (*13*), GCL1 (*15*) and LEA7 (*16*) (**Figures 2C – 2E**). While mannitol and NaCl performed somewhat better, both still achieved only 58 % concordance in gene expression, and thus held many genes that were differentially expressed in opposite direction seen under true drought stress. Examples of drought marker genes which followed this pattern of mis regulation are RAB18 (*17*), RD21 (*18*), and ANNAT4 (*19*) (**Figure 2F – 2H**).

We wondered whether the repression of drought inducible genes by PEG, mannitol and NaCl was an artifact caused by comparing osmotic stress assays performed on plates to a drought stress conducted in pots. To ensure this was not the case, we performed an additional experiment by treating plate-grown seedlings with different doses of the ABA. Since ABA is a key drought signaling hormone, we would expect to see good agreement between the ABA transcriptional responses elicited by plate grown plants and the drought signaling responses found in pot grown plants. Indeed, we found this to be the case - genes found differentially expressed by ABA on plates overlapped significantly with drought responsive genes in a highly concordant manner (**Supplementary Figure 3**).

Why does PEG down-regulate rather than up-regulate drought responsive genes in the root? We hypothesize that the large number of genes that were repressed by PEG is the result of hypoxia. We base this on PEG's ability to impeding oxygen solubility (*20, 21*). This appears to be reflected at the molecular level, where genes downregulated by PEG are over-represented in the 'monooxygenase activity', and 'oxygen binding' GO Terms (adj. p-values < 0.01, **Supplementary Table 5**). In contrast, it appeared mannitol and NaCl treatments departed from true drought responses for a different reason. NaCl responsive GO Terms included a specific downregulation of 'phosphorous metabolic processes' (adj. p-value = 5.48×10^{-6}), suggesting that the roots were changing phosphate levels in response to NaCl, a process known to help maintain ion homeostasis (*22*). For mannitol, we observed a specific downregulation of 'cell wall organization or biogenesis' and 'microtubule-based processes' (adj. p-values < 1×10^{-4}), suggesting a unique root developmental response to mannitol, possibly mediated by mannitol acting as a signaling molecule (*23*). Surprisingly, for both mannitol and NaCl stresses, we saw photosynthesis related GO terms aberrantly upregulated in the roots, which may be due to an interaction between the osmotic stress and exposing the roots to light (*24*) (**Supplementary Table 5**).

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

Since PEG, mannitol and NaCl elicited transcriptional responses that ran counter to true drought stress, we were motivated to design a new way of simulating drought on an agar plate. We hypothesized that instead of adding a compound, we could simulate drought by simply adding less water to agar media (leading to both higher agar and nutrient concentration). We called this

media 'low-water agar' (LW), and by testing three different doses, found that it limited plant growth in a similar way to PEG, mannitol and NaCl (**Figure 1A & B, Supplementary Figure 1**). At the molecular level RNA-seq revealed 868 and 2,169 genes differentially expressed in the roots and shoots respectively (**Figure 1D & 1E**), many of which responded in a dose-responsive way comparable to other treatments (**Figure 1F**).

Crucially, we found that these gene expression responses overlapped significantly with those found under drought stress (Fisher adj. p-values < 1×10^{-32}) (**Figure 2A & 2B**). Unlike PEG, mannitol and NaCl, we found genes down regulated in the root by low-water agar treatment were similarly down regulated by drought stress. By these means, the correct directional expression elicited by low-water agar led to a higher overlap with the bona-fide drought response (91 % concordance). The improved performance of low-water agar can be seen in canonical drought marker expression of genes such as RAB18, RD21 and ANNAT4 (**Figure 2F – 2H**). In line with this, low-water agar gene expression responses compared better to those induced by ABA treatment, suggesting that low-water agar stimulates an ABA-mediated drought signaling response (**Supplementary Figure 3**). Indeed, unlike the other osmotic stress assays, root GO Term analysis of low-water agar revealed the over-represented term 'response to abscisic acid stimulus' (adj. p-value < 1×10^{-4}, **Supplementary Table 5**).

Finally, we wanted to ensure that our low-water agar assay was sensitive enough to detect variability in drought-responsive phenotypes. To test this, we grew 20 different *Arabidopsis* ecotypes on 50 % low-water agar, where ecotypes were selected from a previous drought study (*25*). By comparing the total shoot area after 3 weeks of growth, we found that our assay was able to indicate which accessions arrest shoot growth under drought, versus those that do not (e.g. line 5151 vs. line 9590, **Figure 2I**). Furthermore, we found that the relative impact low-water agar had on an accession's shoot size was associated with the relative impact drought had on its fitness, as measured under field conditions (Spearman rho = -0.46, p = 0.04, **Supplementary Figure 4**) (*25*). This gives us confidence that our assay could be useful for screening for novel drought responses among a wider group of accessions or mutants.

In summary, compared to PEG, mannitol or NaCl treatments, we believe our low-water agar assay presents a better alternative for simulating responses comparable to genuine drought. Low-water agar may be superior because it creates a harder substrate for plants to absorb water from. Another contributing factor may be the increase in nutrient concentration in the agar

media. This may help mimic true drought stress since when water evaporates from the soil it can lead to an increase in the soil nutrient concentration, which can in turn impact gene expression (*26*). We note that due to its simplicity, our assay cannot mimic many additional effects drought stress can have on gene expression in real environments – such as those that arise from changes in soil structure, or the root microbiome (*27*). However, by inducing growth arrest and gene expression responses comparable to true drought, low-water agar offers a simple high-throughput method to screen phenotypes and probe gene regulatory networks that mediate drought responses. We describe the simple way to make low-water agar media in the **Supplementary Methods**.

Supplementary

Supplementary Methods

Supplementary Figure 1 – Plant growth images under osmotic stress.
Supplementary Figure 2 – Gene expression profiles of canonical drought markers in shoot tissue.
Supplementary Figure 3 – Overlapping drought responsive and ABA responsive genes.
Supplementary Figure 4 – Associating low-water agar's impact on shoot size with plant fitness.

Supplementary Table 1 – normalized gene expression counts.
Supplementary Table 2 – list of differentially expressed genes.
Supplementary Table 3 – osmotic stress assay biomass measurements.
Supplementary Table 4 – drought stress assay biomass measurements.
Supplementary Table 5 – GO Terms.
Supplementary Table 6 – Plant Growth Tracker image values.

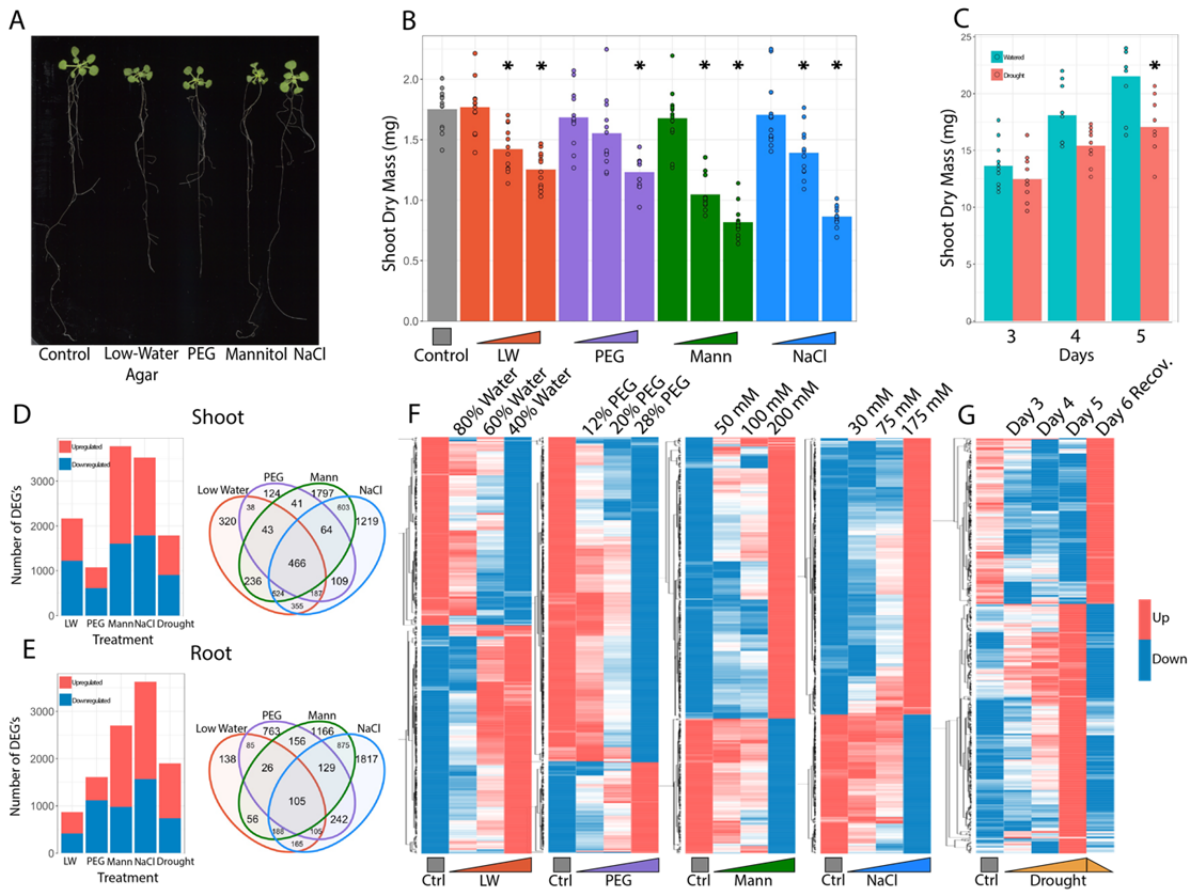


Figure 1 - Benchmarking the impact different osmotic assays have on *Arabidopsis* biomass and gene expression. A: *Arabidopsis* growth on plates under low-water agar, PEG, mannitol and NaCl treatments. **B**: Dry weight of *Arabidopsis* seedlings under different doses of each stress treatment (n = 12, * t-test adj. $p < 0.01$). **C:** *Arabidopsis* rosette dry weight after 3 to 5 days of withholding water (n = 9 - 11, * paired t-test $p < 0.01$). **D - E:** Number and intersect of differentially expressed genes (DEGs) in response to each osmotic stress treatment within root and shoot tissue (adj. p-value < 0.05). **F:** Heatmap displaying the top 500 most significantly differentially expressed genes in each osmotic stress assay in the root. **G:** Heatmap displaying the top 500 most significantly differentially expressed genes in response to drought stress in the root.

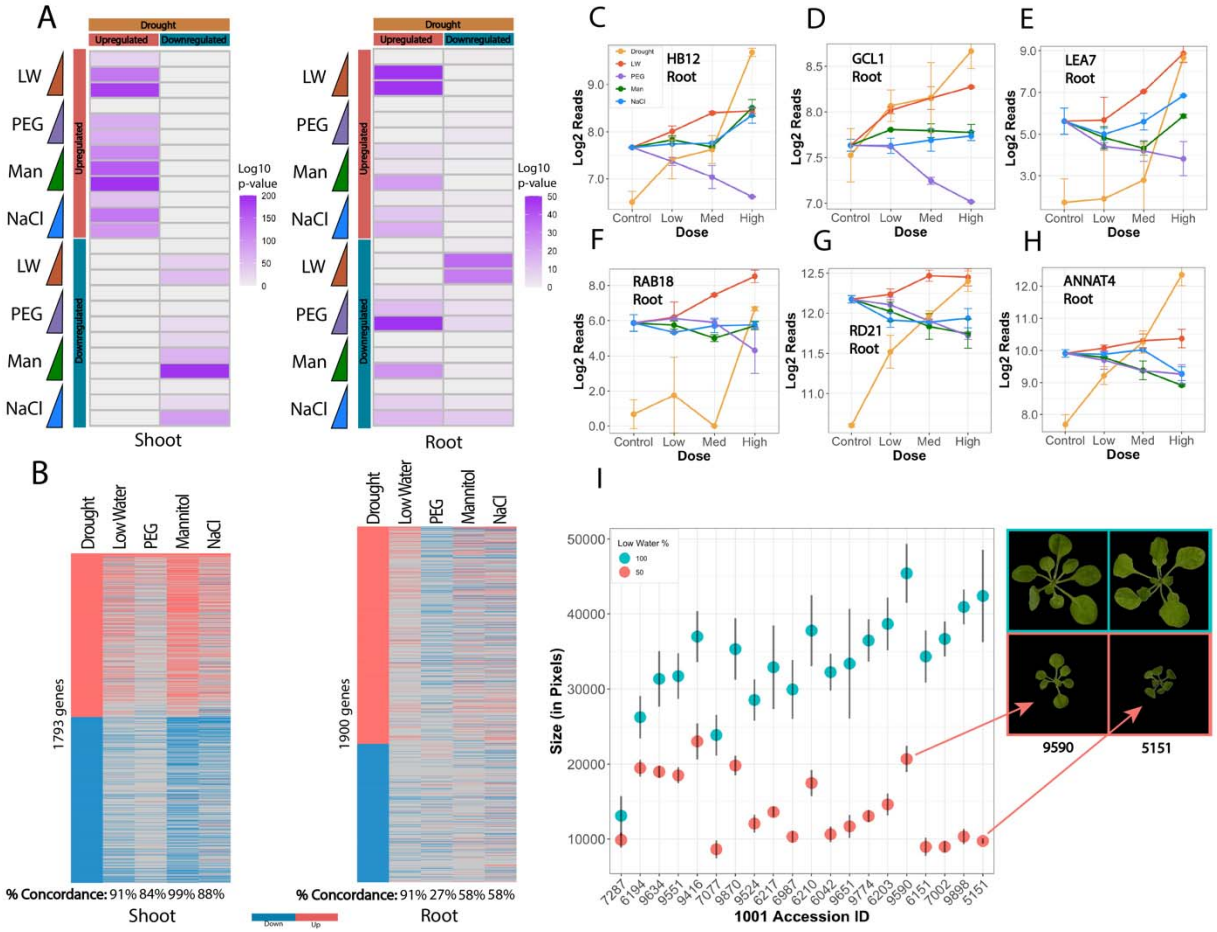
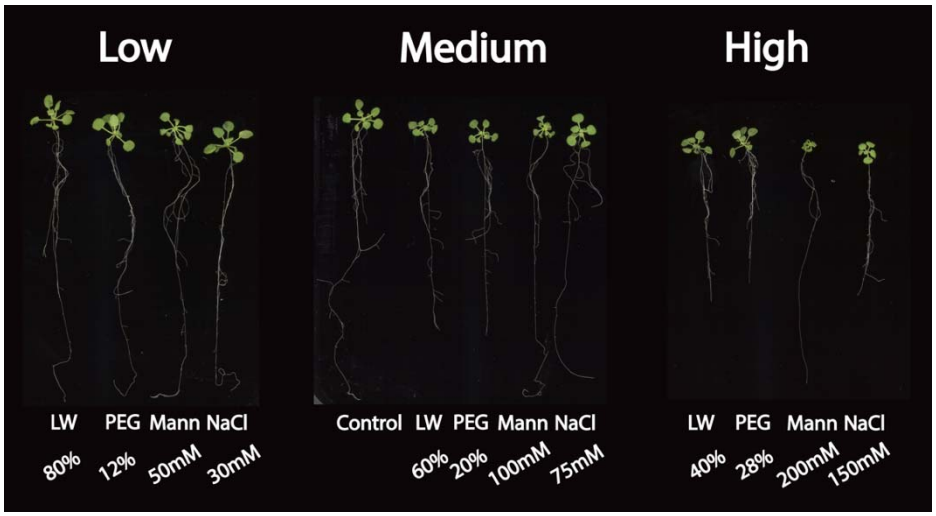
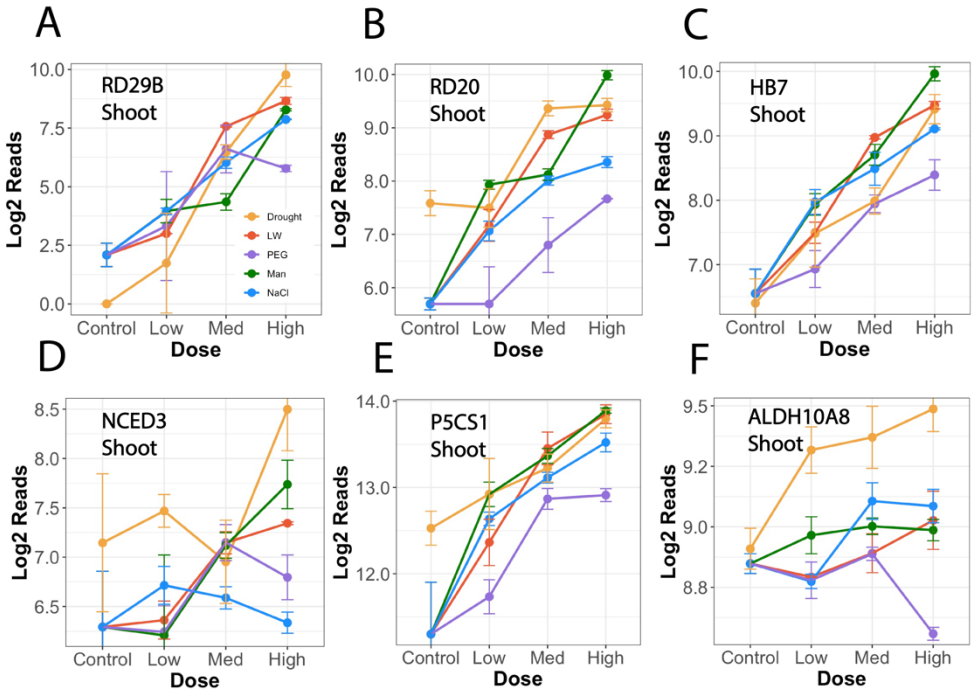


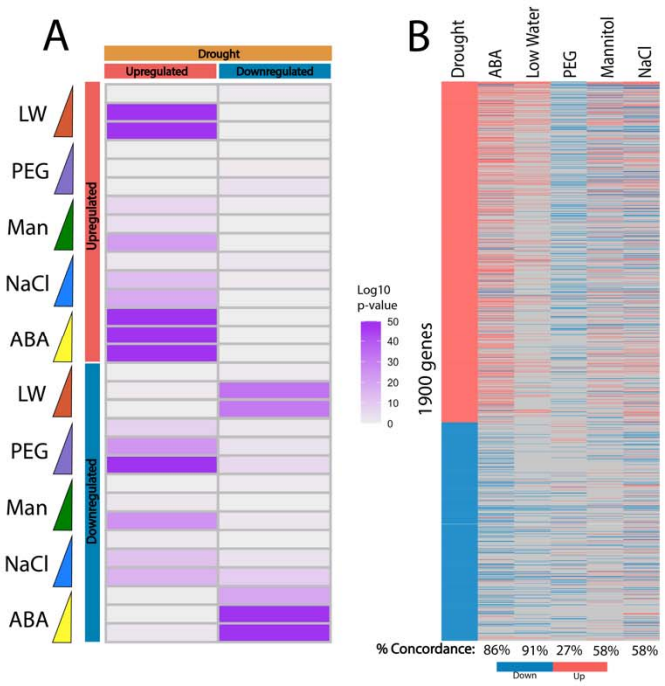
Figure 2 - Comparative transcriptomic analysis reveals PEG, mannitol and NaCl downregulate drought inducible genes in the root. **A:** Overlap analysis of genes found differentially expressed under drought treatment, compared to those under either PEG, mannitol, NaCl or low-water agar assays in both root and shoot (Fisher exact test adj. $p < 0.05$). **B:** Heatmap displaying genes differentially expressed under drought stress in root or shoot tissue compared to their expression under the highest dose of each osmotic stress assay. **C - H**: Expression patterns of drought marker genes under low, medium and high doses of each assay (where drought doses were Day 3, Day 4 and Day 5 respectively): HB12 (AT3G61890), GCL1 (AT5G65280), LEA7 (AT1G52690), RAB18 (AT1G43890), RD21 (AT1G47128), ANNAT4 (AT2G38750). **I:** Total rosette area of 20 *Arabidopsis* lines grown under either 100 % or 50 % low-water agar treatment.



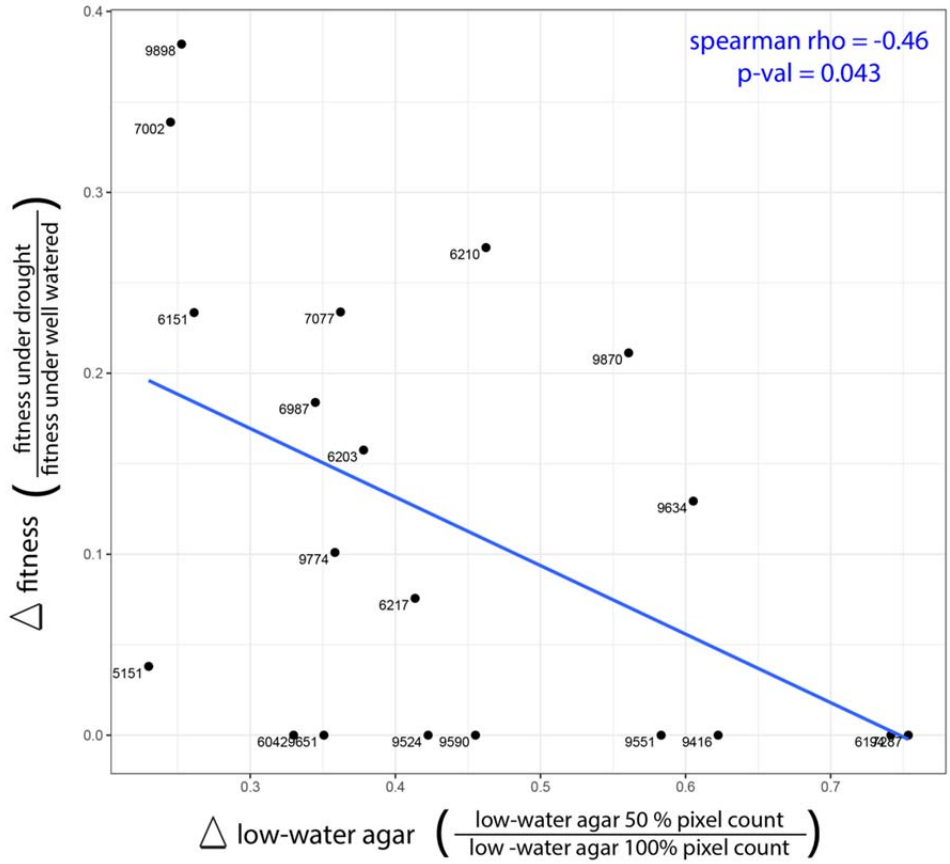
Supplementary Figure 1 - Images of plants grown under different doses of each osmotic stress assay. *Arabidopsis* growth on plates under low-water (LW) agar, PEG-6000, mannitol and salt (NaCl) treatments.



Supplementary Figure 2 - Gene expression profiles of canonical drought markers in shoot tissue. A - F: Expression patterns of drought marker genes under low, medium and high doses of each assay (where drought doses were Day 3, Day 4 and Day 5 respectively). RD29B (AT5G52300), RD20 (AT2G33380), HB7 (AT2G46680), NCED3 (AT3G14440), P5CS1 (AT2G39800), ALDH10A8 (AT1G74920).



Supplementary Figure 3 - Comparative transcriptomic analysis reveals ABA induced differential expression is comparable to drought and low-water (LW) agar signaling A: Intersect analysis of genes found differentially expressed under drought treatment, compared to those under either ABA, PEG, mannitol, NaCl or low-water agar assays in the root (Fisher exact test adj. $p < 0.05$). **B:** Heatmap displaying genes differentially expressed under drought stress in root tissue compared to their expression under the highest dose of each osmotic stress assay.



Supplementary Figure 4 - Associating low-water 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 (*25*).

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Data Availability

Raw sequencing data can be found 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. Our statistical pipeline (performed in R) is available upon request.

Materials and Methods

Low-Water Agar Assay – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media), before transfer to 'low-water' plates that contained varying water content. The 100% treatment plate, where water is not lacking, contained 75 mL of 2% agar and 1x 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 1.25x LS media. Plants were grown upon 3 different treatments (80%, 60%, and 40% water amount) for 14 days. On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

To test different *Arabidopsis* accessions on low-water 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 (2 - 5 plants per plate) (**Supplementary Table 6**). Shoot area was calculated from images using Plant Growth Tracker (GitHub - https://github.com/jiayinghsu/plant_growth_tracker).

Vermiculite Drought Assay – *Arabidopsis* seedlings were grown on vertical plates for 17 days (short day, 1x LS, 1% sucrose, 2% agar media), before transfer to vermiculite (0.75x LS media). Plants were then grown on vermiculite at 100% field capacity (FC) for 12 days. On the 13th day, the first time point was sampled (4.5 hours after subjective dawn) where tissue was flash frozen in liquid nitrogen. After this, excess solution was drained from each pot, and then each pot was calibrated to 1x FC. Plant tissue was harvested each day on subsequent days at the same time of day. After the 5th day sample was taken, water was re-added to the remaining pots to an excess of 1x FC. ~ 15 plants were sampled per time point. Dry weight rosette and FC measurements can be found in **Supplementary Table 4**.

Polyethylene Glycol (PEG) Osmotic Stress Assay – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media) before transfer to polyethylene glycol (PEG) media of varying concentrations. PEG media plates were prepared by dissolving crystalline 6000 MW PEG into freshly autoclaved 1x LS media and pouring 50mL of PEG media solution onto 1x LS, 2% agar media plates, letting the PEG solution diffuse into the solid media overnight, then pouring off excess and transferring seedlings to PEG infused media plates. Plants were grown under 3 different treatments (12%, 20%, and 28% PEG solution) for 14 days. On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

Mannitol and NaCl Osmotic Stress Assays – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media) before transfer to either mannitol or salt (NaCl) media of varying concentrations. Mannitol and NaCl media plates were prepared by adding respective volume of stock solution to 1x LS, 2% agar media before autoclaving for desired molar concentration. Plants were grown under 3 different treatments of mannitol or NaCl (50mM, 100mM and 200mM for mannitol, 30mM, 75mM, and 150mM for NaCl) for 14 days. On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

Abscisic Acid (ABA) Exogenous Treatment Assay – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media) before transfer to 1x

LS, 2% agar, control media and grown for 14 days. On day 14, four abscisic acid (ABA) solutions of 10uM, 5uM, 1uM, and 0uM concentration were prepared from 10mM ABA dissolved in ethanol stock, the 0uM solution containing the same 0.1% ethanol concentration as the highest ABA dose. 30 min after subjective dawn, 15 mL of each solution was dispersed onto the roots of the seedlings. After 1 min of treatment, the ABA solution was removed from the plates, and the plates returned to the growth chamber. 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

RNA-extraction and Library Preparation – Plant tissue was crushed using the TissueLyser (Agilent) and RNA extracted using RNeasy Mini Kit (Qiagen). RNA quality was assessed using Tape station High Sensitivity RNA assay (Agilent). 0.5 - 1 ug of total RNA proceeded to library preparation, where libraries were prepared using TruSeq stranded mRNA kit (Illumina). Resulting libraries were sequenced on the NovaSeq 6000 (Illumina) with 2x150 bp paired-end read chemistry. Read sequences were aligned to the *Arabidopsis* TAIR10 genome using HISAT2 (*28*), and gene counts called using HT-seq (*29*), by relying on Araport11 annotation (*30*). Normalized counts can be found in **Supplementary Table 1**. For each organ, libraries from all experiments were normalized together before calling differential expression.

Statistical Analysis – Differential gene expression was called using DESeq2 (*31*). Specifically, for plate based assays, we called differential expression by comparing the control treatment to the highest treatment dose (i.e. either PEG 28%, Mannitol 200 mM, NaCl 175 mM, Dry Plate 40 %, ABA 10 uM), using an adjusted p-value threshold of 0.05. To detect differential expression in our drought assay on vermiculite, we called differential expression using a linear model, using *design ~ water-loss*, where 'water-loss' was the amount of water that had evaporated from the pot. The complete list of differentially expressed genes for each experiment can be found in **Supplementary Table 2**. Resulting heatmaps were generated using Morpheus (Broad Institute). Overlap analyses were performed using Fisher exact tests, with an adjusted p-value threshold of 0.05. The background for these intersect was all expressed genes within the respective organ. GO Term analysis was performed in VirtualPlant (*32*), with all expressed genes within the respective organ used as background.

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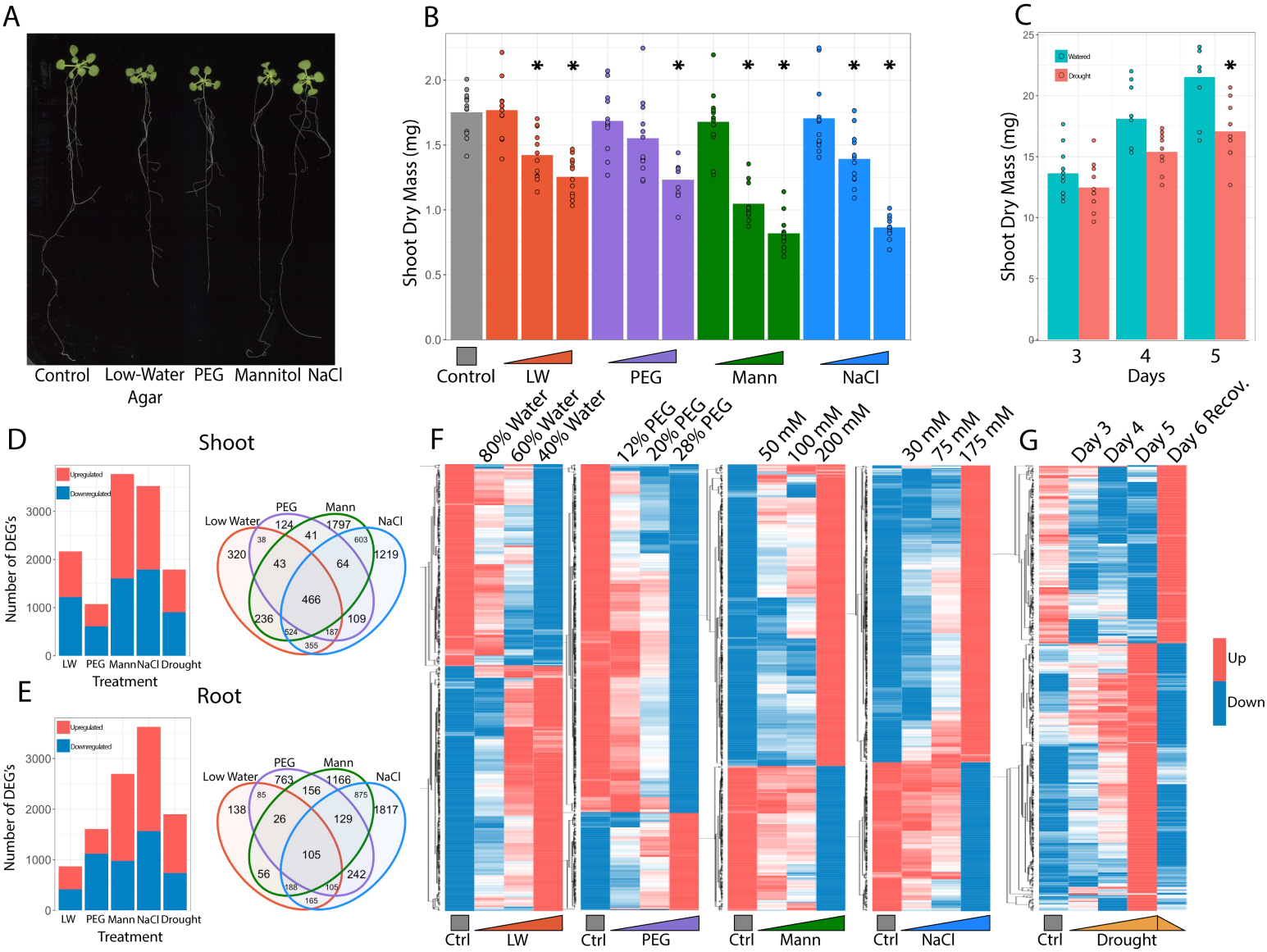

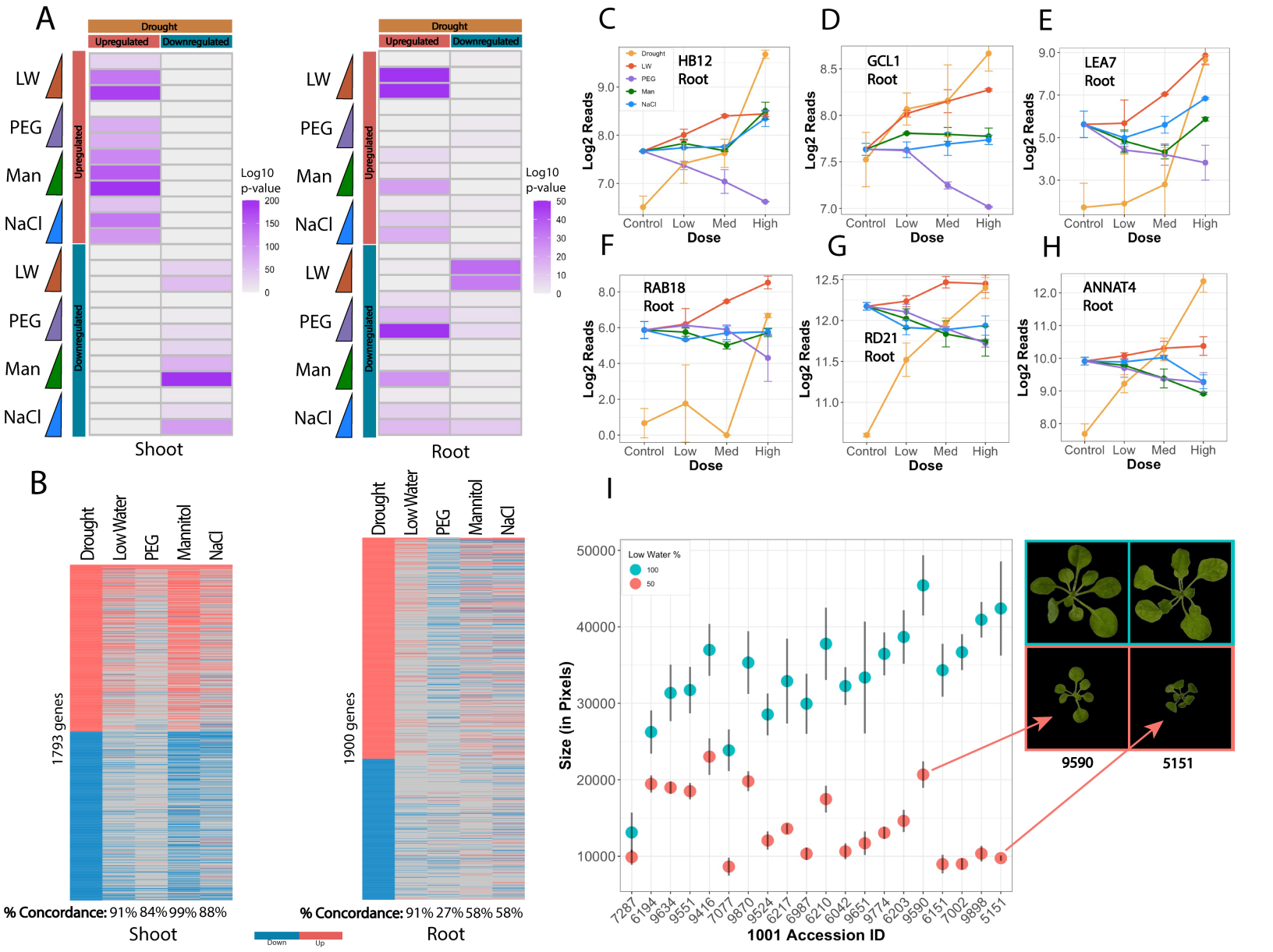

Supplementary

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Supplementary Figure 1 – Plant growth images under osmotic stress.

Supplementary Figure 2 – Gene expression profiles of canonical drought markers in shoot tissue.

Supplementary Figure 3 – Overlapping drought responsive and ABA responsive genes.

Supplementary Figure 4 – Associating low-water agar's impact on shoot size with plant fitness.

Supplementary Table 1 – normalized gene expression counts.

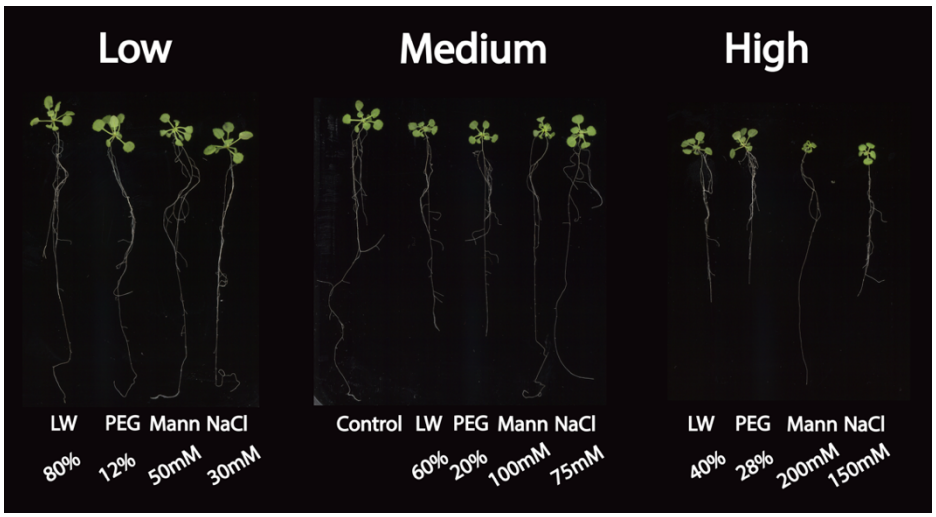
Supplementary Table 2 – list of differentially expressed genes.

Supplementary Table 3 – osmotic stress assay biomass measurements.

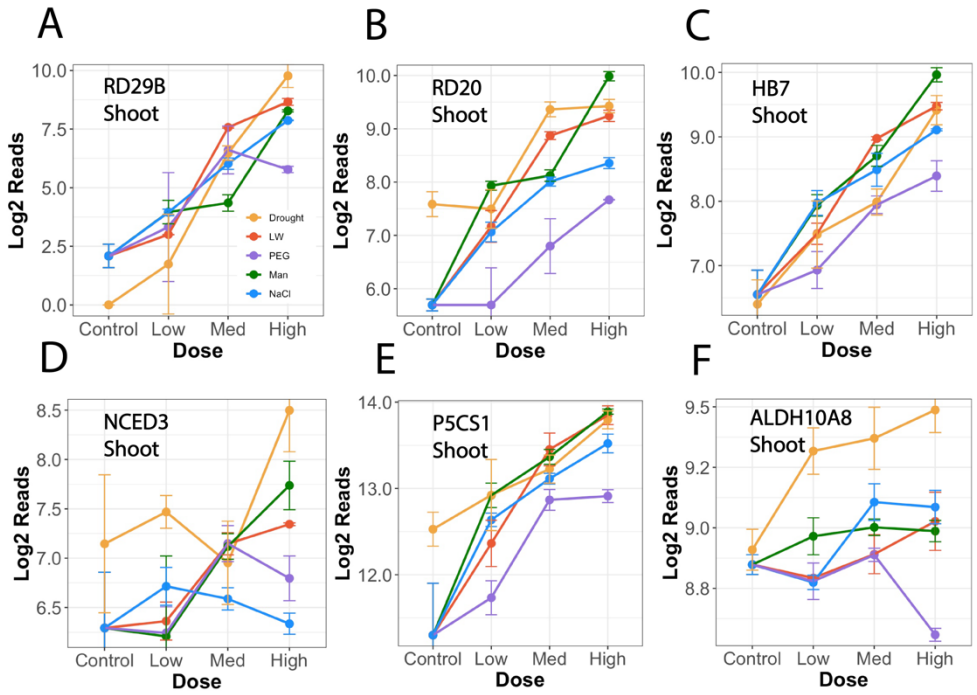
Supplementary Table 4 – drought stress assay biomass measurements.

Supplementary Table 5 – GO Terms.

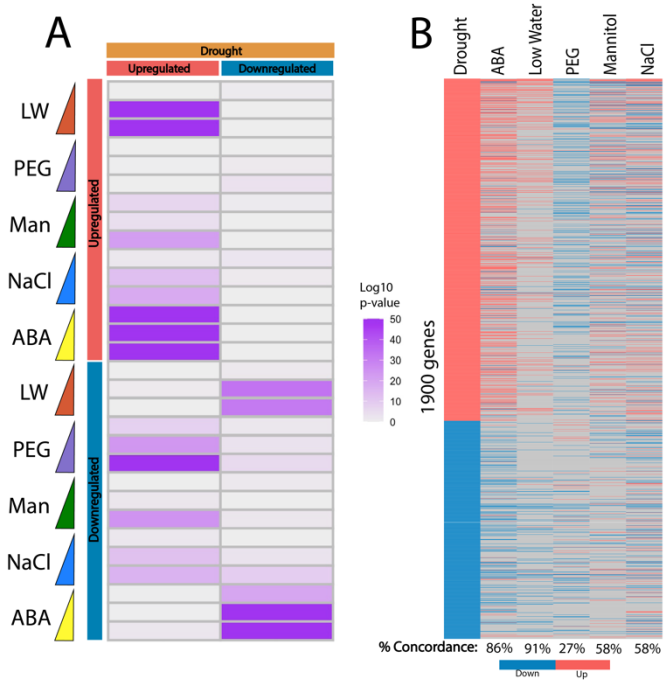
Supplementary Table 6 – Plant Growth Tracker image values.



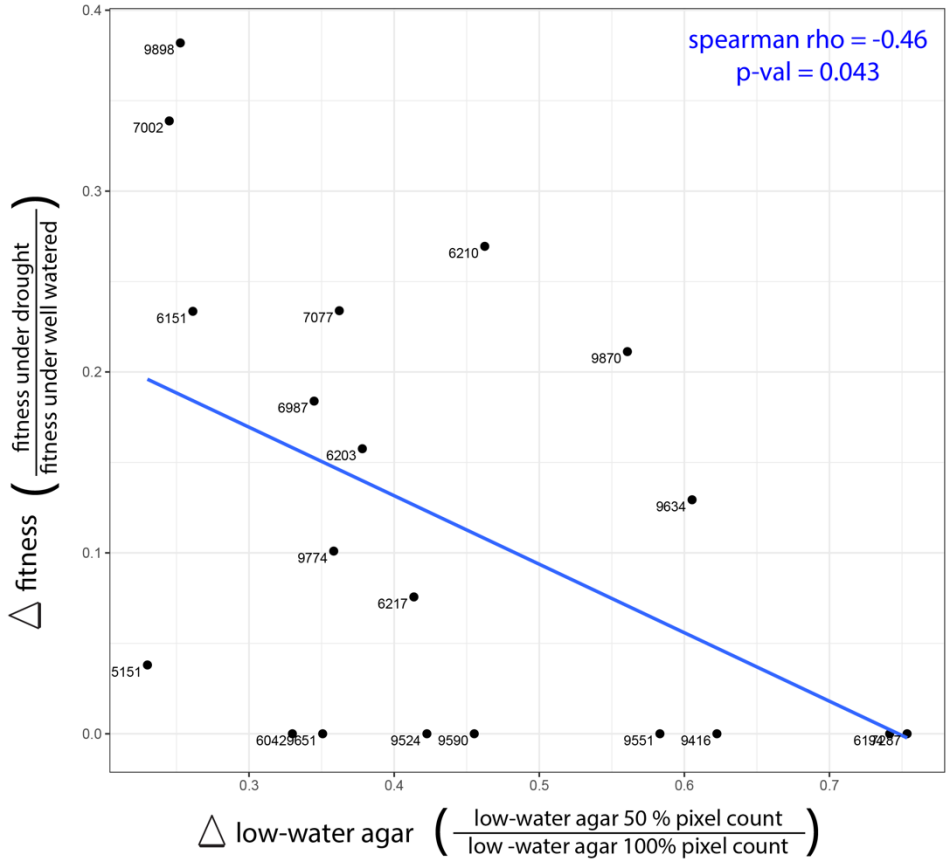
Supplementary Figure 1 - Images of plants grown under different doses of each osmotic stress assay. *Arabidopsis* growth on plates under low-water (LW) agar, PEG-6000, mannitol and salt (NaCl) treatments.



Supplementary Figure 2 - Gene expression profiles of canonical drought markers in shoot tissue. A - F: Expression patterns of drought marker genes under low, medium and high doses of each assay (where drought doses were Day 3, Day 4 and Day 5 respectively). RD29B (AT5G52300), RD20 (AT2G33380), HB7 (AT2G46680), NCED3 (AT3G14440), P5CS1 (AT2G39800), ALDH10A8 (AT1G74920).



Supplementary Figure 3 - Comparative transcriptomic analysis reveals ABA induced differential expression is comparable to drought and low-water (LW) agar signaling A: Intersect analysis of genes found differentially expressed under drought treatment, compared to those under either ABA, PEG, mannitol, NaCl or low-water agar assays in the root (Fisher exact test adj. $p < 0.05$). **B:** Heatmap displaying genes differentially expressed under drought stress in root tissue compared to their expression under the highest dose of each osmotic stress assay.



Supplementary Figure 4 - Associating low-water 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 (*1*).

Materials and Methods

Low-Water Agar Assay – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media), before transfer to 'low-water' plates that contained varying water content. The 100% treatment plate, where water is not lacking, contained 75 mL of 2% agar and 1x 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 1.25x LS media. Plants were grown upon 3 different treatments (80%, 60%, and 40% water amount) for 14 days. On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

To test different *Arabidopsis* accessions on low-water 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 (2 - 5 plants per plate) (**Supplementary Table 6**). Shoot area was calculated from images using Plant Growth Tracker (GitHub - https://github.com/jiayinghsu/plant_growth_tracker).

Vermiculite Drought Assay – *Arabidopsis* seedlings were grown on vertical plates for 17 days (short day, 1x LS, 1% sucrose, 2% agar media), before transfer to vermiculite (0.75x LS media). Plants were then grown on vermiculite at 100% field capacity (FC) for 12 days. On the 13th day, the first time point was sampled (4.5 hours after subjective dawn) where tissue was flash frozen in liquid nitrogen. After this, excess solution was drained from each pot, and then each pot was calibrated to 1x FC. Plant tissue was harvested each day on subsequent days at the same time of day. After the 5^{th} day sample was taken, water was re-added to the remaining pots to an excess of 1x FC. ~ 15 plants were sampled per time point. Dry weight rosette and FC measurements can be found in **Supplementary Table 4**.

Polyethylene Glycol (PEG) Osmotic Stress Assay – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media) before transfer to polyethylene glycol (PEG) media of varying concentrations. PEG media plates were prepared by dissolving crystalline 6000 MW PEG into freshly autoclaved 1x LS media and pouring 50mL of PEG media solution onto 1x LS, 2% agar media plates, letting the PEG solution diffuse into the solid media overnight, then pouring off excess and transferring seedlings to PEG infused media plates. Plants

were grown under 3 different treatments (12%, 20%, and 28% PEG solution) for 14 days. On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

Mannitol and NaCl Osmotic Stress Assays – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media) before transfer to either mannitol or salt (NaCl) media of varying concentrations. Mannitol and NaCl media plates were prepared by adding respective volume of stock solution to 1x LS, 2% agar media before autoclaving for desired molar concentration. Plants were grown under 3 different treatments of mannitol or NaCl (50mM, 100mM and 200mM for mannitol, 30mM, 75mM, and 150mM for NaCl) for 14 days. On day 14, 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

Abscisic Acid (ABA) Exogenous Treatment Assay – *Arabidopsis* seedlings were grown on vertical plates for 8 days (short day, 1x LS, 1% sucrose, 2% agar media) before transfer to 1x LS, 2% agar, control media and grown for 14 days. On day 14, four abscisic acid (ABA) solutions of 10uM, 5uM, 1uM, and 0uM concentration were prepared from 10mM ABA dissolved in ethanol stock, the 0uM solution containing the same 0.1% ethanol concentration as the highest ABA dose. 30 min after subjective dawn, 15 mL of each solution was dispersed onto the roots of the seedlings. After 1 min of treatment, the ABA solution was removed from the plates, and the plates returned to the growth chamber. 2 hours after subjective dawn, shoot and root samples were flash frozen (6 plants per replicate). Dry weight measurements can be found in **Supplementary Table 3**.

RNA-extraction and Library Preparation – Plant tissue was crushed using the TissueLyser (Agilent) and RNA extracted using RNeasy Mini Kit (Qiagen). RNA quality was assessed using Tape station High Sensitivity RNA assay (Agilent). 0.5 - 1 ug of total RNA proceeded to library preparation, where libraries were prepared using TruSeq stranded mRNA kit (Illumina). Resulting libraries were sequenced on the NovaSeq 6000 (Illumina) with 2x150 bp paired-end read chemistry. Read sequences were aligned to the *Arabidopsis* TAIR10 genome using HISAT2 (*2*), and gene counts called using HT-seq (*3*), by relying on Araport11 annotation (*4*). Normalized counts can be found in **Supplementary Table 1**. For each organ, libraries from all experiments were normalized together before calling differential expression.

Statistical Analysis – Differential gene expression was called using DESeq2 (*5*). Specifically, for plate based assays, we called differential expression by comparing the control treatment to the highest treatment dose (i.e. either PEG 28%, Mannitol 200 mM, NaCl 175 mM, Dry Plate 40 %, ABA 10 uM), using an adjusted p-value threshold of 0.05. To detect differential expression in our drought assay on vermiculite, we called differential expression using a linear model, using *design ~ water-loss*, where 'water-loss' was the amount of water that had evaporated from the pot. The complete list of differentially expressed genes for each experiment can be found in **Supplementary Table 2**. Resulting heatmaps were generated using Morpheus (Broad Institute). Overlap analyses were performed using Fisher exact tests, with an adjusted p-value threshold of 0.05. The background for these intersect was all expressed genes within the respective organ. GO Term analysis was performed in VirtualPlant (*6*), with all expressed genes within the respective organ used as background.

References

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