Arabidopsis species deploy distinct strategies to cope with drought stress

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Summary

- Little is known about how the physiological response to water deprivation differs among closely related plant species with different ecologies. In particular, how the relatives of the model species *Arabidopsis thaliana* react to drought stress is unknown.

- We conducted a dry-down experiment that mimics a period of missing precipitation and monitors plant reactions to the progressive decrease in soil water content (SWC) in *Arabidopsis thaliana*, and its close relatives *A. lyrata* and *A. halleri* at phenotypic and transcriptomic levels.

- The three species differed significantly in their reaction to decreasing soil water content. *A. thaliana* withstood low SWC but did not survive wilting. *A. lyrata* and *A. halleri* wilted at higher SWC but differed in water consumption rate and tolerance levels. Transcriptome data collected just before wilting and after recovery corroborated the phenotypic analysis, with *A. halleri* and *A. lyrata* showing a stronger activation of stress- and recovery-related genes, respectively.

- We conclude that these *Arabidopsis* species have evolved distinct strategies to face drought stress. *A. lyrata* employed both avoidance and tolerance mechanisms, whereas *A. thaliana* showed stronger avoidance reactions but no tolerance. *A. halleri* is the least able to protect itself from the stress imposed by drought.

Key words: *Arabidopsis*, avoidance and tolerance strategies, drought stress response, ecological speciation, plant wilting.

Introduction

All physiological and cellular plant aspects depend on water, so limitation in its supply is a major abiotic stress restricting plant growth and crop yield (Stebbins, 1952; Boyer, 1982; Bohnert *et al.*, 1995; Bray, 1997, Lambers *et al.*, 1998; Bray *et al.*, 2000). Water limitation is also a crucial determinant of the distribution, abundance and diversity of plant species (Hoffmann & Sgró, 2011).

All spermatophytes possess the molecular toolkit to tolerate intense cellular dehydration in seeds (Golovina *et al.*, 1997; Kermode, 1997; Wehmeyer & Vierling, 2000). Adult plants can draw from this toolbox to tolerate a certain degree of dehydration in vegetative organs (Ludlow, 1989; Shinozaki & Yamaguchi-Shinozaki, 2007). This tolerance strategy relies on osmotic adjustment via the accumulation of an array of solutes, such as amino-acids, sugars, or dehydrins (Close, 1996). The expression of heat shock proteins, chaperones, or late embryogenesis abundant (LEA) proteins can further help to protect the cell against damages imposed by low internal water potential (Ingram & Bartels, 1996; Reddy *et al.*, 2004, Yue *et al.*, 2006; Szabados, 2010).

However, plants have evolved additional strategies to handle drought stress: escape and avoidance (Ludlow, 1989; Fukai & Cooper, 1995; Verslues & Juenger, 2011; Fang & Xiong, 2015). The escape strategy is based on the adjustment of developmental transitions to elude direct exposure to drought. With an increase in the duration of seed dormancy or a shortening of the life cycle, the plant is simply not facing dry seasons (Fox, 1990; Bewley, 1997; Tonsor *et al.*, 2005; Franks *et al.*, 2007; *Kronholm et al.*, 2012; Lovell *et al.*, 2013). The avoidance strategy, instead, seeks to maintain water levels within tissues through a reduction of water loss and the enhancement of water uptake, so that the plant bypasses the

damaging effects of drought (Levitt, 1980; Ludlow, 1989; Price *et al.*, 2002; Farooq *et al.*, 2009; Munemasa *et al.*, 2015).

The relative importance of strategies to cope with drought stress is expected to be intimately linked to the life history and ecology of species. Indeed, tolerance, avoidance, and escape strategies are not independent in evolution (Grime, 1977). Trade-offs between growth and tolerance can constrain their optimization (McKay *et al.*, 2003, Steven, 2011). Annual species prioritize the escape strategy, which in turn can release the need for tolerance mechanisms (Kooyers, 2015). Perennial species, by contrast, must maintain tolerance mechanisms to increase long-term survival.

Dehydration triggers dramatic responses in plant cells, as indicated by the fast and extensive changes in gene transcript levels (Shinozaki & Yamaguchi Shinozaki, 2000; Iuchi *et al.*, 2001; Seki *et al.*, 2001; Shinozaki & Yamaguchi, 2007; Matsui *et al.*, 2008; Harb *et al.*, 2010). Part of this response is regulated by the key drought-stress hormone abscisic acid (ABA), but ABA-independent transcriptional regulation also plays an important role (Iuchi *et al.*, 2001; Seki *et al.*, 2001; Sakuma *et al.*, 2006; Yoshida et *al.*, 2014; Urano *et al.*, 2017). The complex architecture of gene regulatory responses to stress is believed to contribute to restricting the reactions at cell and whole-plant levels when the internal water potential drops (Bray, 1997; Szabados, 2010; Osakabe *et al.*, 2014). By articulating growth and stress responses, transcriptomic changes take part in both the deployment of avoidance strategies and the promotion of recovery from stress, yet they also reveal the degree of stress sensed by the organisms. Distantly related annual species, such as wheat and *Arabidopsis*, show common patterns of stress responses. Much less is known about how responses to stress are reshaped in

121 closely related species with strongly divergent ecologies and
122 life-histories.

123 Comparison of *A. thaliana* to its close relatives can help
124 disentangle the molecular changes contributing to tolerance and
125 avoidance mechanisms, because different species in the genus
126 have evolved distinct ecologies with contrasting demands on
127 tolerance and avoidance (Clauss & Koch, 2006). The model
128 species *A. thaliana* shows a broad distribution range from north
129 of Scandinavia to Africa (Hoffmann, 2005, Durvasula *et al.*,
130 2017). Its response to severe or mild drought stress has been
131 described in detail (Seki *et al.*, 2002; Bray, 2004; Verslues &
132 Juenger, 2011; Des Marais *et al.*, 2012; Juenger, 2013;
133 Bechtold *et al.*, 2015; Lovell *et al.*, 2015). Several studies point
134 to the adaptive relevance of its variation (Kesari *et al.*, 2012;
135 Exposito-Alonso *et al.*, 2017). This annual species can also rely
136 on modifications of its life cycle to adjust the timing of escape
137 and/or avoidance strategies to drought threats (McKay *et al.*,
138 2003; Kronholm *et al.*, 2012; Wolfe & Tonsor, 2014). The two
139 sister species *Arabidopsis lyrata* and *A. halleri*, by contrast, are
140 less likely to rely on escape strategies because year-to-year
141 survival is of major importance for these perennials. *A. lyrata* is
142 probably the most exposed of the two to natural selection by
143 drought due to its preference for low competitive communities
144 in soils that do not retain water (Clauss & Koch, 2006;
145 Ellenberg & Leuschner, 2010; Sletvold & Agren, 2012). *A.*
146 *halleri*, instead, must grow to out-compete other species in
147 crowded habitats (Clauss & Koch, 2006; Ellenberg &
148 Leuschner, 2010; Stein *et al.*, 2017). Its specific ability to
149 accumulate heavy metals enhances its defenses against
150 herbivores but sets strong constitutive demands on detoxifying
151 systems which are important for reestablishing homeostasis
152 after stress (Mittler, 2002; Becher *et al.*, 2004; Krämer &
153 Clemens, 2006; Stolpe *et al.*, 2016). The contrasted ecologies

of these three species thus predict major consequences on their strategies to face up with the challenges imposed by water limitations.

To test this prediction, we set up an experiment to infer the response strategy to drought of 10-15 accessions representative of the three species *A. thaliana*, *A. halleri* and *A. lyrata*. For this, we measured plant drought reaction at both phenotypic and transcriptomic levels in a dry-down experiment that mimics the progression of water depletion in natural conditions. Our data showed that species deploy different avoidance and tolerance strategies in response to decreasing levels of SWC.

Materials and Methods

Plant material and growth conditions

16 to 22 and 12 to 17 central European *A. lyrata* and *A. halleri* accessions, respectively, were included in the dry down experiments. The accessions were taken from populations representative of the diversity described in these species (Table S1, Pauwels *et al.*, 2005; Ross-Ibarra *et al.*, 2008; Novikova *et al.*, 2016; Stein *et al.*, 2017). They were compared to 16 *A. thaliana* accessions from Spain with European genomic background (The 1001 Genomes Consortium 2016). This sample was chosen because i) the populations are among the most drought resistant in *A. thaliana* (Exposito-Alonso *et al.*, 2017) and ii) are late flowering (Arapheno database, FT16, DOI: 10.21958/phenotype:262) so that the stress exposure cannot be circumvented by life cycle termination. For each accession, five replicates (vegetatively propagated clones for the self-incompatible species, single-descent seeds for *A. thaliana*) were distributed in 5 randomized complete blocks.

Plants were grown in 7x7x8 cm pots filled with 150 g of a well-homogenized mixture of VM soil (60 to 70% of peat and 30 to

40% of clay), perlite and seramis (clay granules) in a CLF controlled growth chamber (Perkin Elmer, USA). Growth conditions were 10 h (20°C): 14 h (16°C), light: dark, at a photon flux density (PFD) of 100 µmol m^{-2} s^{-1} supplemented with 10 min of dark-red light at the end of the day. Relative humidity was set to 60%.

Dry-down experimental design

Plants were grown for five weeks in the greenhouse, re-potted in weighed pots filled with the initial soil mixture, and transferred to the growth chamber. Soil moisture was quantified every day (X_t) by monitoring pot mass with a precision balance with an accuracy of 0.01 g. To calculate the soil moisture, several pots were fully dried down in an oven to estimate the weight of dry soil (X_0) in the initial soil mixture and subsequently saturated with water to determine the weight of 100% wet soil (X_f). The percentage of soil moisture was calculated as $[(X_t - X_0) / (X_f - X_0)] \times 100$. For acclimation, plants were grown for two weeks in pots with 60% soil moisture. After acclimation, plants were not watered until showing first symptoms of wilting. Plants were re-watered two days after wilting. One to two weeks later survival and symptoms of damage were scored.

Three independent biological experiments were performed, with slight differences in the number of replicates and/or accessions (for details see Table S1-S3). The two first experiments were used for phenotypic characterization and the third for sampling of leaf material for RNA extraction. In the experiment, plants were re-watered on the day of wilting to allow collecting leaf material after recovery.

Phenotypic trait measurements

Phenotypic differences between species in well-watered conditions

Three phenotypes were measured in *A. halleri* and *A. lyrata* in glasshouse-grown plants under well-watered conditions: stomatal density, stomata length, and carbon isotope discrimination ($\delta^{13}C$). Stomatal density and length were quantified in fully-developed leaves of five replicates of nine accessions per species following protocol described by Paccard *et al.,* (2014). $\delta^{13}C$ in one fully developed leaf was quantified for 4 replicates of the same nine accessions of each species according to the method used by Gowik *et al.,* (2011).

Phenotypic variation in response to soil dry-down

Eight phenotypes were measured during the dry-down experiment. Rosette leaf area was quantified on day zero of the dry-down experiment, using ImageJ to separate green pixels from the background images and RosetteTracker (Vylder *et al.,* 2012) to convert total green pixel into mm^2. The day when leaves lost their turgidity was scored as wilting day. Soil moisture was measured every day until the day of wilting. The rate of soil water loss was calculated for each pot over the first seven days after water withdrawal. Leaf lamina thickness was measured on one ink-marked medium-size leaf every second day using a digital ruler (HOLEX, Hoffmann Group, Knoxville, USA) with an accuracy of 0.03 mm. Efficiency of the photosynthetic light reaction was measured by Pulse-Amplitude-Modulation (PAM) fluorometry (Schreiber *et al*., 1986) using the IMAGING-PAM-Series (M-Series-Maxi version, Heinz Walz GmbH, Effeltrich, Germany). In order to gain information on the intactness of photosystem II (PSII) and hence its potential photosynthetic capacity, the maximum quantum efficiency of open PSII reaction centers ($F_v : F_m$, i.e. the ratio of variable to maximum Chl*a* fluorescence) was

247 determined (Genty *et al.*, 1989; Maxwell & Johnson, 2000).
248 Before the application of a saturating light flash (duration 0.8
249 s), plants were dark-adapted for 30 min. Intact and non-stressed
250 plants usually show an $F_v : F_m$ ratio of around 0.8. Plants that
251 developed new leaves within two weeks after re-watering were
252 scored as having survived and the damage caused by wilting
253 was quantified on a damage severity scale from one to five,
254 reflecting the percentage of damaged leaf area, leaf color and
255 leaf strength. The number of days of tolerated wilting was
256 scored on plants that survived the first dry-down experiment.
257 For this, plants were dried down a second time until wilting and
258 re-watered after three, four, five, or six days of wilting.
259 Photosynthetic activity and duration of tolerated wilting were
260 measured in the first experiment, whereas rosette area and leaf
261 thickness were measured only in the second experiment (Table
262 S2).

263 ## Statistical analysis of phenotypic variation

264 All plots were created using the *CRAN-package ggplot2*
265 (Wickham, 2009). We used generalized linear models and
266 multiple comparison tests using the *Simultaneous Inference in*
267 *General Parametric Models* package named *multcomp* and
268 Tukey's Honest Significant Difference test (Tukey HSD). For
269 each phenotype, we ran several models. As we did not detect
270 any block effect for the different measured traits, we removed it
271 from our models. Following are the different tested models, and
272 later in the results part, we will mention which was the best
273 model:

274 (M1) tests the accessions nested within species effect

275 $Y_{ijk}=\mu + \alpha_i$ species $+ \beta_{ij}$ (species $_i$ accession $_j$) $+ \varepsilon_{ijk}$

276 (M2) tests only the species effect when the accession effect is
277 not significant

278 $Y_{ij}=\mu + \alpha_i \text{ species}_i + \varepsilon_{ij}$

279 (M3) tests the interaction between species and time effect

280 $Y_{ijk}=\mu + \alpha_i \text{ species}_i + \beta_j \text{ time}_j + \gamma_{ij} (\text{species}_i \text{ time}_j) + \varepsilon_{ijk}$

281 (M4) tests the effect of interaction between species and the
282 cofactor of interest

283 $Y_{ijk}=\mu + \alpha_i \text{ species}_i + \beta_j \text{ cofactor}_j + \gamma_{ij} (\text{species}_j \text{ cofactor}_j)$
284 $+\varepsilon_{ijk}$

285 Where:

286 Y: quantitative dependent variable e.g. measured phenotypic
287 trait; μ: is the overall mean; α, β, and γ: regression
288 coefficients; species; accession; time; cofactor (e.g. initial
289 rosette size, desiccation rate, initial leaf thickness, damage
290 scores, days after wilting etc.): independent variables with the
291 different levels i, j, and k; ε: prediction error.

292 We performed an ANOVA using Fisher's test (or Chi test for
293 the binomial distribution of error) to identify the best model (P-
294 value $\leq$ 0.05). Different error distributions were specified
295 depending on the phenotypic trait. A negative binomial was
296 used for number of days until wilting, soil moisture, initial
297 rosette area, initial leaf thickness, damage scores, relative leaf
298 water loss, stomatal density and stomata length. A Gaussian
299 distribution was used for the desiccation rate and $\delta^{13}C$, a quasi-
300 Poisson for the photosynthesis activity and quasi binomial for
301 survival rate.

302 Analysis of transcriptome variation during dry-
303 down

304 In the third dry-down experiment, three to four young leaves of
305 'hal2.2' and 'Plech61.2a', typical accessions of *A. halleri* and
306 *A. lyrata*, respectively, were sampled from three replicate

307 individuals at three time points: 1) before water withdrawal
308 (soil moisture around 60%), 2) before wilting symptoms
309 appeared (20% to 25% of soil moisture), and 3) leaves formed
310 during the recovery phase (10-15 days after re-watering). These
311 two accessions are representative of the phenotypic diversity
312 observed in the dry-down experiment. RNA extraction was
313 performed using the *PureLink™ RNA Ambion Mini Kit*
314 (Thermofisher, Darmstadt, Germany). RNA quality and
315 quantity were checked by Agilent 2100 bioanalyzer (Agilent
316 Technologies, Palo Alto, Calif.) using RNA nano chips. RNA
317 of 18 leaf samples was sequenced on Illumina *HiSeq4000* by
318 the Cologne Center for Genomics.

319 We used the *fastx-tool-kits* from the *FastQC* package (V0.11.4)
320 for raw sequence quality trimming and filtering following He et
321 al. (2016). Low quality nucleotides were removed from the 3´-
322 ends of the sequences using 20 as a phred score threshold (t)
323 and 50 as minimum length (l). Sequences were reverse
324 complemented using fastx_reverse_complement to cut the other
325 end as we did for the 3´-end. Reads with less than 90% bases
326 above the quality threshold and paired-end reads with a single
327 valid end were discarded. The resulted trimmed and filtered
328 reads were mapped to the *A. lyrata* reference genome V1 (Hu *et*
329 *al.*, 2011) using the software package *STAR* with standard
330 parameters (Dobin & Gingeras, 2015). Transcriptome
331 sequencing yielded a total of 15 million read pairs per sample,
332 with a read length of 75 bp. We used 'samtools view –q 10' to
333 select the uniquely and high quality mapping reads with a
334 probability of correct mapping of 90%.

335 On average, more than 80% of all reads were uniquely mapped
336 and around 20% of unmapped and multiple mapped reads (Fig.
337 S1). R scripts developed by He F. were used to verify that reads
338 covered the whole length of genes (and confirm that we had no
339 sign of RNA degradation) and for counting the number of reads

mapped to each. The *DESeq2* Bioconductor package from *R (Bioconductor version: Release 3.5)* was used to find genes that were differentially expressed (DE) between the different conditions (Love *et al.*, 2014). We used Wald test to compute P values and the following design: ~ species/sample point, with two levels for the factor species (*A. halleri* and *A. lyrata*), and three levels for the factor sample point (leaves sampled at 60% of soil moisture, at 20-25% of soil moisture, and after recovery). Genes with a P value < 0.1 after Benjamini-Hochberg correction for false discovery rate (FDR) and $\log_2$-fold change ≤ -0.5 or ≥0.5 were considered as DE.

Gene ontology analysis

Functional enrichments among DE genes were performed using *org.At.tair.db* data package of *Bioconductor* and the rank test of the *TopGO* package (Alexa & Rahnenfuhrer, 2010) was used to identify enriched gene ontology terms. The *elim* algorithm followed by a *Fisher* test were used with a cut-off of 0.01. As background all expressed genes were used (around 12220 genes). Enrichments were analyzed separately for: 1) all responsive genes, 2) down-regulated genes, and 3) up-regulated genes. The hyper-geometric test was used to test for the significance of gene overlap with a set of stress responsive genes (Matsui *et al.*, 2008).

Results

Interspecific differences in stomatal density and stomata length but not in water-use efficiency

We evaluated whether, under well-watered conditions, constitutive physiological differences between *A. lyrata* and *A. halleri* can influence their potential to face limiting SWC. Variation in stomatal density on the leaf surface was explained by both within and between species variance (M1: $F_{18,\ 469}$ =

36.15, P-value < $2e^{-16}$; $F_{1, 487}$=256.59, P-value < $2.2e^{-16}$, respectively, Fig. 1a). In *A. lyrata* stomatal density on the abaxial leaf surface was lower than in *A. halleri* (on average 80 and 150 stomata mm^{-2} in *A. lyrata* and *A. halleri*, respectively). In *A. thaliana*, it was reported that stomatal density varies from 87 to 204 stomata mm^{-2} and it is negatively correlated with stomata length (Dittberner *et al.*, 2018). Stomata were larger in *A. lyrata* compared to *A. halleri* (P-value< $2e^{-16}$) and the genetic variation in stomata length was significant both within and between species (M1: $F_{16, 1370}$ = 53.68, P-value < $2e^{-16}$; $F_{1, 1386}$=3801.39, P-value < $2.2e^{-16}$, respectively). These differences however did not coincide with differences in carbon isotope discrimination ($\delta^{13}C$), a commonly used proxy for water-use efficiency (WUE, Farquhar & Richards, 1984; Farquhar *et al.*, 1989; Lambers *et al.*, 1998; Dawson *et al.*, 2002). In non-stressed conditions, leaf $\delta^{13}C$ showed significant genetic variation within species, but not between *A. halleri* and *A. lyrata* (M1: $F_{16, 54}$= 7.440, P-value= $9.76e^{-09}$, and $F_{1, 70}$ = 0.005, P-value =0.969, respectively Fig. 1b).

Wilting-related phenotypes revealed different drought response strategies

The day of first appearance of wilting symptoms differed significantly between species in the first experiment, although accessions within species also differed (M1: $F_{2, 214}$=316.48, P-value < $2.2e^{-16}$, Fig. 3a, $F_{48, 166}$ =3.51, P-value=$1.159e^{-09}$, for species and accessions within species, respectively). The same result was observed in the second experiment (M1: $F_{2, 201}$= 115.27, P-value < $2.2e^{-16}$, $F_{33, 168}$= 1.97, P-value= 0.0029, Fig. S2a). Wilting manifested differently in the three species. In *A. thaliana*, leaves became pale and curled laterally, in *A. lyrata*, they curled apically, and in *A. halleri* leaves changed to darker green and collapsed (Fig. 2). On average, *A. halleri* accessions wilted around five to seven days after water withdrawal, *A.*

lyrata accessions after 12 days and *A. thaliana* accessions after 18 days (Fig. 3a, Table S4). Differences in the timing of wilting did not exactly coincide with SWC differences. At wilting, *A. halleri* and *A. lyrata* showed similar soil moisture (18-20%), whereas *A. thaliana* only wilted after soil moisture dropped below 10% (Fig. 3b, Table S5). Again, these effects were consistent across experiments (Fig. S2b). Significant differences were detected between species for soil moisture at wilting (M1: $F_{2,\ 214}$ =44.27, P-value=3.982e^{-16}, $F_{2,\ 201}$ =181.60, P-value < 2.2e^{-16} for the first and second experiment respectively), and within species (M1: $F_{48,\ 166}$ =1.52, P-value=0.02, $F_{33,\ 168}$ =2.23, P-value=0.00049 for the first and second experiment respectively).

A. halleri plants exhaust SWC faster

To understand why *A. halleri* plants wilted around one week earlier than *A. lyrata* but at a similar soil moisture, we evaluated the rate of soil water loss for each species. We detected a significant interaction between species and time on soil moisture before wilting which showed that soil moisture decreased faster in pots where *A. halleri* accessions grew (Fig. S3a, M3: $F_{12,\ 1194}$ = 97.026, P-value < 2.2e^{-16}). *A. halleri* thus consumed water significantly faster than *A. thaliana* and *A. lyrata*. Here again, this observation was replicated in the second biological experiment (M3: $F_{4,\ 1224}$= 761.07, P-value < 2.2e-16, Fig. S3b).

To examine the impact of plant size on the rate of soil water loss, we measured initial plant size and estimated the desiccation rate, defined as the rate of soil water loss per day over the seven days following the water withdrawal in the second experiment of the dry-down experiment. *A. lyrata* and *A. halleri* accessions started with similar rosette size, but *A. thaliana* rosettes were initially larger (M2: $F_{2,\ 173}$=10.85, P-

value= 3.65e-05, Fig. S4a, Table S6). We detected a significant effect of the initial rosette area on the desiccation rate (M4 $F_{1, 170}$=16.10, P-value=8.97e^{-05}) but no significant interaction between initial rosette area and species on desiccation rate (M4: $F_{2, 170}$=1.89, P-value=0.15). Therefore, the consumption of soil water does not scale with plant size even though significant correlations between desiccation rate and initial rosette size were detected in *A. halleri*, less in *A. thaliana* but not in *A. lyrata* (Fig. 4a).

A. lyrata has the lowest relative loss of leaf water content before wilting

To estimate changes in leaf water content during the water-limited phase, we monitored leaf thickness (Lambers *et al.*, 1998) during soil dry-down phase in the second biological experiment. Initial leaf thickness was significantly higher in *A. lyrata* plants compared to *A. thaliana* and *A. halleri* (M1: $F_{2, 140}$=9.38, P-value=0.00015, Fig. S4b, Table S7). We also detected a significant accessions effect within *A. lyrata* on the initial leaf thickness ($F_{33, 140}$= 1.642, P-value=0.02548).

The significant interaction effect of soil desiccation rate and species (M4, $F_{2, 818}$=11.15, P-value=1.667e-05) on leaf thickness change over time revealed that the correlation between leaf thickness and soil desiccation rate was significant only for *A. halleri* (Fig. 4b, Table S9). Furthermore, this analysis showed that *A. thaliana* leaves were able to hold higher amounts of water at lower soil moisture, compared to *A. lyrata* and *A. halleri* (Fig. 5), an indication that this species can effectively avoid the effects of drought by maintaining a comparatively higher water content in its leaves.

A. thaliana and *A. halleri*, however, lost similar amounts of water in the days preceding wilting. The relative loss of leaf water content before wilting was calculated by the ratio of leaf

thickness two days before wilting by leaf thickness seven days before wilting (Fig. 6). There was no significant accessions effect on the decrease of leaf thickness in the seven days before wilting (M1: $F_{33, 138}$= 0.9401, P-value=0.5663) but the relative decrease before wilting was significantly higher in *A. thaliana* and *A. halleri*, compared to *A. lyrata* (M1: $F_{2,171}$=6.628, P-value= 0.001688, Fig. 6, Table S8). This pattern indicates that leaf water content in the days preceding the onset of wilting decreased more slowly in *A. lyrata* plants compared to *A. halleri* and *A. thaliana*. This suggests that wilting *A. lyrata* leaves experience lower loss of turgor.

High photosynthesis efficiency in wilted A. halleri and A. lyrata plants

Photosynthesis efficiency was measured to evaluate the physiological status of plants at wilting. We used F_v : F_m ratio, as indicator for the potential capacity of non-cyclic electron flow in the photosynthetic light reaction. Despite the collapsed or rolled leaves observed at wilting in *A. halleri* and *A. lyrata*, respectively, both still had a high photosynthetic capacity: on average 83 and 90%, respectively. By contrast, the photosynthetic capacity had significantly dropped in wilted *A. thaliana* rosettes (Fig. S5, Table S10).

A. thaliana has the lowest survival rate

Individual plants were re-watered two days after observing symptoms of wilting. Two to three weeks after re-watering, we scored survival. The proportion of survivors was significantly lower in *A. thaliana* compared to *A. halleri* and *A. lyrata* (9, 85 and 84%, respectively, Fig. 7, Table S11). These differences were consistent across the two experiments (Fig. S6).

To evaluate and compare the tolerance to wilting in *A. lyrata* and *A. halleri*, we ran an additional experiment examining

whether extending the time from wilting to re-watering impacted survival. We detected a significant interaction effect of species and time to re-watering on survival (M4: Chi-Squared=234, DF= 1, DF residuals=252, P-value=$1.615e^{-04}$). We observed that 70-85% of *A. lyrata* plants survived 3 to 6 day-long wilting periods (Fig. 7). In comparison, this percentage dropped to 10% for *A. halleri* plants after five days of wilting and this was significantly different between species (Fig. 7, M2: $F_{1,\ 26}$= 20.681, P-value = 0.0001109). These results indicate that *A. lyrata* is more tolerant to wilting than its sister species *A. halleri*.

Efficient post-drought recovery in A. lyrata plants

We further, assessed the tolerance to wilting by comparing damage exhibited by plants that survived two days of wilting in *A. lyrata* and *A. halleri*. The interaction between species and the damage score was found to be significant (M4, $F_{3,\ 100}$=2.96, P-value= 0.035). In *A. lyrata*, about 70% of plants showed a very low degree of damage in leaves, whereas in *A. halleri*, only 30% of plants had low damage levels (Fig. 8, $F_{1,\ 25}$= 24.063, P-value= $4.761e^{-05}$). We did not include *A. thaliana* in the statistical analysis because only 10 out of 60 plants survived wilting. These results confirmed that *A. lyrata* tolerates soil dehydration and wilting better than *A. halleri*.

Transcriptome analysis confirms that A. halleri is more sensitive to low SWC

A. lyrata and *A. halleri* both wilted at the same SWC but they differed in their survival following wilting. In order to gain insight into the molecular changes underpinning these differences, we performed a third dry-down experiment to collect leaf material in one representative accession of each of the sister species *A. halleri* and *A. lyrata* and examined the reaction to stress and recovery at the transcriptome level.

For each species, we compared transcript abundance at three time points during the dry-down experiment, i.e., at soil moisture 60%, soil moisture 20-25% and after recovery. The two species wilted at around 18% of soil moisture, as observed in the first two experiments, i.e., just below the soil moisture level at which leaf material was sampled. 107 and 976 genes changed expression level at 20-25 vs. 60% soil moisture in *A. lyrata* and *A. halleri*, respectively (FDR 0.1; fold-change >1.6). Only three genes were responsive in both species to the decrease in SWC and this was a random overlap (*hypergeometric test, P-value=0.993*).

After recovery, 275 and 20 *A. lyrata* and *A. halleri* genes, respectively, had changed expression level compared to the non-stress SW (Table 1). Since both species had similarly high survival rates upon two days of wilting and because new undamaged leaves were sampled, these differences are not due to survival differences. We conclude that *A. halleri* displayed a comparatively sharpened response to low SWC, whereas the transcriptome of *A. lyrata* was comparatively more altered after recovery.

In a previous study, 2975 and 5445 genes were shown to be responsive to two and 10 hours of dehydration in *A. thaliana* respectively (Matsui *et al.*, 2008). These drought-responsive genes were enriched in all sets of responsive genes identified in our study, either in *A. halleri* or in *A. lyrata*, at 20% soil moisture or after recovery (Table 2, *hypergeometric test*, maximum $p \leq$ 8-77E-19). This confirmed that our protocol succeeded in activating dehydration responsive genes. The list of significantly differentially expressed genes (including only AGI codes) is provided in Table S12.

Different GO categories are regulated in the two species

Analysis of enrichment in Gene Ontology (GO) categories confirmed that different sets of genes were activated in the two species at each sampling stage. In *A. halleri* many genes involved in growth and development were down regulated when SWC decreased to 20-25%, (Table 3). These functions were not enriched in *A. lyrata* samples collected at the same time, instead genes involved in response to water deprivation and in ethylene and ABA signaling pathways were up regulated in *A. lyrata* after recovery (Table 3). Several GO terms appeared enriched, including isopentenyl diphosphate metabolic process, response to water deprivation, hyperosmotic salinity response, photosynthesis light reaction, response to chitin, photosystem II assembly, and maltose metabolic process (Table 3). They were also enriched among genes responding to mild drought stress in *A. thaliana*, although the direction of the gene expression change was not the same (Des Marais *et al.*, 2012). We further observed that genes with altered expression in *A. halleri* were enriched for genes functioning in plastid organization, pentose-phosphate shunt and photosystem II assembly. These three GO categories harbor an excess of *cis*-acting changes in the *A. halleri* lineage in response to dehydration stress (He *et al.*, 2016).

Discussion

In our experimental design, we have used several accessions per species as we were interested in comparing the drought stress response of the three related species, while accounting for variation within species. Our results showed genotypic differences in initial leaf thickness, initial stomatal density or initial rosette area, but the response to depletion in SWC did not reveal significant differences between accessions. Differences in the response to water depletion therefore

revealed fixed interspecific differences in avoidance and tolerance strategies to drought stress.

Critical SWC does not reflect ecological differences between A. halleri and A. lyrata

The sister species *A. lyrata* and *A. halleri* have separated recently and gene flow between the clades is still detectable (Novikova *et al.*, 2016). Yet, the two species display marked differences in ecological preference (Clauss & Koch, 2006). Ellenberg indices, which are reliable estimates of ecological preferences in Central Europe, show that *A. lyrata* is found in very dry areas with a soil humidity index (F) of 3, while *A. halleri* occurs in habitats where water is less limiting (F= 6) (Ellenberg & Leuschner, 2010). We were therefore surprised to observe that *A. halleri* and *A. lyrata* individuals wilted at identical soil water content. In addition, contrary to our expectations, the ruderal species *A. thaliana* tolerated markedly lower levels of soil water content than its perennial relatives. Altogether, these observations show that the ecological preferences of *A. lyrata*, *A. halleri* and *A. thaliana* are not explained by the SWC threshold at which wilting symptoms appear.

A. halleri is directly exposed to stress caused by low SWC

We observed that *A. halleri* was the fastest to consume the water contained in the soil. In pots where *A. halleri* individuals grew, SWC decreased significantly faster (Fig. S3). *A. halleri* also displayed the strongest correlation between plant size and the rate of water consumption and an accelerated decrease in leaf thickness preceding the onset of wilting (Fig. 4-6). At 25% soil water content, i.e. shortly before the appearance of the first wilting symptoms, the rate of decrease in leaf thickness accelerated in *A. halleri* compared to *A. lyrata*. This turning point coincided with a change in the expression levels of a

larger number of genes belonging to stress-repressed GO categories such as leaf morphogenesis, cell proliferation, or photosynthesis. The down-regulation of growth-related genes we observed, even before wilting symptoms appear, indicates that the plant experiences direct stress at the cellular level as SWC approaches the limiting threshold. In agreement with the high levels of stress it experienced, *A. halleri* also showed a comparatively higher damage when survivors resumed growth after stress.

A. halleri thus displayed the weakest levels of drought avoidance of the three species. *A. halleri* thrives in more competitive habitats than its relatives (Clauss & Koch, 2016; Stein *et al.*, 2017), and competitive ability generally evolves in a trade-off with stress tolerance in plant species (Grime *et al.*, 1977; Sreenivasulu *et al.*, 2012). In addition, high stomatal density has been associated with elevated growth rates and lower drought resistance (Doheny-Adams *et al.*, 2012; Liu *et al.*, 2012). *A. halleri* indeed displayed higher stomatal density than *A. lyrata*. It is therefore possible that improved competitive ability was selected in this lineage at the expense of tolerance and avoidance mechanisms. Such evolutionary scenarios have been documented in several grass species (Fernández & Reynolds, 2000; Liancourt *et al.*, 2005; Sugiyama, 2006). Interestingly, we have previously observed that an excess of *cis*-acting changes up-regulating gene expression after one hour of dehydration had accumulated in the *A. halleri* lineage in several functions that the more tolerant species *A. lyrata* down-regulates during recovery (He *et al.*, 2016). It is therefore possible that the loss of tolerance and avoidance of drought stress was advantageous in the context of selection for increased competitive ability.

A. lyrata displays avoidance and tolerance responses to soil dehydration

By comparison with *A. halleri*, *A. lyrata* displayed a more parsimonious use of water. On the leaf surface, lower stomatal density (Fig. 1a) suggests a greater ability to maintain water levels in water-limiting conditions. In monkey flowers, for example, low stomatal density was associated with higher WUE (Wu *et al.*, 2010). Although under well-watered conditions *A. lyrata* did not show a globally higher WUE than *A. halleri* (Fig. 1b), its decreased stomatal density may allow better performance in drought stress conditions. *A. lyrata* plants displayed both a lower rate of water consumption and markedly lower damage levels after resuming growth. In addition, we observed that *A. lyrata* plants had the ability to survive longer durations of wilting than both *A. halleri* and *A. thaliana* (Fig. 7). It is also the only species that showed adaxial leaf rolling, a phenotype favoring drought avoidance in plants (Oppenheimer, 1960; O'Toole & Moya, 1978; Jones, 1979; Clarke, 1986). Leaf rolling indeed reduces transpiration rate by reducing the effective leaf area. Altogether, this indicates that exposure to limiting SWC is comparably less damaging in *A. lyrata*.

The transcriptome response to decreasing SWC corroborated this observation, by documenting lower levels of cellular stress in *A. lyrata* immediately before wilting, compared to *A. halleri*. Only a few genes changed expression before wilting in *A. lyrata*. We further observed that among genes down-regulated after recovery, the most enriched GO category is 'pentose-phosphate shunt' ($p<5.10^{-5}$), a metabolic pathway involved in the scavenging of reactive oxygen intermediates that is strongly activated by abiotic stress (Mittler, 2002; Kruger & von Schaewen, 2003). Several additional GO functions associated with drought stress, such as 'hyperosmotic salinity response', 'response to water deprivation', 'abscisic acid-activated signaling pathway', 'ethylene-activated signaling pathway', and 'response to chitin' were up- regulated in *A. lyrata* during

692 recovery. The latter functions seem to have a dynamic role in
693 drought stress. In *A. thaliana*, they were up-regulated by severe
694 fast wilting (Matsui et al. 2008) but down-regulated by mild
695 stress (Des Marais *et al.*, 2012). Their up-regulation after
696 recovery in *A. lyrata*, in the absence of obvious stress, shows
697 that the reaction of this species to lowering SWC contrasts not
698 only with that displayed by *A. halleri* but also with that known
699 for *A. thaliana*. The absence of a strong modification of the
700 expression of drought-stress responsive genes at SWC
701 approaching critical levels in *A. lyrata*, combined with a high
702 survival rate, further indicates that this species has the ability to
703 i) minimize its exposure to the stressful consequences of low
704 soil water content and ii) maximize its ability to survive severe
705 dehydration. It thus deploys both avoidance and tolerance
706 strategies.

707 ***High levels of stress avoidance associate with low tolerance to***
708 ***drought in A. thaliana***

709 In annual species, seasonal drought can be a potent source of
710 selection for accelerated flowering and faster cycling (Franks *et*
711 *al.*, 2007; Fitter & Fitter, 2002). *A. thaliana* was therefore
712 expected to maximize its resource investment into fast cycling
713 and show a lower level of stress tolerance compared to its
714 perennial relatives. Here, we focused on late flowering *A.*
715 *thaliana* accessions that in the conditions we imposed could not
716 accelerate their development to escape drought. This allowed
717 comparing their ability to avoid or tolerate wilting. Contrary to
718 expectations, we observed that our sample of accessions could
719 persist at lower SWC than both of their perennial relatives, *A.*
720 *lyrata* and *A. halleri* (Fig. 3a). In addition, the delayed decrease
721 in leaf thickness observed in *A. thaliana* shows that, compared
722 to the other two species, it is able to maintain its leaf water
723 content at lower SWC (Fig. 5). This therefore suggests that the
724 annual species *A. thaliana* also employs stress avoidance

mechanisms. The ability of this annual species to escape stress by accelerating development has therefore not led to the loss of mechanisms favoring the maintenance of internal water potentials. Indeed, the production of proline, which is both an osmoprotectant and an anti-oxidant, $\delta^{13}C$, a proxy measuring WUE, as well as the maintenance of photosynthesis during terminal drought have been documented to play a role in local adaptation in this species (Verslues & Juenger, 2011; Kesari *et al.*, 2012; Exposito-Alonso *et al.*, 2017; Dittberner *et al.*, 2018).

A. thaliana, however, was not able to tolerate wilting. We observed a marked decrease in the photosynthetic capacity at wilting in this species, as previously reported in several species such as *Hordeum vulgare*, *Hibiscus rosa-sinensis*, and *Andropogon gerardii* (Golding & Johnson, 2003; Muñoz & Quiles, 2013; Maricle *et al.*, 2017). In addition, *A. thaliana* did not survive after two days of wilting, although its perennial relatives displayed markedly higher survival rates. The annual species therefore appears to have evolved lower levels of tolerance to wilting.

We detected no significant variation for the response to decreasing SWC between the *A. thaliana* accessions included in this study, however, we cannot conclude that the avoidance capacity and the low tolerance to wilting we observed is fixed in the species. The *A. thaliana* population we used consisted of a set of late-flowering accessions from Spain that could not accelerate flowering fast enough to escape stress. This set of accessions is not necessarily representative of the whole species. *A. thaliana* is broadly distributed and its accessions can form ecotypes with markedly different levels of stress resistance (May *et al.*, 2017). Furthermore, two recent studies indicate that Swedish accessions have a comparatively greater capacity to face dry conditions, probably because the short favorable season of Scandinavia constrains them to face

limiting water availability when it strikes (Exposito-Alonso *et al.*, 2017, Dittberner *et al.*, 2018).

This study documents the contrasting reactions deployed by *Arabidopsis* species in response to lowering SWC. In the face of their respective ecologies, these diversified reactions likely reflect the priority shifts imposed by divergent ecologies and life cycles. Future studies aiming at dissecting the genetic and molecular underpinning of these differences promise to teach us much about the processes accompanying ecological diversification in plant species.

785 ***Table 1:*** Number of significantly differentially expressed genes
786 in *Arabidopsis halleri* and *A. lyrata* during the dry-down
787 experiment at 20% of soil moisture or after recovery compared
788 to expression before stress (60% of soil moisture).

		A. halleri	***A. lyrata***
20% vs 60% of soil moisture	Up	253	36
	Down	676	71
recovery vs 60% of soil moisture	Up	8	111
	Down	12	156

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811 ***Table 2:*** Percentage of differentially expressed genes that
812 overlap with differentially expressed genes reported in Matsui
813 *et al.*, (2008) after 2 h (dh2) and 10 h (dh10) of dehydration
814 stress (N.S.: not significant). The random expectation of
815 overlap % is indicated in bold on the top row.

		dh2 **expected:** **up 7.39%** **down 10%**	**dh10** **expected:** **up 10%** **down 7.5%**
***A. halleri* 20% vs 60% of soil moisture**	Up (127 ATG genes)	27.5% *P*= 1.09E-12	47.2% *P*= 7.82E-28
	Down (385 ATG genes)	12.4% *P*= 6.03E-23	36.3% *P*= 1.17E-59
***A. halleri* recovery vs 60% of soil moisture**	Up (6 ATG genes)	0 N.S.	0 N.S.
	Down (7 ATG genes)	0 N.S.	28.5% *P*= 1.20E-02
***A. lyrata* 20% vs 60% of soil moisture**	Up (15 ATG genes)	40% *P*= 4.52E-05	46.6% *P*= 3.34E-05
	Down (37 ATG genes)	5.4% N. S.	18.9% *P*= 5.7E-03
***A. lyrata* recovery vs 60% of soil moisture**	Up (61 ATG genes)	63.9% *P*= 1.06E-30	54% *P*= 8-77E-19
	Down (90 ATG genes)	11.1% N. S.	32.2% *P*=1.63E-12

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826 ***Table 3:*** GO Categories Showing a Significant Enrichment (P
827 < 0.01) among differentially expressed genes between 20% and
828 60% of soil moisture and between recovery and 60% of soil
829 moisture for *Arabidopsis halleri* and *A. lyrata*.

	GO.ID	Term	pvalue	Gene regulation
A. halleri 20% vs 60% of soil moisture	GO:0015979	photosynthesis	0.0011	down
	GO:1901576	organic substance biosynthetic process	0.0013	down
	GO:0044711	single-organism biosynthetic process	0.0014	down
	GO:0051188	cofactor biosynthetic process	0.0023	down
	GO:0008283	cell proliferation	0.0035	down
	GO:0006098	pentose-phosphate shunt	0.0041	down
	GO:0009965	leaf morphogenesis	0.0048	down
	GO:0009657	plastid organization	0.0059	down
	GO:0042254	ribosome biogenesis	0.0059	down
	GO:0006084	acetyl-CoA metabolic process	0.0064	down
A. lyrata recovery vs 60% of soil moisture	GO:0006098	pentose-phosphate shunt	0.000043	down
	GO:0010200	response to chitin	0.000051	up
	GO:0010207	photosystem II assembly	0.00007	down
	GO:0000023	maltose metabolic process	0.00017	down
	GO:0009873	ethylene-activated signaling pathway	0.0002	up
	GO:0019252	starch biosynthetic process	0.00039	down
	GO:0009612	response to mechanical stimulus	0.0015	up
	GO:0009414	response to water deprivation	0.0029	up
	GO:0042538	hyperosmotic salinity response	0.0043	up
	GO:0051707	response to other organism	0.005	up
	GO:0009657	plastid organization	0.00571	down
	GO:0050790	regulation of catalytic activity	0.00763	down
	GO:0042742	defense response to bacterium	0.00784	down
	GO:0009738	abscisic acid-activated signaling pathway	0.0086	up

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Author Contributions

M.B., J.M. and G.S. designed the experiments; M.B. performed the experiments; M.B., F.H., G.S. and J.M. interpreted the data; R.E.H. supervised the photosynthesis measurements; A.P.M.W. and T.M. quantified $\delta^{13}C$; M.B. and J.M. wrote the manuscript with input from all co-authors.

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Supporting Information

Figure S1: Summary of short read mapping to the *A. lyrata* reference genome V1 (Hu et al., 2011).

Figure S2: Wilting day and soil moisture at wilting for the two first biological experiments of the drying-down experiments.

Figure S3: Soil water content during the first 7 days after water withdrawal.

Figure S4: Initial rosette area and leaf thickness of the plants used in the second biological experiments of the drying-down experiment.

Figure S5: Photosynthesis efficiency at wilting.

Figure S6: Proportion of surviving *A. halleri*, *A lyrata*, and *A. thaliana* plants 2 days after re-watering for the two first biological experiments.

Table S1: List of accessions used for the dry-down experiments.

Table S2: Phenotypes measured in the three drying-down experiments.

Table S3: Number of accessions used in the three drying-down experiments.

Table S4: Summary statistics of the multiple comparison of the wilting day between species.

Table S5: Summary statistics of the multiple comparison of the soil moisture at wilting between species.

Table S6: Summary statistics of the multiple comparison of the initial rosette area between species.

Table S7: Summary statistics of the multiple comparison of the initial leaf thickness between species.

Table S8: Summary statistics of the multiple comparison of the relative leaf water loss 7 days before wilting between species.

Table S9: Summary statistics of glm testing the effect of interaction between species and desiccation rate on the relative loss of leaf water content before wilting.

Table S10: Summary statistics of the multiple comparison of the photosynthetic efficiency at wilting between species.

Table S11: Summary statistics of the multiple comparison of the survival rate 2 days after re-watering between species.

Table S12: Differentially expressed genes identified for each of *Arabidopsis halleri* and *A. lyrata* between 20 and 60% of soil moisture and bewteen recovery and 60% of soil moisture.

Figures

Figure 1: Stomata density and $\delta^{13}C$ measured in *Arabidopsis halleri* and *A. lyrata* grown under well-watered conditions. (a) Abaxial stomatal density. (b) $\delta^{13}C$ measured for the same plants. Violin plots with the same letter are not significantly different according to Tukey's HSD (P value <0.05).

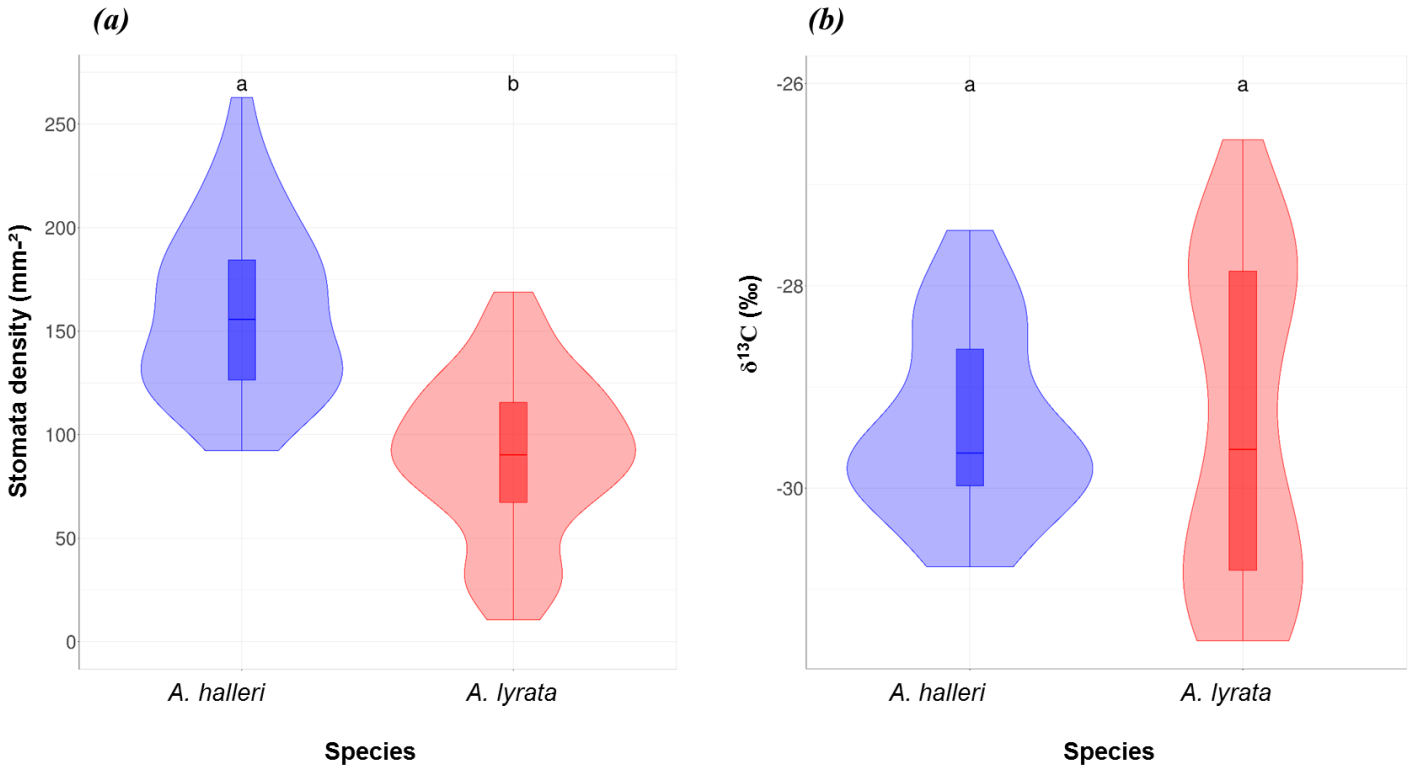


Figure2: Typical phenotypes of wilting observed in *Arabidopsis halleri*, *A. lyrata*, and *A. thaliana*. Plant morphology before the water withdrawal treatment (top row) and at wilting (bottom row) for A. halleri (a, d), A. lyrata (b, e) and A. thaliana (c, f). All plants were grown in 7cm pots.

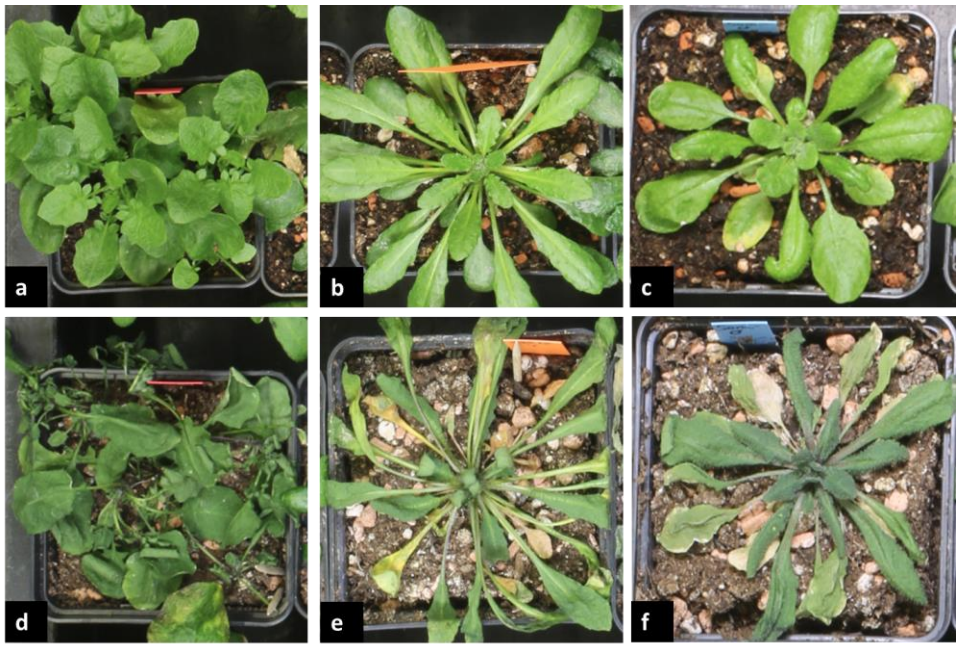


Figure 3: Wilting day and soil moisture at wilting for *Arabidopsis halleri*, *A. lyrata*, and *A. thaliana*. ***(a)*** Number of days between initiation of soil dry-down treatment and wilting. ***(b)*** Soil moisture at wilting. Letters above violin plots indicate significant differences between species (*Tukey's HSD test, P value <0.05*). Results are shown for the first biological experiment.

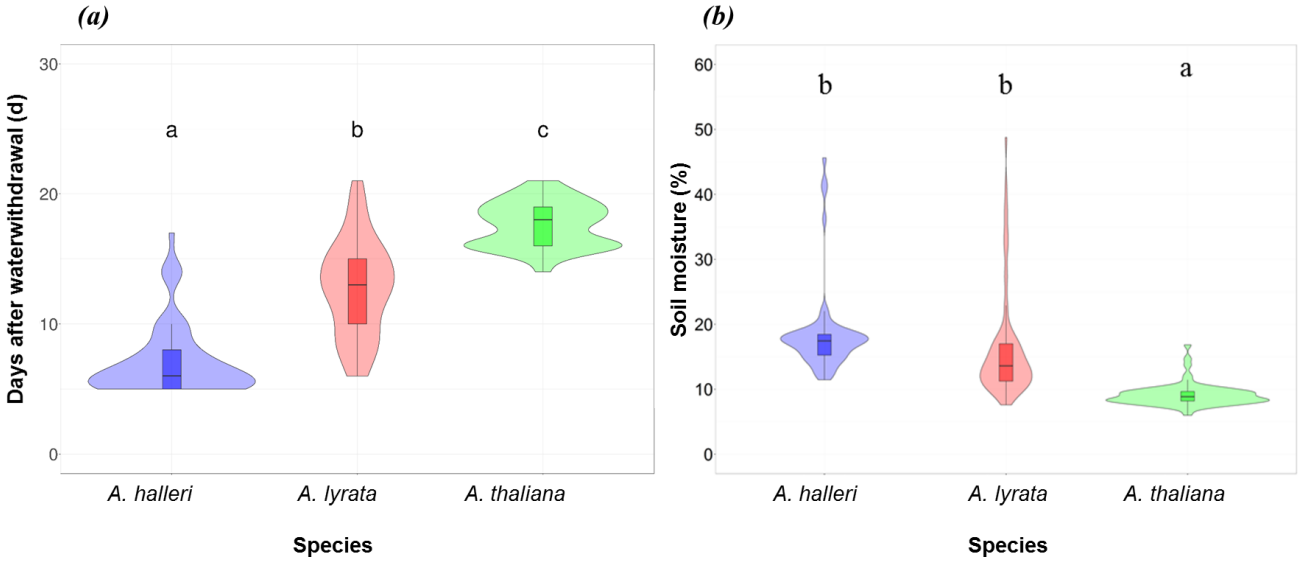
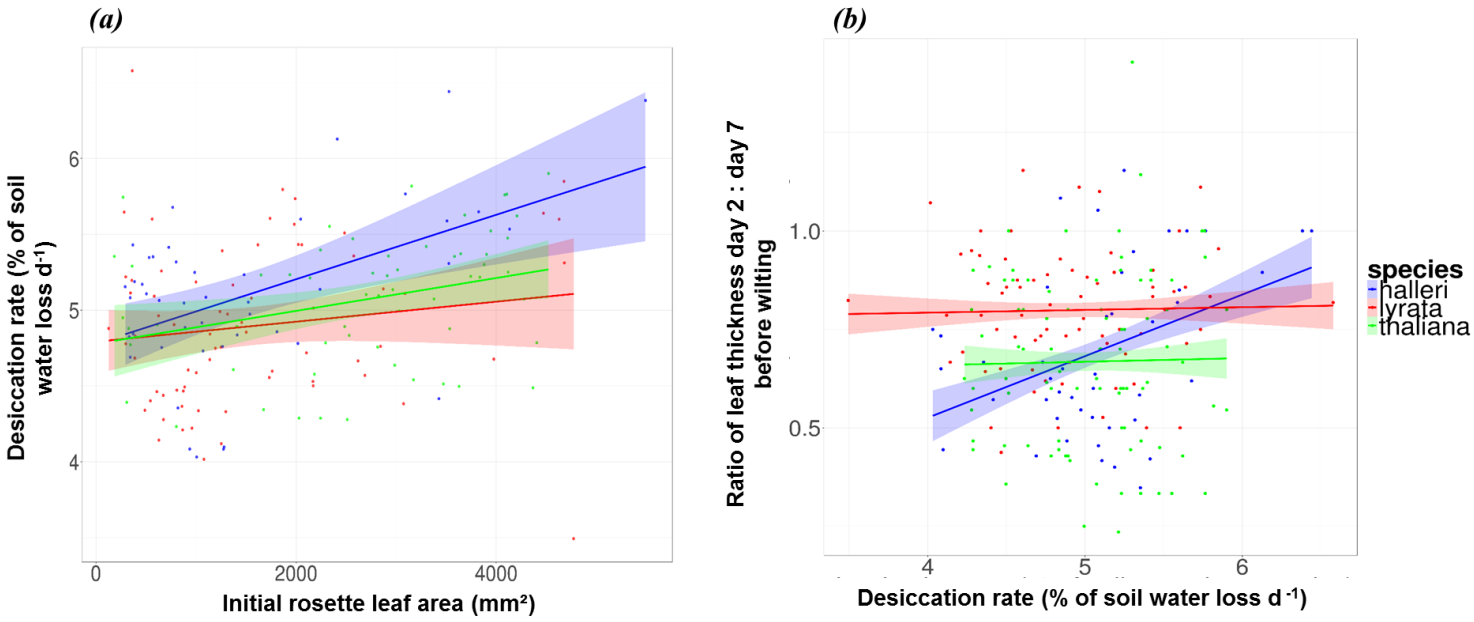


Figure 4: Correlations between desiccation rate and initial leaf size and desiccation rate and the relative leaf water loss. ***(a)*** Correlation between the initial rosette leaf area (at 60% of soil moisture) and the percentage of soil desiccation rate (Pearson correlation coefficients and p values for: *Arabidopsis thaliana* (r = 0.32, P value = 0.013); *A. lyrata* (r = 0.14, P value = 0.22) and *A. halleri* (r = 0.48, P value = 0.00072). ***(b)*** Correlation between the relative water loss in leaves before wilting (equivalent to the ratio of leaf thickness day 2 : day 7 before wilting) and the desiccation rate (Pearson correlation coefficients and p values for: *A. thaliana* (r = 0.018, P value = 0.732); *A. lyrata* (r = 0.023, P value = 0.692) and *A. halleri* (r = 0.39, P value = $4.282.10^{-08}$). Results are shown for the second biological experiment. Lines represent a linear regression

1280 smoothing where the shaded ribbons represent the standard
1281 error.



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1283 **Figure 5:** Leaf thickness in response to decrease of soil
1284 moisture for *Arabidopsis thaliana*, *A. halleri*, and *A. lyrata*.
1285 Results were collected in the second biological experiment.
1286 Shaded ribbons represent the standard deviation. Filled
1287 triangles correspond to the average wilting soil moisture for the
1288 different species.

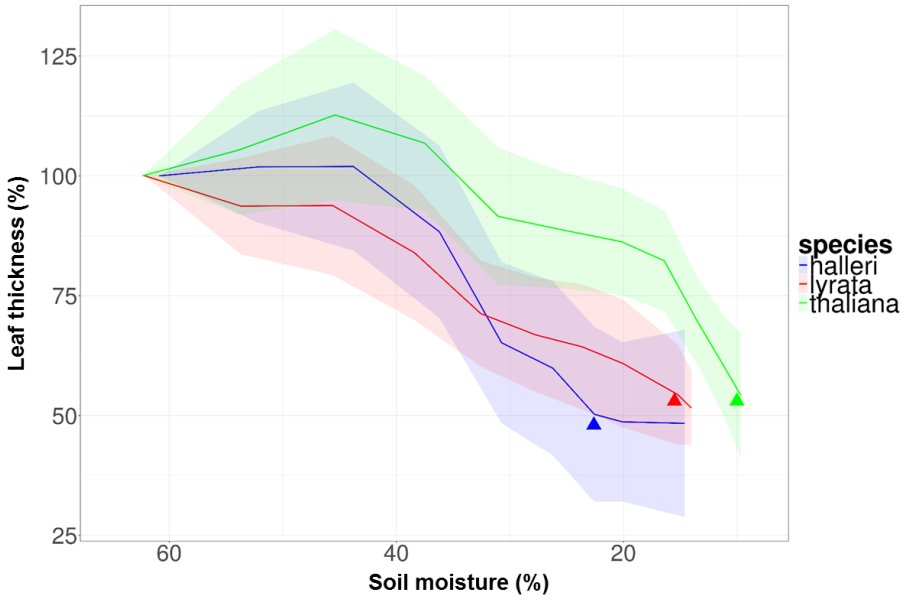

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Figure 6: Relative leaf water loss seven days before wilting in *Arabidopsis halleri*, *A. lyrata*, and *A. thaliana*. This is equivalent to the ratio of leaf thickness at day two vs day seven before wilting. Boxplots with the same letter are not significantly different (*Tukey's HSD, P value <0.05*). Results are shown for the second biological experiment.

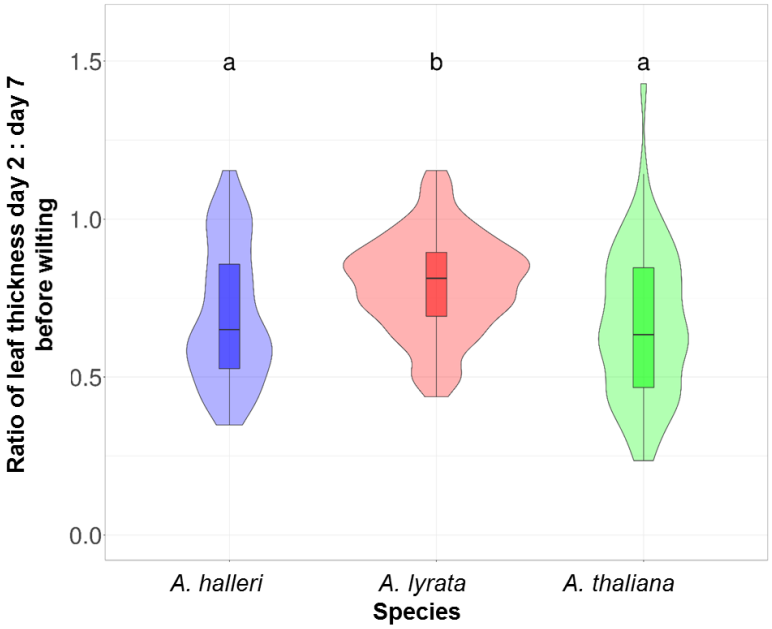

Figure 7: Average survival rate after re-watering following two to six days of wilting for *Arabidopsis halleri*, *A. lyrata*, and *A. thaliana*. Results are shown for the first biological replicate. Barplots with one star or more are significantly different (*Tukey's HSD, Signif. codes: ·, P < 0.1 ; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant*).

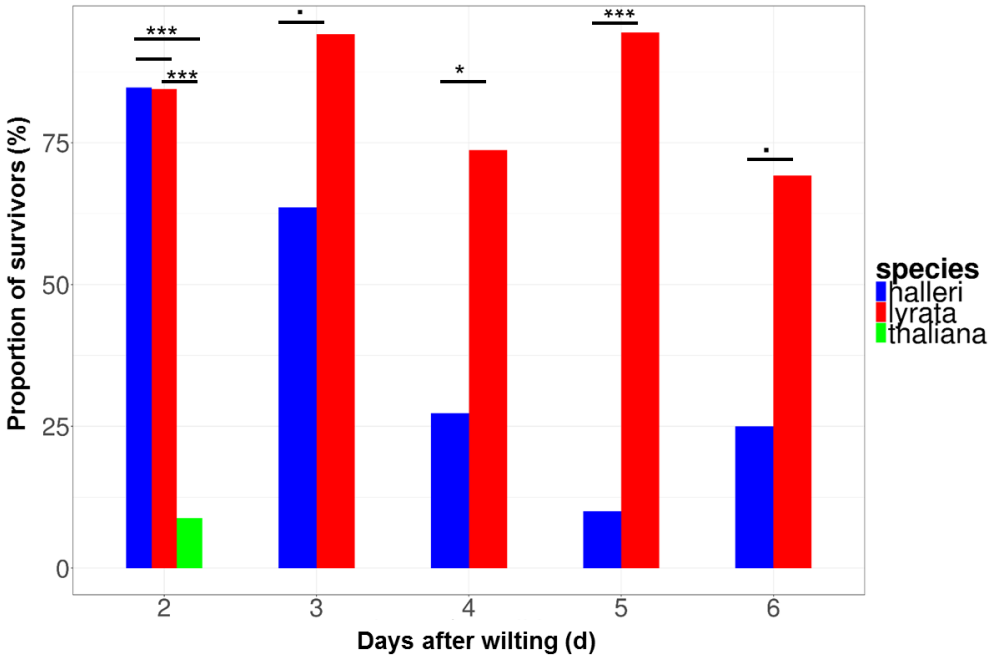

Figure 8: Damage scored on survivors to two days of wilting after resuming growth for *Arabidopsis halleri*, *A. lyrata*, and *A. thaliana*. . Results are shown for the second biological experiment. Barplots with one star or more are significantly different (*Tukey's HSD, Signif. codes: ·, P < 0.1 ; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant*).