1 **Distinctive phytohormonal and metabolic profiles of *Arabidopsis thaliana* and *Eutrema***

2 ***salsugineum* under similar soil drying**

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4 Carla Pinheiro[1,2,*], Elizabeth Dickinson[3], Andrew Marriott[3], Isa C. Ribeiro[1], Marta Pintó-

5 Marijuan[1,5], Carla António[1,3], Olfa Zarrouk[1], Maria Manuela Chaves[1], Ian C. Dodd[6], Sergi

6 Munné-Bosch[5], Jane Thomas-Oates[3], Julie Wilson[3,4#].

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8 [1] Instituto de Tecnologia Química e Biológica, Universidade NOVA de Lisboa, Av. da

9 República, EAN, 2781-901 Oeiras, Portugal

10 [2] DCV - Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, 2829-516

11 Caparica, Portugal

12 [3] Department of Chemistry, University of York, Heslington, York YO10 5DD, United

13 Kingdom

14 [4] Department of Mathematics, University of York, Heslington, York YO10 5DD, United

15 Kingdom

16 [5] Department of Evolutionary Biology, Ecology and Environmental Sciences, Facultat de

17 Biologia, Universitat de Barcelona, Avinguda Diagonal 643, 08028 Barcelona, Spain

18 [6] The Lancaster Environment Centre, Lancaster University, Lancaster, United Kingdom

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21 * Corresponding author: pinheiro@itqb.unl.pt

22 [#] Corresponding author: julie.wilson@york.ac.uk

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25 Running title: **Arabidopsis and Eutrema responses to soil drying**

26

27 **Abstract**

28

29 Although plants respond to soil drying via a series of concurrent physiological and molecular

30 events, drought tolerance differs greatly within the plant kingdom. While *Eutrema salsugineum*

31 (formerly *Thellungiella salsuginea*) is regarded as more stress tolerant than its close relative

32 *Arabidopsis thaliana*, their responses to soil water deficit have not previously been directly

33 compared. To ensure a similar rate of soil drying for the two species, daily soil water depletion

34 was controlled to 5-10 % of the soil water content. While partial stomatal closure occurred

35 earlier in Arabidopsis (Day 4) than Eutrema (from Day 6 onwards), thereafter both species

36 showed similar stomatal sensitivity to drying soil. However, both targeted and untargeted

37 metabolite analysis revealed greater response to drought in Arabidopsis than Eutrema. Early

38 peaks in foliar phytohormone concentrations and different sugar profiles between species were

39 accompanied by opposing patterns in the bioactive cytokinin profiles. Untargeted analysis

40 showed greater metabolic adjustment in Arabidopsis with more statistically significant changes

41 in both early and severe drought stress. The distinct metabolic responses of each species during

42 early drought, which occurred prior to leaf water status declining, seemed independent of later

43 stomatal closure in response to drought. The two species also showed distinct water usage, with

44 earlier reduction in water consumption in Eutrema (Day 3) than Arabidopsis (Day 6), likely

45 reflecting temporal differences in growth responses. We propose Arabidopsis as a promising

46 model to evaluate the mechanisms responsible for stress-induced growth inhibition under the

47 mild/moderate soil drying that crop plants are typically exposed to.

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49 **Keywords**: Bioactive cytokinins; Drought resilience; Metabolite profiles; Redox state;

50 Rewatering; Stomatal conductance; Unsupervised multivariate analysis.

51

Abbreviations

52	**Abbreviations**	
53	2-IP	2-isopentenyl adenine
54	ABA	Abscisic acid
55	ACC	Ethylene precursor 1-amino-cyclopropane-1-carboxyic acid
56	AscA	Ascorbate (reduced)
57	Chla	Chlorophyll a
58	CKs	Cytokinins
59	DHA	Dehydroascorbate (oxidized)
60	DHZ	Dihydrozeatin
61	DHZR	Dihydrozeatin riboside
62	DTT	Dithiothreitol
63	Fv/Fm	Leaf chlorophyll fluorescence
64	GAs	Gibberellins
65	IAA	Indole-3-acetic acid
66	IPA	Isopentenyl adenosine
67	JA	Jasmonic acid
68	LC-MS	Liquid chromatography - mass sectrometry
69	LRWC	Leaf relative water content
70	OA	Osmotic adjustment
71	OP	Osmotic potential
72	PCA	Principal components analysis
73	QC	Quality control
74	Z	*Trans*-zeatin
75	ZR	*Trans*-zeatin riboside
76	OP100	Osmotic potential at full turgor
77	ROS	Reactive oxygen species
78	RRWC	Root relative water content
79	SA	Salicylic acid
80	SWC	Soil water content
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Introduction

In view of climate change, a major goal for the plant biology community is to understand the mechanisms that allow some plants to withstand drought or hot weather. Knowledge of how plants survive and reproduce in challenging environmental conditions can allow novel targets to be tested in crop-breeding programs. The well-known model species *Arabidopsis thaliana* provides information that can be applied to crop systems (Piquerez et al. 2014; Gilliham et al. 2017). Using the Columbia accession (Col-0) and its mutants has allowed many stress regulatory and responsive pathways to be deciphered (Koornneef and Meinke, 2010; Osakabe et al. 2014), although its stress resilience has not been fully established. Despite wide ecotypic variation (Montesinos-Navarro et al. 2011; Clauw et al. 2016), Arabidopsis is not expected to cope well in extreme environments (Zhu et al. 2015). Instead, Arabidopsis relatives such as *Eutrema salsugineum* have been proposed as stress-tolerant models (Orsini et al. 2010; Zhu et al. 2015). Eutrema seems prepared for stress, as its stress-related genes are upregulated in comparison to Arabidopsis even when grown under optimal conditions (Taji et al. 2004; Gong et al. 2005). As with *Arabidopsis*, *Eutrema salsugineum* ecotypes from different geographical regions show significant genetic variation (Lee et al. 2016). However, physiological and metabolic responses of Arabidopsis and its stress tolerant relatives to soil water deficit have not been directly compared.

Physiological responses to water deficit are modulated by the intensity, duration, and rate of progression of imposed drought (Pinheiro and Chaves, 2011). Extensive research on the stomatal regulation of water loss demonstrates a trade-off between carbon assimilation, efficient water use and leaf cooling capacity (Chaves et al. 2016). Plants can be grouped according to whether they avoid heat (keeping their stomata open for longer) or use water efficiently (closing their stomata sooner, a typical drought-avoidance strategy). However, if plants can avoid the deleterious effects of heat by keeping their stomata open for longer, while maintaining a favourable water status by extracting more water (e.g. by having deep roots), this strategy benefits carbon uptake in addition to the cooling effect. Under drought, Arabidopsis Col-0 closes its stomata at higher soil moisture levels than other Arabidopsis genotypes (Meyre et al. 2001). The two well-studied ecotypes of Eutrema, Shandong and Yukon, can grow under limited soil water availability (Xu et al. 2014; Macleod et al. 2015), but their drought performance, relative to Arabidopsis, is unknown.

The two plant species seemingly have distinct water consumption strategies, although it may

be difficult to separate species *versus* accession variation. Arabidopsis (Col-0) had relatively higher total transpiration than Eutrema (Shandong) under non-challenging conditions, which was related to its higher relative growth rate (Orsini et al. 2010). Salinity decreased transpiration to a larger extent in Arabidopsis than Eutrema. In addition to these different water consumption strategies, Eutrema and Arabidopsis also had different biochemical composition under non-challenging growth conditions, with foliar sucrose and glucose content higher in Eutrema, while the hormones salicylic acid (SA) and jasmonic acid (JA) were higher in Arabidopsis (Arbona et al. 2010; Pilarska et al. 2016). Furthermore, Eutrema expressed more stress and defence genes than Arabidopsis under non-challenging conditions, which is described as stress priming (e.g. Taji et al. 2004; Gong et al. 2005; Lee et al. 2016). It is uncertain whether these biochemical differences regulate differences in transpiration, and consequently different rates of soil water depletion.

The metabolic features associated with the initial stages of soil drying are not clear. In Arabidopsis, soil drying partially closes the stomata well before any decrease in carbon assimilation rate (Hummel et al. 2010; Bechtold et al. 2016) or any significant increase in foliar abscisic acid (ABA) content (Bechtold et al. 2016). ABA is described as the main driver controlling plant performance under limited water availability since it induces stomatal closure, but more comprehensive recent studies demonstrated that most of the plant hormones are involved in stress signalling (Müller and Munné-Bosch, 2015). In addition, during the very early stages of water limitation, effects on carbon metabolism (CO_2 assimilation, and sucrose and starch formation and allocation) may be decoupled from stomatal closure (Pinheiro et al. 2011; Bechtold et al. 2016). Many players are involved in stress perception and signal transduction leading to large alterations in carbon metabolism (Golldack et al. 2014; Urano et al. 2017). The metabolic balance between several molecules triggers adjustment mechanisms, and when several thresholds are achieved, physiological responses to drought occur (Pinheiro et al. 2011). The integration of multiple environmental signals by sugars, hormones, and reactive oxygen species (ROS) adjusts plant growth and determines whether plants survive or perish under given environmental conditions (Pinheiro and Chaves, 2011; Osakabe et al. 2014). The precise chain of events is not yet defined, and although some pathways and interactions are understood, others are more elusive (Rivas-San Vicente and Plasencia, 2011; Munné-Bosch and Müller, 2013; Ruan, 2014; de Ollas and Dodd, 2016). Although recent reports highlight that stomatal closure is one of the initial events in response to soil drying, many other metabolic adjustments also take place.

This research aimed to elucidate the impact of gradually declining soil water availability on leaf metabolism by directly comparing Arabidopsis (Col-0) and Eutrema (Shandong) under slowly imposed progressive soil water deficit. As small changes in soil water content (10-15 %) affect not only leaf conductance but also plant metabolism (Davies et al. 1990; Pinheiro et al. 2011), we used both untargeted metabolite analysis and targeted metabolite/biochemical analyses to explore the physiological and metabolic adjustments prior to significant stomatal closure. Although Arabidopsis and Eutrema show distinctive responses, Arabidopsis is able to keep spending water for longer and could therefore provide a good model to study stress responses under the soil drying conditions that crop plants are typically exposed to.

Materials and methods

Arabidopsis thaliana (Col-0) and *Eutrema salsugineum* (Shandong) seeds were soaked and stratified at 4 ℃ for 4 or 14 days, respectively. Eutrema seeds were kindly donated by Arie Altman (The Hebrew University of Jerusalem) and Arabidopsis seeds were purchased from ABRC (Arabidopsis Biological Research Center, Columbus USA). *Eutrema salsugineum* is the current designation of *Thellungiella salsuginea* (Integrated Taxonomic Information System on-line database, www.itis.gov; The International Plant Names Index, www.ipni.org). Seeds were then transferred to pots (300 mL) containing a 1:1 mixture of coarse sand and peat (Shamrock). Plants were grown under controlled conditions, under a 12 h photoperiod, temperatures ranging from 20 to 24 ˚C, with a 60-70 % relative humidity and photosynthetically active radiation (PAR) of 250-300 μmol m^{-2} s^{-1} (SON-T Agro 400w, Phillips). Plants were watered every day with demineralized water to 85 % of soil water content (SWC). SWC was monitored daily and is defined as: SWC = [(pot weight – pot weight with totally dried substrate)] / [(pot weight at drained capacity – pot weight with totally dried substrate)] x 100. Drought stress treatments were imposed when plants had 8 to 10 fully expanded leaves (40 days for Eutrema and 36 days for Arabidopsis) and had covered the surface of the pots (thereby minimising evaporation from the soil). Plant growth increased during the experiment by 2.9 g fresh weight (FW) for Arabidopsis and 2.5 g FW for Eutrema (on average), corresponding to less than 0.8 % error in estimating SWC (Fig. 1).

Preliminary drought experiments, in which water was withheld, showed faster soil water depletion and more rapid stomatal closure in Arabidopsis (Suppl. Fig. S1). Similarly, higher transpiration rates of Arabidopsis were previously reported (Orsini et al. 2010). Stomatal

182 conductance of the two species was differentially sensitive to soil drying (Suppl. Fig. S1c and

183 c). Within the 45-55 % SWC range, Arabidopsis showed greater stomatal closure than Eutrema,

184 but below 40 % SWC both species showed similar stomatal sensitivity to soil water deficit and

185 were severely affected by drought. However, analysis of covariance demonstrated no

186 significant species x SWC interaction, with both species showing a similar relationship

187 between % gs *vs* soil water content (Suppl. Fig. S1c).

188 To compare stress duration and intensity effects on plant responses, the rate of soil water

189 depletion was controlled to 5-10 % of the SWC per day by pre-dawn irrigation (Fig. 1). Even

190 when controlling the SWC, Arabidopsis consumed more water than Eutrema, as indicated by

191 the greater divergence between SWC measured at maximum soil water deficit (symbols) and

192 the SWC to which the pot was re-turned to pre-dawn ("stress" line in Fig. 1). This greater water

193 use of Arabidopsis was most prominent between Days 3 and 7.

194 Plants were harvested 0 (last day of watering), 1, 3, 5 and 12 days after beginning the

195 experiment, corresponding to 75 %, 66 %, 45 %, and 12 % SWC, respectively. Samples were

196 also taken the day after re-watering (1 d). Six biological replicates were obtained at each time-

197 point, except for Day 1 controls for which there were only five biological replicates, providing

198 65 samples of each plant species. At the beginning of the assay, the most recently expanded

199 two-three leaflets were identified and used for physiological and water status measurements.

200 For the biochemical analysis, and when analysing severe drought and early rewatering, only

201 non-senescent leaflets were used, i.e. the younger leaflets. Samples for biochemical (hormone,

202 carbohydrate, pigment and oxidative status) analysis were immediately frozen in liquid

203 nitrogen and kept at -80 ℃ until further extraction and analysis. Samples for osmotic potential

204 and for RWC were then collected.

205 **Leaf conductance, water status and osmotic adjustment**

206 Stomatal conductance was measured 2-3 h after the beginning of the photoperiod in five plants

207 per treatment using a portable gas exchange photosynthesis system coupled to a 6400-15

208 chamber (1 cm^2 diameter cuvette, Li-6400, Li-Cor, Lincoln, Nebraska, USA). Three to five

209 measurements were made per plant on the most recently expanded leaf.

210 Leaf and root samples were taken 4 h after the beginning of the photoperiod. Leaf discs (3 mm

211 diameter) and total roots were weighed to obtain fresh weight (FW), placed in darkened petri

212 dishes containing distilled water for 2 h to fully hydrate, then re-weighed to obtain turgid

213   weight (TW), and then dried at 80 ºC for 48 h to obtain dry weight (DW). Leaf (LRWC) and

214   root (RRWC) relative water content were calculated as: RWC = [(FW – DW) x 100 / (TW –

215   DW)].

216   Leaf osmotic potential (ψs) was evaluated from leaf disks (8 mm, $n = 5$–6), frozen and stored

217   at -80 ºC. The leaf osmotic potential was measured with an HR-33T dew point microvoltmeter

218   and C-52 sample chambers (Wescor, Inc., Logan, UT, USA). The osmotic potential was

219   adjusted to the LRWC to calculate the osmotic potential at full turgor (OP100), and the osmotic

220   adjustment was calculated as previously described (Turner et al. 2007).

221   **Phytohormone quantification via LC-MS targeted analysis**

222   Freeze-dried shoots (50 mg) were used to extract and quantify the following hormones (Müller

223   and Munné-Bosch, 2011): auxin (indole-3-acetic acid: IAA), gibberellins (GA1, 4, 9, 19, 20,

224   24), cytokinin (CK) compounds (*trans*-zeatin: Z; *trans*-zeatin riboside: ZR; 2-isopentenyl

225   adenine: 2iP; isopentenyl adenosine: IPA; dihydrozeatin: DHZ; dihydrozeatin riboside:

226   DHZR), and stress-related phytohormones (ABA; JA; SA; and ethylene precursor 1-amino-

227   cyclopropane-1-carboxyic acid: ACC).

228   Extraction was performed in methanol solutions containing 1 % glacial acetic acid, using the

229   following standards: d_5-IAA, d_6-2-isopentenyl adenine (d_6-2iP), d_6-IPA, d_6-ABA, d_5-JA, d_4-

230   SA, d_4-ACC, d_2-GA$_1$, d_2-GA$_4$, d_2-GA$_9$, d_2-GA$_{19}$, d_2-GA$_{20}$ and d_2-GA$_{24}$; d_5-Z and d_5-ZR were

231   used as standards for Z, DHZ, ZR, and DHZR. After adding 170 µL of the extraction solution

232   and 30 µL of a solution containing 100 ppm of the standards in the same solvent, the materials

233   were mixed in a vortex mixer for 5 s and exposed to ultrasound for 30 min, followed by

234   centrifugation at 9,500 *g* for 10 min. The supernatant was removed and the residue was washed

235   twice with 100 µL of the solvent solution. The supernatant and washes were combined and

236   filtered through PTFE 0.22 µm filter paper (Waters, Milford, MA) and 5 µL aliquots were

237   analysed using a UPLC-ESI-MS/MS (Acquity UPLC System from Waters, Milford, MA) and

238   tandem MS/MS experiments were performed on an API 3000 triple quadrupole mass

239   spectrometer (PE Sciex, Concord, Ont., Canada) using a HALO™ C18 column (2.1 × 75 mm,

240   2.7 µm) (Advanced Materials Technology, Inc. Wilmington, DE) and a binary mobile phase

241   system composed of (A) water modified with 0.05 % glacial acetic acid and (B) acetonitrile

242   modified with 0.05 % glacial acetic acid. Quantification was performed by preparing a

243   calibration curve including each of the analysed compounds and calculating the

244   compound/standard ratio using Analyst™ software (Applied Biosystems, Inc., Foster City,

245   CA). The results were expressed on a dry weight (DW) basis.

Ascorbate oxidative status

247   Ascorbate reduced and oxidized forms were determined by a plate-reader method (Queval and
248   Noctor, 2007) with slight modifications. Briefly, lyophilised leaves (20 mg DW) were placed
249   in a microcentrifuge tube with two tungsten balls and ground under liquid nitrogen in a Retsch
250   MM300 Bead Mill Cell Disrupter (Retsch GmbH & Co Haan Germany). Subsequently, 1 mL
251   of extraction buffer (6 % meta-phosphoric acid) was added, vortexed for 1 min and clarified
252   by centrifugation at 10,000 g (10 min, 4ºC). Finally, extracts were neutralized and adequately
253   diluted before spectrophotometric readings on a 96 well quartz microplate (Hellma Hispania
254   SL, Badalona, Spain). The levels of ascorbate (AscA) (reduced) and dehydroascorbate (DHA)
255   (oxidized) were determined using ascorbate oxidase (AO) and dithiothreitol (DTT),
256   respectively (Foyer et al. 1983). AO specifically oxidizes all AscA in the sample. Therefore,
257   the decrease in O.D. at 265 nm is related to AscA content. Alternatively, when the samples are
258   incubated with DTT, DHA is reduced to AscA and the increase in O.D. is proportional to the
259   initial DHA content. The ascorbate oxidative status was estimated as DHA/(DHA + AscA).

Photosynthetic pigments quantification

261   For pigment extraction, lyophilised leaf samples (15 mg DW) were placed in a microcentrifuge
262   tube with two tungsten balls, ground under liquid nitrogen in a Retsch MM300 Bead Mill Cell
263   Disrupter (Retsch GmbH & Co Haan Germany), and extracted with ice-cold 80 % acetone
264   (v/v). After centrifuging at 6,500 g for 10 min at 4 °C, the supernatant was collected and the
265   pellet was re-extracted with the same solvent until it was colourless. Then, supernatants were
266   pooled and analysed spectrophotometrically. Specific absorption coefficients in 80 % acetone
267   previously reported were used to quantify chlorophyll a, chlorophyll b and carotenoids
268   (Lichtenthaler and Buschmann, 2001).

Extraction of water soluble carbohydrates and starch

270   Water-soluble carbohydrates were extracted from freeze-dried leaf material following a
271   chloroform:methanol method previously described (Antonio et al. 2008). Briefly, 50 mg DW
272   of leaf material was ground in liquid nitrogen and extracted with 250 µL ice-cold chloroform:
273   methanol (3:7, v/v), vortex-mixed and incubated at -20 ºC for 2 h. After incubation, samples
274   were extracted twice with ice-cold water, and after centrifugation at 17,900 g at 4 ºC for 10
275   min, the upper phases were collected and pooled. The combined supernatants containing the

9

276 water-soluble carbohydrates were evaporated to dryness using a centrifugal concentrator

277 (Savant SpeedVac Plus SC110A, Thermo Electron Corporation, Runcorn, UK). Samples were

278 reconstituted in 100 μL water and centrifuged at 6,800 g at 20 ˚C for 30 min, followed by LC-

279 MS analysis.

280 For starch analysis, the pellet resulting from the chloroform:methanol extraction was washed

281 twice with water. Ten volumes of water were added to the pellet, boiled for 3 min, and

282 autoclaved at 130 ˚C for 1 h. After cooling, samples were incubated with 6 U amyloglucosidase

283 (Roche Applied Science, Amadora, Portugal) for 2 h at pH 4.8 and 60 ˚C. Starch was quantified

284 in the supernatant using a starch enzymatic quantification kit (nº 10207748035, R-Biopharm

285 Aktiengesellschaft, Darmstadt, Germany) and by making use of the Hatterscheid and

286 Willenbrink modification as previously described (Pinheiro et al. 2001).

287 **Untargeted LC-MS analysis of the water-soluble carbohydrate fraction**

288 Arabidopsis and Eutrema samples were analysed as separate cohorts. In each case, samples

289 were randomized and run in batches of eight or nine with the injection of a pooled sample

290 between batches for quality control (QC). LC-MS analyses were performed on a Dionex U3000

291 2D HPLC system coupled to a Bruker maXis UHR-Q-TOF MS with an ESI interface. Analytes

292 were detected in the negative ion mode using the following MS parameters: capillary voltage,

293 4500 V; nebulizer gas, 2 Bar; drying gas, 8.0 L/min; drying temperature, 200ºC, and collision

294 energy, -10.0 eV. Mass spectra were acquired over the scan range m/z 50-1000.

295 Chromatographic separation was carried out using a porous graphitic carbon (PGC)

296 Hypercarb™ column (5 μm, 100 mm × 4.6 mm; Thermo Electron, Runcorn, Cheshire, UK) at

297 a flow rate of 600 µL min^{-1}. All samples were reconstituted with 500 µL deionised water with

298 a further 50-fold dilution in deionised water to prevent signal saturation and to minimise matrix

299 effects. The sample injection volume was 20 μL and the PGC column was used at ambient

300 temperature (25 ºC). The binary mobile phase was composed of (A) water modified with 0.1

301 % (v/v) formic acid (FA) and (B) acetonitrile modified with 0.1 % FA. The gradient elution

302 was as follows: 0-4 min maintained at 2 % B; 4-7 min, 2 to 8 % B; 7-10 min 8-25 % B and

303 maintained for 3 min, followed by column regeneration and re-equilibration: 13-19 min, 25 to

304 40 % B; 19-19.5 min, 40 to 50 % B held for 1 min; 20.5-21 min 50 to 99 % B held for 2 min;

305 23-25 min 99 to 2 % B and maintained for 10 min. All solvents were purchased from Fisher

306 Scientific except FA, which was purchased from Sigma Aldrich.

307 **Statistical analysis**

10

308 Raw LC-MS data were pre-processed using Progenesis QI (Nonlinear Dynamics, Newcastle
309 Upon Tyne, UK). Mass spectra were aligned by retention time and normalized to the same total
310 ion count before peak picking was performed to provide a matrix of potential metabolites for
311 each observation, annotated by the accurate mass (m/z between 50 and 1000) and retention time
312 (between 1 and 30 min) of the corresponding peak. In total, 53208 and 33032 peaks were
313 recorded for Arabidopsis and Eutrema, respectively, and were used as variables in multivariate
314 and univariate analyses.

315 When the Eutrema data were scaled to unit variance to allow smaller variables to contribute to
316 the analysis, differences between batches became apparent, with the last two batches differing
317 substantially from the rest (Suppl. Fig. S2a). Liquid chromatography-mass spectra are often
318 acquired batch-wise to allow necessary calibrations and cleaning of the instrument. However,
319 this may introduce further sources of variation, such as differences in the conditions under
320 which data for individual batches is acquired. Quality control (QC) samples are frequently
321 employed to both judge and correct for this variation.

322 However, batch correction using the QC observations increased inter-batch variation as the
323 change in observations between batches was often not well-represented by the change in
324 corresponding QCs. Therefore, background correction for each variable was performed
325 (Rusilowicz et al. 2016; Wehrens et al. 2016). This method identifies a background trend, using
326 experimental observations as well as the QCs, with which to adjust the intensities. The run
327 order for data collection was randomized, but by chance a disproportionate number of early-
328 stress observations occurred in batch 3 and several late-stress observations in batch 4. With the
329 exception of these two batches, which were combined, we used a separate trend for each batch,
330 obtained as a moving median with a window width of 5 observations. The effectiveness of
331 batch correction was assessed using the Bhattacharrya distance (Wehrens et al. 2016). In
332 addition, an outlier that dominated the variance after scaling was removed before calculating
333 the trend. Control correction was also performed on each variable to remove differences due
334 to growth. For each day of harvest, this was achieved by subtracting the median over the six
335 control replicates from the corresponding variable in the water-stressed observations for that
336 day. The Arabidopsis data showed no obvious differences between batches (Suppl. Fig. S3),
337 and therefore, batch correction was deemed unnecessary but control correction was performed
338 to prevent differences due to growth from masking early-stress characteristics. Principal
339 components analysis (PCA) was used for unsupervised multivariate analysis with both

340 unscaled data and after scaling to unit variance to prevent high content metabolites dominating
341 the analysis.

342 To identify patterns in metabolites over time, k-means cluster analysis was performed with the
343 control-corrected time-series for both datasets. The initial clusters obtained were filtered using
344 the sum of squared values to remove the time-series for metabolites that did not differ
345 appreciably between drought and control observations, i.e. where all values in the control-
346 corrected time-series were close to zero. Cluster analyses of the remaining time-series (with
347 various values of k) showed the largest cluster to consist of time-series with small random
348 fluctuations (essentially flat with random noise) rather than any temporal trend. We therefore
349 introduced an iterative filtering process to reduce the number of time-series, leaving small
350 clusters of time-series with very consistent patterns over time. In each iteration, k-means
351 clustering with $k = 15$ was performed and the largest cluster removed before the next analysis.
352 After four iterations, 46 time-series remained and were clustered using k-means with $k = 9$.

353 Univariate analyses were performed using the non-parametric Mann-Whitney U-test with
354 Benjamini-Hochberg correction for multiple comparisons (Benjamini and Hochberg, 1995).
355 Three-way group comparisons were carried out (early stress/late stress/rewatered and Days 1,
356 3 and 5 for each species) with one-way ANOVA and Tukey's honest significant difference
357 (HSD) correction for multiple pairwise testing. Data correction methods were implemented
358 using C code written in-house and statistical analyses were performed in the R platform,
359 version 2.13.1 (R Core Team, 2016) or in Matlab (The MathWorks Inc., Natick, MA, USA).

360 Analysis of covariance (ANCOVA) discriminated possible species difference in stomatal
361 sensitivity to drying soil.

362

363 **Results**

364 **Stomatal sensitivity to drying soil and plant water status**

365 Under well-watered conditions, stomatal conductance (gs) of both species exceeded 0.11 mol
366 m^{-2} s^{-1} (Suppl. Fig. S4a). Since gs of well-watered plants varied from day to day, gs of plants
367 in drying soil was normalised according to the average well-watered values of each species.
368 As the soil dried (Fig. 2), partial stomatal closure of Arabidopsis and Eutrema was detected on
369 Days 4 and 6, respectively (Fig. 2). Within the 45-55 % SWC range, Arabidopsis showed
370 greater stomatal closure than Eutrema, but below 40 % SWC both species showed similar

stomatal sensitivity to soil water deficit and were severely affected by drought. Stomatal conductance responded sluggishly to re-watering, with limited recovery (Suppl. Fig. S4a). Across the entire experiment, both species showed a similar relationship between % gs *vs* soil water content, with analysis of covariance demonstrating no significant species x SWC interaction (Suppl. Fig. S4b). Thus, both species showed similar stomatal sensitivity to drying soil.

Initial stomatal closure was not associated with decreased leaf water status, i.e. lower cell volume did not trigger early stomatal closure (Sack et al. 2018). On imposing soil water deficit, leaf RWC transiently decreased on Day 3 in Arabidopsis (supplementary table S1), but no significant differences in Eutrema leaf (and root) RWC were detected until Day 5 (supplementary table S2). Although statistically significant (at the 95% confidence level), its small magnitude (~4%) could be within the method error or due to daily fluctuations.

In contrast to plant water status, the water consumption patterns changed very early on, but were not temporally correlated with stomatal closure. Compared with its well-watered control, Eutrema started to lose less water from Day 3 onwards (3 days before any significant stomatal closure), as indicated by the slope of the soil RWC% line for plants in drying soil (Fig. 1). In contrast, Arabidopsis used less water from Day 6 onwards (two days after partial stomatal closure occurred). This suggests that earlier growth inhibition of Eutrema decreased whole plant water loss was independent of changes in plant water status.

By Day 12, leaf RWC of both species had declined to very low values (< 20 %) and leaflets selected for water status measurements (those most recently expanded at the onset of the assay) were severely wilted and exhibited senescence symptoms. Lower leaf chlorophyll fluorescence (Fv/Fm) and lower chlorophyll a content indicated photoinhibition and/or leaf senescence (Kalaji et al. 2016).

Despite the severity of the stress imposed, root water status of both species recovered within 24 h of re-watering. Root RWC of Eutrema was similar to those of the well-watered controls, while the root RWC of Arabidopsis was ~90 % of that of the controls. However, leaf RWC remained low, only ~50 % and ~40 % of the well-watered control values in Eutrema and Arabidopsis respectively (supplementary tables S1 and S2). In addition, Fv/Fm tended to increase in Eutrema, but values were unaffected in Arabidopsis (supplementary tables S1 and S2).

Untargeted metabolite analysis

The responses to soil water depletion in Arabidopsis and Eutrema were analysed via untargeted LC-MS, making use of the water-soluble fraction. After batch correction of the Eutrema data, PCA of the control corrected and scaled data grouped according to drought-stress duration for both species (Fig. 3). Moreover, PCA of unscaled data showed that most of the variance is due to large differences between early-stress (Days 1, 3 and 5) and late-stress (Days 12 and 13) observations. Statistical separation of late-stress effects was not related to differing sample water content, since comparable dry weights were used and the resulting data normalised before statistical analysis.

In addition, an iterative k-means algorithm filtered out the largest clusters to leave those comprising more unusual, and potentially more informative, patterns (Suppl. Fig. S5). Hierarchical clustering with the 46 time-series selected by the k-means analysis (Fig. 4) allowed the similarities (or differences) between the associated metabolites to be visualised.

Eutrema responds with small changes to early drought

When considering only the early-stress observations, the PCA scores plot shows clear grouping by stress duration for both plant species (Fig. 5). Distinctive metabolic signatures were obtained even for early days with limited soil drying (< 20 % change in SWC at Day 3).

In both Arabidopsis and Eutrema, inspection of the PCA loadings showed that many variables contribute to the separation of each of the early stress days. Thus, metabolic separation between sampling dates is due to the cumulative changes arising from small contributions of many metabolites. However, the two species react differently to similar decrease in the soil water availability. More metabolites responded to early drought stress in Arabidopsis, with 428 variables showing statistically significant differences between Days 1, 3 and 5 ($P < 0.01$; 36 with $P < 0.001$) in comparison to 35 in Eutrema ($P < 0.01$; 4 with $P < 0.001$). However, none of the variables that consistently differed between the early days corresponded to those identified as late-stress markers (such as sucrose), showing different metabolism during early and late drought.

Severe drought causes larger metabolic alterations in Arabidopsis than in Eutrema

Late-stress markers for both Arabidopsis and Eutrema included peaks that were identified as the carbohydrates sucrose and raffinose, by comparison with authentic standards of these molecules (Table 1). Sucrose significantly increased and raffinose significantly decreased (P

14

433 < 0.001) in late stress (Day 12) and on re-watering (Day 13). A significant decrease was found
434 for features with m/z values of 341 and 387, most probably a hexose disaccharide. A feature
435 with m/z 711, also decreasing significantly, is tentatively assigned to stachyose, known to co-
436 elute with raffinose (Antonio et al. 2008). Soil water deficit significantly ($P < 0.00001$)
437 decreased two co-eluting features (with m/z 191 and m/z 405) in both plant species The feature
438 with m/z 191 was assigned to citric acid, following tandem mass spectrometry (MS^2) analysis
439 and comparison of the fragmentation pattern in both METLIN (www.metlin.scripps.edu) and
440 PRIMe (www.prime.psc.riken.jp) metabolomics databases. The co-eluting feature at m/z 405
441 on MS^2 produced a single fragment at m/z 191.0185, that was tentatively assigned as the
442 [2M−2H+Na]⁻ charge-sharing dimer of citric acid (accurate mass 405.0287). Univariate
443 analyses (after multiple test correction) indicated that 607 variables significantly ($P < 0.0001$)
444 differed between late-stress observations and controls in Arabidopsis, in comparison to just
445 171 in Eutrema.

446 In the cluster analysis, three clusters tend to decrease over time, including the response of
447 raffinose (Suppl. Fig. S5e-g), which was more extreme in Arabidopsis than Eutrema, therefore
448 occurring in a different cluster. Although the different ionic forms of citric acid from both
449 Arabidopsis and Eutrema group together (Suppl. Fig. S5f), a difference in the trend between
450 the two different plant species can be seen, with Eutrema showing an early increase before the
451 overall decrease. Citric acid decreased in response to late and severe drought, as previously
452 observed in lupin and Eutrema (Pinheiro et al. 2004; MacLeod et al. 2015). The final two
453 clusters (Suppl. Fig. S5h and i) show the response profiles of (unknown) compounds that are
454 significantly greater than or lower than the controls throughout the time-series, notably all from
455 Arabidopsis, and are good candidates for further studies.

456 In contrast, four clusters tended to increase rapidly in late drought; the scale of the response
457 accounts for the difference between these four clusters. They mostly comprise the differing
458 ionic forms of sucrose. In Arabidopsis, unknown compounds with m/z 133 and m/z 288
459 exhibited a very similar pattern to sucrose (Suppl. Fig. S5a-d). The most extreme responses
460 result in separate clusters consisting of just one or two observations Suppl. (Fig. S5c and d).
461 For each sucrose ionic species, the response for Days 12 and 13 is more extreme for
462 Arabidopsis than for Eutrema. In both plant species, a further unknown with m/z 195 also
463 clusters with sucrose, and re-watering causes a greater response than during late stress.

464 **Targeted biochemical analysis**

465 Although severe drought decreased the biomass of both species, Arabidopsis (22% decrease)
466 was less sensitive than Eutrema (38% decrease) (supplementary tables S1 and S2). The growth
467 reduction was accompanied by starch remobilization, supporting the hypothesis of carbon
468 reserve reallocation. Although osmotic adjustment was detected under severe drought and
469 rewatering in Eutrema (supplementary table S2), it was only detected in Arabidopsis on
470 rewatering (supplementary table S1).

471 To characterize in more detail the responses to soil water depletion in Arabidopsis and
472 Eutrema, various biochemical parameters (Table 2) were measured during early drought. PCA
473 analysis with all biochemical parameters for both species (Suppl. Fig. S7) showed the greatest
474 source of variance to be the separation of late/severe drought and re-watered observations, as
475 in the untargeted analyses. Without variable scaling, loadings plots showed a large influence
476 of the variables with the greatest mean values (leaf RWC, osmotic potential (OP) and starch)
477 in the total variance. After scaling to unit-variance, the separation of late stress/re-watered
478 observations is still seen along the first principal component, although accounting for far less
479 of the total variance. In Arabidopsis, variables from re-watered samples were closer to those
480 from early-day observations. In Eutrema, the difference between late stress and re-watering is
481 only apparent along the second component, which represents less variance and more similar
482 metabolic status. These findings suggest: 1) Arabidopsis responds faster to soil water
483 availability; and/or 2) Eutrema requires prolonged stimulus to reprogram its metabolism.

484 **Consistent biochemical changes in both Arabidopsis and Eutrema**

485 Under severe stress, some parameters, including ascorbic acid (AscA), leaf chlorophyll
486 fluorescence (Fv/Fm) and chlorophyll a (Chla), have similar patterns in the two species (Fig.
487 S8 & S9). We did not detect significant changes in carotenoid content, but decreased
488 chlorophyll a content indicates that chlorophyll degrades faster than carotenoids (Lichtenthaler
489 and Buschmann, 2001). Ascorbate content significantly decreased under severe drought (43%
490 in Eutrema; 24% in Arabidopsis), suggesting senescence programs were already activated
491 (Noctor et al. 2014) although the sampled leaves did not show visible symptoms of senescence.
492 A further decrease in ascorbate on rewatering (55% in Eutrema; 52% in Arabidopsis) indicates
493 the senescence program was still active.

494 In contrast to most hormone responses to soil drying, which are quite distinct in the two species
495 (Table 2), SA was found to decrease significantly in both species.

Distinct biochemical changes between Arabidopsis and Eutrema

While some metabolites showed minimal (< two-fold) differences between species, starch, JA and ZR were more abundant in Arabidopsis, and IAA and DHA were more abundant in Eutrema (supplementary tables S1 and S2). Severe drought increased content of the ethylene precursor ACC by 70 % in Eutrema, but had no effect in Arabidopsis, suggesting ethylene-independent stomatal closure as both species showed similar stomatal sensitivity to drying soil. In contrast, re-watering Eutrema returned ACC levels to well-watered values, while profoundly increasing ACC content in Arabidopsis.

Several CK species including ZR and 2-iP, long distance translocation forms of CKs (Kieber and Schaller, 2014), as well as IPA (2-iP precursor) accumulated in Arabidopsis but not in Eutrema during late stress (Fig. 6). In contrast, re-watering returned content of these CKs to well-watered values in Arabidopsis, while stimulating their accumulation in Eutrema. IPA and 2-iP are precursors of Z, one of the most active CK forms (Hirose et al, 2008; Kieber and Schaller, 2014). However, the mobilization (metabolism and/or translocation) of these CKs in Arabidopsis was not reflected in higher Z levels.

Species-dependent hormonal responses during early stress

ABA, JA, SA and GA profiles are clearly different for the two plant species between Days 1 and 5 (Table 3, Fig. 7). Despite daily irrigation to ensure a similar rate of soil drying in the two species, soil water deficit increased foliar ABA content of Arabidopsis, but not Eutrema, on Day 5. Foliar JA content transiently increased on Day 3 only in Arabidopsis, preceding increased ABA accumulation on Day 5. Similarly, SA content transiently increased on Day 3 in Arabidopsis (Fig. 7).

In Eutrema, changes in leaf RWC and ABA occurred after Day 5, with foliar ABA accumulation in Eutrema occurring below 45 % SWC. Species differences could be associated with the osmotic potential (OP) and the redox state regulation, as significant changes were observed in Eutrema, but not in Arabidopsis (Fig. 8). Decreased OP in Eutrema at Day 3 may maintain turgor, thereby removing the stimulus for ABA synthesis (Sack et al. 2018). The opposing trends seen in AscA and DHA for Days 3 and 5 in Eutrema may induce signalling patterns that prevent ABA accumulation. In Arabidopsis, ABA increased at Day 5, but there were no significant changes in AscA or DHA until Day 5.

Altered GA metabolism also supports the hypothesis that Arabidopsis responds differently than

17

527   Eutrema to soil water availability. Two precursors of the bioactive GA4 (GA24, GA9; Fig.

528   7&8, Table 3) showed altered profiles in Arabidopsis but not in Eutrema; with increased GA24

529   and GA9 contents at Day 5 indicating GA4 deactivation, a growth inhibitory signal.

530

531   **Discussion**

532   Transpiration data indicate more conservative water use in Eutrema than Arabidopsis although

533   Arabidopsis had greater stomatal sensitivity to drying soil within a certain SWC range.

534   Decreased transpiration of Eutrema prior to any significant stomatal closure supports the

535   hypothesis that growth inhibition is the first response to soil water deficit as transpiration is

536   considered a proxy for growth (Tardieu et al. 2010; Maurel et al. 2016). The soil water content

537   threshold perceived as a stress signal is higher in Eutrema, which may be a result of stress

538   priming. While instantaneous measurements of gs at the same time of the day indicate no

539   stomatal response in Eutrema, the number of hours per day that stomata are open may be

540   affected. Leaf expansion is also under biophysical control, and decreased water fluxes to

541   expanding cells will reduce growth (Tardieu et al. 2010; Maurel et al. 2016). Together, these

542   data suggest species differences in regulating water consumption, implying distinct integration

543   of environmental signals and regulation of stomatal closure in Eutrema and Arabidopsis.

544   The significantly higher water consumption of Arabidopsis between Days 3 and 7 triggered

545   enhanced foliar ABA accumulation, potentially mediating stomatal closure. However, a

546   temporal decoupling of foliar ABA accumulation from stomatal closure was detected, as in

547   previous reports (Pinheiro et al. 2011; Bechtold et al. 2016). For both species, partial stomata

548   closure occurred before ABA concentration changed significantly. Direct hydraulic regulation

549   of stomatal conductance, or water-deficit stimulation of localised foliar ABA accumulation

550   provide alternative hypotheses for stomatal closure. Thus ABA quantification at the guard cell

551   level (Harris and Outlaw, 1991) is needed to better understand the regulation of stomatal

552   conductance. Several other hormones, notably JA and SA, may also regulate stomatal

553   conductance (Arbona et al. 2010; Rivas-San Vicente and Plasencia, 2011; de Ollas and Dodd,

554   2016). While early stress affects ABA, JA and SA concentrations in Arabidopsis, only SA

555   concentrations change in Eutrema. Thus under similar rates of soil drying, the two species show

556   distinct hormonal balance.

557   The distinct metabolic responses between the two species can also be related to phytohormonal

responses. Eutrema's limited metabolic response can be related to slower metabolism, reflecting a stress priming effect. An alternative hypothesis could be that Eutrema slows its metabolism much earlier as a stress avoidance strategy (Tardieu, 2012). Taken together with the differing transpiration response, the larger changes in Arabidopsis suggest different metabolic strategies to deal with the progressive decline in soil water availability. Compared to Eutrema, the more "optimistic" strategy of Arabidopsis Col-0 maintains biomass production under mild stress and/or under deficit irrigation (Skirycz et al. 2011). It will be important to determine whether growth is maintained, both above and below ground, and if reserves are reallocated as the mechanisms that limit biomass accumulation under mild stress are poorly understood (Pinheiro and Chaves, 2011; Skirycz et al. 2011).

During severe and prolonged drought, more than three times as many variables differed significantly in Arabidopsis than Eutrema, suggesting that Arabidopsis adjusts its metabolism more extensively. An alternative view is that larger changes in Arabidopsis indicate less active metabolism, since metabolites accumulate because the plant has no capacity to use them. Thus greater sucrose accumulation in Arabidopsis is a typical drought response (Peters et al. 2007; Antonio et al. 2008; Pinheiro and Chaves, 2011; Granda and Camarero, 2017). Greater sugar availability occurs since CO_2 assimilation is not limited as much as growth. Thus carbon is available but plants are unable to use it, termed "sink limitation" or passive accumulation (Granda and Camarero, 2017). Alternatively, higher sugar content may reflect their use in osmoregulation, maintaining cell integrity and providing readily available carbon to resume growth (active reserve storage concept; Granda and Camarero, 2017) when re-watered. This regulatory mechanism integrates carbon availability and its use within the plant (Pinheiro and Chaves, 2011), diverting photoassimilates to other biochemical pathways (than growth) to withstand severe drought and/or resume growth whenever possible.

Traditionally, it has been argued that only resurrection plants can survive such severe drought, i.e. recover from leaf RWC values below 20% (Dinakar and Bartels, 2013). Since leaf RWC was determined in the most recently expanded leaves at the beginning of the assay (see Materials and Methods), these older leaves were severely wilted and senescent after 12 days, while younger leaves visually maintained turgor. Several reports indicate that Arabidopsis Col-0 plants are able to recover from severe drought, with 30% of Col-0 plants surviving exposure to 15% SWC and severe wilting (Sun et al. 2013) while 20% of severely wilted Col-0 plants survived SWCs < 20% (Zhao et al. 2016). Moreover, Col-0 plants with 40-50% leaf RWC

recovered from drought (Meyre et al. 2001; Tran et al. 2007; Kosma et al. 2009; Koffler et al. 2014) while some plants recovered from 20% leaf RWC although the survival percentage was very low (Lü et al. 2012; Nguyen et al. 2016). In contrast to Arabidopsis, Eutrema Shandong plants recovered from drought if the leaf RWC declined to 50%, but not 30% (Dedrick 2007). Since our measurements were made only 1 day after rewatering and no plants were available to evaluate long-term recovery, irreversible damage cannot be ruled out.

Nevertheless, the two species showed opposing CK profiles, suggesting distinct metabolic status. During late stress, bioactive CKs, like ZR and 2-iP (Hirose et al, 2008; Kieber and Schaller, 2014), as well as IPA (2-iP precursor) accumulated in Arabidopsis but not in Eutrema. In contrast, re-watering returned the content of these CKs to well-watered values in Arabidopsis, while stimulating their accumulation in Eutrema. Decreased levels of bioactive CKs due to severe and prolonged drought stress have been associated with better performance under drought, in mutants with decreased levels of bioactive CKs achieved via overexpression of *CKX* genes or by inactivating *IPT* genes (Ha et al. 2012). Since these mutant lines show reduced growth under optimal conditions, it can be argued that their water requirements are lower than those of the WT. However, while lower transpiration is described for some *CKX* mutants (Farber et al. 2016), *ipt* mutants show similar water consumption (Nishiyama et al. 2011). On the other hand, senescence-induced *IPT* overexpression maintained bioactive CK content as the soil dries (Rivero et al. 2007; Xu et al. 2017), without reducing growth (Rivero et al. 2007). Nevertheless, re-watering increased bioactive CKs in these drought-tolerant transgenics (Rivero et al. 2007) and similarly Eutrema had CK profiles concordant with a drought tolerant plant. As Arabidopsis and Eutrema showed similar stomatal sensitivity to re-watering, the differential CK profiles suggests CK-independent stomatal regulation at that time.

Conclusions

Slowly imposed drought induced different physiological and metabolic responses in Arabidopsis and Eutrema. Arabidopsis showed greater metabolic adjustment with ABA, JA and SA contents increasing early in Arabidopsis. Although greater soil drying was necessary to initiate partial stomatal closure in Eutrema, water use (in comparison to controls) decreased earlier than in Arabidopsis, with growth differences likely responsible. Eutrema rapid response possibly occurring because it is already primed against low-level stress. Under severe and

622 prolonged drought, conserved metabolic responses (increased sucrose and decreased raffinose
623 and citric acid) co-occurred with near-complete stomatal closure in both species.

624 Species differences in physiological and metabolic responses and their timing indicate
625 alternative strategies to physiologically adjust to soil drying, likely reflecting adaptations to
626 their respective niches. Better understanding these mechanisms is crucial to select genotypes
627 with more stable growth under stress, with favourable ideotypes depending on where the plant
628 is to be grown. Conservative water use allowing greater survival is a relevant selection criterion
629 in arid or semi-arid regions. Alternatively, in moderate climates with milder droughts, plant
630 production can be boosted if stress has little impact on growth (Skirycz et al. 2011; Tardieu,
631 2012), with higher stomatal conductance in these conditions maintaining growth and biomass
632 accumulation (Tardieu, 2012). Thus Arabidopsis seems a promising model to evaluate the
633 mechanisms responsible for stress-induced growth inhibition under the mild/moderate soil
634 drying that crop plants are typically exposed to.

635

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821

Figure Legends

823 **Fig. 1** Soil water content (SWC, %) after imposing water deficit and on re-watering (shaded

824 area). To ensure a similar rate of soil drying for the two species, daily soil water depletion was

825 controlled to 5-10 % of the soil water content by partial water replacement. Dashed lines show

826 SWC after this partial water replacement, whereas solid lines show SWC before partial water

827 replacement to visualise daily water consumption. Data show the means ± standard error of 6

828 pots (except Day 1 with 5 pots). For pre-irrigation SWC, significance levels were calculated

829 using the Mann–Whitney U test. Significant differences are denoted by asterisks (* $P < 0.05$,

830 ** $P < 0.01$, *** $P < 0.001$).

831 **Fig. 2** Leaf stomatal conductance (as a % of the control plants) plotted against SWC. Mean

832 values (of 3 to 5 biological replicates) are shown with only positive standard errors for clarity.

833 Significant results, as determined by Mann-Whitney U test, are denoted by asterisks (* $P <$

834 0.05, ** $P < 0.01$, *** $P < 0.001$). ANCOVA for each main effect (treatment and species) and

835 their interaction is presented in supplementary Fig. S4B.

836 **Fig. 3** PCA plots showing the scores for the first two principal components obtained for the

837 untargeted metabolomic analysis coloured by experimental group and the day of harvest, for

838 Arabidopsis (**a**) and Eutrema (**b**). For both plant species, the data have been scaled to unit

839 variance and control corrected. In the case of Eutrema only, batch correction has also been

840 performed.

841 **Fig. 4** Dendrogram obtained from hierarchical clustering of the 46 time-series selected by the

842 iterative k-means analysis of the metabolite data. The clusters are coloured and annotated A-I

843 according to the clusters identified in the k-means analysis (Suppl. Fig. S5). Metabolites within

844 clusters are labelled as follows: S= sucrose; R = raffinose; St = stachyose; CA = citric acid; U

845 = unassigned hexose disaccharide.

846 **Fig. 5** PCA scores plots for the first two principal components obtained from scaled early-

847 stress observations (Days 1, 3 and 5) in the untargeted analysis after control correction for **a**

848 Arabidopsis and **b** Eutrema. The observations are coloured according to the day of harvest,

849 showing that the clustering of observations is related to drought duration.

850

851   **Fig. 6** Cytokinins during early (Days 1, 3, 5) and late (Day 12) stress and on re-watering (Day

852   13). Mean values and ± standard error of 6 biological replicates (except for Day 1 where $n =$

853   5). The mean values after control correction (i.e. the mean value for the controls has been

854   subtracted) are represented. In Arabidopsis, ZR, 2iP and IPA peak at late stress and decrease

855   on re-watering. However, in Eutrema, these hormones show a slight decrease in late stress and

856   increase dramatically on re-watering. Arabidopsis: dark grey; Eutrema: light grey. Significant

857   results are shown in Table 2.

858   **Fig. 7** Biochemical parameters with a statistically significant change in early drought stress in

859   Arabidopsis but not in Eutrema. The mean difference from well-watered plants for leaf RWC

860   and the hormones ABA, JA, SA and GA24 are shown with error bars representing the standard

861   error. The mean values after control correction (i.e. the mean value for the controls has been

862   subtracted) are represented. Arabidopsis: dark grey; Eutrema: light grey. Significant results are

863   shown in Table 3.

864   **Fig. 8** Biochemical parameters with a significant change in early stress in Eutrema but not in

865   Arabidopsis. The mean measurement for osmotic potential, DHA, AscA, 2iP and GA9 are

866   shown with error bars representing the standard error of the observations. The mean values

867   after control correction (i.e. the mean value for the controls has been subtracted) are

868   represented. Arabidopsis: dark grey; Eutrema: light grey. Significant results are shown in Table

869   3.

870

871 **Suppl. Table S1** Biochemical parameters for both control (WW) and stressed (WD)
872 observations in Arabidopsis. Data are the means ± standard error of 6 biological replicates,
873 except for Day 1 ($n = 5$). Asterisks in the third row show parameters with a significant
874 difference between WW and WD for a particular day (obtained using Mann-Whitney tests).
875 Asterisks in the final column show days that are significantly different from earlier days (using
876 Tukey's HSD test) with the specific days given in parentheses. Here, asterisks denote *** $P <$
877 0.001, ** $P < 0.01$ and * $P < 0.05$.

878 **Suppl. Table S2** Biochemical parameters for both control (WW) and stressed (WD)
879 observations in Eutrema. Data are the means ± standard error of 6 biological replicates, except
880 for Day 1 ($n = 5$). Asterisks in the third row show parameters with a significant difference
881 between WW and WD for a particular day (obtained using Mann-Whitney tests). Asterisks in
882 the final column show days that are significantly different from earlier days (using Tukey's
883 HSD test) with the specific days given in parentheses. Here, asterisks denote *** $P < 0.001$,
884 ** $P < 0.01$ and * $P < 0.05$.

885 **Suppl. Table S3** Biochemical parameters for both control (WW) and stressed (WD)
886 observations in Arabidopsis. Samples were control-corrected (see Methods section). Data
887 shown are the means ± standard error of 6 biological replicates, except for Day 1 ($n = 5$).

888 **Suppl. Table S4** Biochemical parameters for both control (WW) and stressed (WD)
889 observations in Eutrema. Samples were control corrected (see). Data shown are the means ±
890 standard error of 6 biological replicates, except for Day 1 ($n = 5$).

891 **Suppl. Fig. S1**. Preliminary drought assay. **a** Soil water content (SWC, %) progression during
892 the assay for Eutrema and Arabidopsis. **b** Leaf stomatal conductance (% of the control gs) as a
893 function of the SWC. For controls, percentage gs was calculated relative to day 0; for
894 treatments, percentage gs was calculated relative to the control for the same day. The 80 % gs
895 level was achieved on different days: by Day 4 in Arabidopsis and by Day 6 in Eutrema. **c**
896 Regression line fit % gs vs soil water content. Each point represents a single measurement.

897 **Suppl. Fig. S2** PCA plots showing the scores for the first two principal components obtained
898 for the Eutrema data after scaling to unit variance with the observations coloured by batch. **a**
899 Before batch correction, clustering within batches can be seen and, in particular, batches 7 and
900 8 cluster separately. **b** After batch correction, differences between batches are no longer

901 apparent.

902 **Suppl. Fig. S3** PCA scores for the first two principal components obtained for the Arabidopsis
903 data after scaling to unit variance. The observations are coloured by data collection batch and
904 no obvious differences between batches can be seen, so that batch correction is not necessary.

905 **Suppl. Fig. S4 a** Leaf stomatal conductance of Arabidopsis and Eutrema after imposing water
906 deficit and on re-watering (shaded area). **b** Regression line fitting % gs vs soil water content.
907 Each point represents a single measurement and p-values were determined by ANCOVA for
908 each main effect (treatment and species) and their interaction (ns: not significant; *** $P <$
909 0.001).

910 **Suppl. Fig. S5** The nine clusters obtained with k-means analysis of the 46 time-series
911 remaining after iterative filtering of the metabolite data. Clusters **a-d** include several sucrose
912 species. Cluster **e** includes raffinose and cluster **f** includes citric acid.

913 **Suppl. Fig. S6** Heatmap showing the similarity of the 46 time-series selected by iterative k-
914 means analysis of the metabolite data. Metabolites are labelled as follows: S = sucrose; R =
915 raffinose; St = stachyose; CA = citric acid; U = unassigned hexose disaccharide.

916 **Suppl. Fig. S7** PCA plots of the biochemical parameters for both control (WW) and treatment
917 (WD) observations in Arabidopsis and Eutrema after control correction. **a** unscaled variables.**b**
918 scaled variables.

919 **Suppl. Fig. S8** Bar charts showing physiological and biochemical parameters in early- (Days
920 1, 3 and 5) and late-drought stress and on re-watering (Day 13) after control correction. Error
921 bars show the standard error between observations ($n = 6$ biological replicates, except for Day
922 1, $n = 5$). Dark grey: Arabidopsis; light grey: Eutrema. ANOVA results are presented in Table
923 2.

924 **Suppl. Fig. S9** Line plots showing physiological and biochemical parameters in early-drought
925 stress (Days 1, 3 and 5) after control correction. Error bars show the standard error between
926 observations ($n = 6$ biological replicates, except for Day 1, $n = 5$). Dark grey: Arabidopsis;
927 light grey: Eutrema. ANOVA results are presented in Table 3.