Evolution of drought resistance strategies following the introduction of white clover (*Trifolium repens* L.)

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ABSTRACT

Background and Aims: Success during colonization likely depends on growing quickly and tolerating novel and stressful environmental conditions. However, rapid growth, stress avoidance, and stress tolerance are generally considered divergent physiological strategies.

Methods: We evaluate how white clover (*Trifolium repens*) has evolved to a divergent water regime following introduction to North America. We conduct RNAseq within a dry-down experiment utilizing accessions from low and high latitude populations from native and introduced ranges, and assess variation in dehydration avoidance (avoidance of wilting) and dehydration tolerance (ability to survive wilting).

Key Results: Introduced populations are better at avoiding dehydration, but poorer at tolerating dehydration than native populations. There is a strong negative correlation between avoidance and tolerance traits and expression of most drought-associated genes exhibits similar tradeoffs. Candidate genes with expression strongly associated with dehydration avoidance are linked to stress signaling, closing stomata and producing osmoprotectants. However, genes with expression linked to dehydration tolerance are associated with avoiding excessive ROS production and toxic bioproducts of stress responses. Several candidate genes show differential expression patterns between native and introduced ranges, and could underlie differences in drought resistance syndromes between ranges.

Conclusions: These results suggest there has been strong selection following introduction for deyhydration avoidance at the cost of surviving dehydration.

INTRODUCTION

Species introductions are occurring at a drastically increasing rate due to globalization and human commerce (Seebens *et al.* 2017; Bonnamour *et al.* 2021). Determining how and why some introduced species become invasive is a persistent goal for biologists that has had relatively limited success (Kolar and Lodge 2001; Pyšek and Richardson 2007). Introduced species almost certainly encounter some level of novel abiotic or biotic environments during initial colonization, and must both establish and spread to become invasive. While historically neglected as an important evolutionary force during invasion, selection is commonly observed during successful invasions - especially for human commensal species with that are introduced repeatedly and/or have high genetic variation among colonists (Huey 2000; Maron *et al.* 2004; Colautti and Lau 2015; Battlay *et al.* 2024).

Selection is key for many of the hypotheses that describe why some species become successful invaders. Stress resistance and phenotypic plasticity have frequently been hypothesized as key attributes for successful invasive species (Richards *et al.* 2006; Gewing *et al.* 2019). Individuals that are able to avoid or tolerate stress can better establish and spread in novel environments. Key to this hypothesis is that selection acts to remove less stress resistant individuals from the populations. Such selection could also occur only in certain areas of the introduced range where selection pressures are strongest. Alternatively, hypotheses like the Evolution of Increased Competitive Ability (EICA; Blossey & Notzold, 1995) suggest that introduced plants are removed from key selective pressures in the native range following colonization and the strongest selection pressure is simply for rapid growth rate and high fecundity to outcompete native species.

One of the most important selection pressures for plants is water availability and associated drought events (Siepielski *et al.* 2017). Three different non-mutually exclusive strategies are hypothesized to evolve in environments with low or inconsistent water availability: drought escape, dehydration avoidance and dehydration tolerance (Ludlow 1989; Kooyers 2015; Volaire 2018). Plants that have evolved to escape drought grow rapidly, reproduce quickly, and finish their life cycles prior to or immediately following drought stress. Plants that are dehydration avoiders actively evade drought conditions with physiological or morphological adaptation or acclimation responses to limit desiccation and thus wilt at a lower soil volumetric water content. While dehydration avoidance can be a constitutive strategy where certain individuals may have high succulence, more trichomes, higher water-use efficiency, or lower stomatal density, it also manifests as a response to reduced water availability by lowering stomatal conductance, raising water-use efficiency, and producing osmoprotectants for osmotic adjustment. Plants with dehydration tolerant strategies limit water-loss during drought conditions by limiting vegetative growth, allocating resources to root growth, and producing antioxidants to limit cellular damage from desiccation. These responses allow plants to recover following intense drought. A common experimental design to tease apart variation in drought strategies between populations and/or species is conducting dry-down experiments (e.g. Des Marais *et al.*, 2014; Bouzid *et al.*, 2019; FitzPatrick *et al.*, 2023). In these experiments, variation in time to first flower can be considered a drought escape metric, differences in soil relative water content at wilting can be considered a measure of dehydration avoidance, and the ability to recover following wilting is a measure of dehydration tolerance (Bouzid *et al.* 2019).

While drought strategies are not mutually exclusive, negative correlations between some strategies are expected due to physiological constraints. For instance, the rapid growth expected for drought escape requires opening stomata in order to increase photosynthetic rate (Geber and Dawson 1990; Dudley 1996). However, open stomata increase transpiration and lower water use efficiency – effectively neutralizing any dehydration avoidance strategy. Yet, the evidence for this tradeoff is equivocal and seemly context dependent (McKay *et al.* 2003; Kooyers *et al.* 2015). Correlations between dehydration avoidance and tolerance strategies are less explored, and are often viewed as compatible responses (Volaire 2018). However, resource allocation tradeoffs could be hypothesized as the functions necessary for maintaining water balance in a limited water environment are not the same resources necessary for surviving and recovering from dehydration.

Although the molecular signals underlying the perception and integration of water stress are becoming better resolved, variation in drought-related responses is rarely understood at the molecular level (VanWallendael *et al.* 2019). For instance, it is largely unknown whether variation in drought resistance arises through differences in sensitivity or perception of drought, integration of signals, or re-wiring of connections within gene-regulatory networks. Molecular responses to low water availability center around the activation of the abscisic acid (ABA) dependent and ABA-independent signal transduction pathways in response to disruption in water potential relative to the surrounding soil and/or atmosphere (Raghavendra *et al.* 2010; Dar *et al.* 2017; Aslam *et al.* 2022). Increases in ABA drive a signal cascade leading to guard cell regulation and stomatal closure (Hsu *et al.* 2021). Notably, production of ABA also induces gene regulatory cascades that lead to increases in antioxidants and osmoprotectants such as proline,

112 trehalose, and raffinose family oligosaccharides (Strizhov *et al.* 1997; Lunn *et al.* 2014). The
113 interactions of ABA with other plant hormones (specifically antagonistic interactions with
114 gibberellic acid and ethylene), lead to cessation of shoot growth and/or increased root growth
115 (Wilkinson and Davies 2010). Not surprisingly, this complex web of gene regulatory cascades
116 has substantial overlap – for instance, products of the SNF1-related kinases 2 (*SnRK2*) gene
117 family integrate growth rate changes, promote stomatal closure, and increase osmoprotectants
118 (Yoshida *et al.* 2015; Belda-Palazón *et al.* 2020). A key goal for the plant science community is
119 determining how these networks are manipulated to create the diversity of responses observed
120 within and among species (Kesari *et al.* 2012; Des Marais *et al.* 2014). Invasive species with
121 divergent responses between introduced and native areas can provide key natural experiments for
122 examining the evolution of drought resistance.
123
124 White clover (*Trifolium repens*) is perennial plant that is native to Eurasia but has been
125 distributed across the world as a cover crop and forage legume during the past 300 years
126 (Kjærgaard 2003). Despite relatively recent introductions, there is little decline in genetic
127 diversity in the introduced ranges of white clover (Wu *et al.* 2021) and extensive evidence for
128 selection and adaptation following introduction (Kooyers and Olsen 2012, 2013; Wright *et al.*
129 2018, 2021; Santangelo *et al.* 2022; Battlay *et al.* 2024). While white clover is most often
130 associated with temperate habitats, there is extensive variation in aridity across both the native
131 and introduced ranges, and water availability seems to be a key selective pressure (Kooyers *et al.*
132 2014). Escape, avoidance, and tolerance responses have been noted by different groups. Drought
133 escape via more annualized life history is favored in lower latitude populations in North America
134 (Wright *et al.* 2021). Dehydration avoidance may be favored in these same populations as clines

135 in cyanogenic glucosides, potentially key sources of nitrogen during drought conditions, have
136 evolved following introduction to several different continents. In each introduced region, the
137 proportion of plants possessing cyanogenic glucosides increases in more arid areas (Kooyers &
138 Olsen 2013, 2014). Finally, agricultural breeders frequently target persistence following drought
139 as a key trait for selection in agricultural lines (Marshall *et al.* 2001; Jahufer *et al.* 2002).
140 However, differences in drought resistance strategies have not been systematically examined
141 across native and introduced populations.
142
143 Here we use a manipulative dry-down experiment to examine how populations across the native
144 and invasive ranges of white clover resist drought. Specifically, we examine the relative degree
145 of dehydration avoidance and tolerance among populations from low and high latitude
146 populations in the native and invasive range using volumetric water content at wilting as a
147 measure of dehydration avoidance and ability to recover following wilting as a measure of
148 dehydration tolerance. We also examine differential expression between well-watered and dry
149 down plants at a timepoint when the dry down lines begin to experience drought stress. We use
150 this data to address four questions: First, do populations from introduced areas or from more arid
151 areas have more substantial drought resistance strategies? The stress tolerance hypothesis
152 suggests that introduced plants should be better stress tolerators, but EICA may suggest the
153 opposite. Second, are there tradeoffs that exist among dehydration avoidance and tolerance
154 strategies? That is, are plants that are good dehydration avoiders also poor dehydration
155 tolerators? Third, do native and introduced populations exhibit different patterns of gene
156 expression in response to early drought stress? That is, are differentially expressed genes
157 between well-watered and drought treatments also variable among ranges and populations?

158 Fourth, how do expression differences control variation in dehydration avoidance and tolerance
159 strategies? Together these experiments provide insight on the molecular mechanisms underlying
160 the evolution of drought resistance across a rapid invasion.
161
162 MATERIALS AND METHODS
163 We performed a manipulative experiment to examine variation among drought strategies and the
164 transcriptomic drivers of such variation within and among the native and introduced ranges of
165 white clover, *Trifolium repens*. We planted seed collected from 3-4 populations from low
166 latitude and high latitude in the native and invasive range (14 total populations; Fig. S1; Innes *et*
167 *al.*, 2022). Seeds for each population had been pooled from >25 different maternal lines. For
168 each population, we used latitude and longitude to extract WorldClim2 variables (Fick and
169 Hijmans 2017) and annual aridity and evapotranspiration data from CGIAR (Zomer *et al.* 2008).
170
171 Seeds were scarified and planted into 4" pots filled with PRO-MIX LP-15 soil pre-saturated with
172 water. Pots were placed in AR-66L2 growth chambers (Percival Scientific; Perry, IA, USA) and
173 misted daily to promote germination. Growth chambers were set to a constant 22°C with a
174 14hr:10hr day:night cycle. Germinants were randomized within well-watered and dry-down
175 treatments (well-watered = 26 individuals, dry-down = 25 individuals; Table S1). Plants were
176 rotated within chambers every three days to minimize microenvironmental variation. Plants were
177 grown for six weeks to accumulate above and below ground biomass. At six weeks, all pots were
178 saturated with water by bottom-watering. Number of leaves on each plant were counted and one
179 pot was excluded from further analysis because it was substantially larger than the other plants.
180 Plants in the well-watered (control) treatment received periodic watering to saturation. Plants in

the dry-down treatment did not receive additional water. Each day, we assessed volumetric water content in each pot using a SMT150T soil moisture meter (Dynamax; Houston TX, USA). Well-watered and dry-down treatments functioned as expected with separation in volumetric water content (VWC) between treatments after control plants were resaturated on day 9 (Fig. S2)

To examine dehydration avoidance, we recorded the day and the soil moisture at which each plant in the dry-down treatment wilted (hereafter, "wilt VWC"). To determine dehydration tolerance, we determined the propensity to recover following wilting (hereafter "wilt survival"). Forty-eight hours after wilting, we resaturated the pots of each wilted plant by bottom watering for an extended period of time (~1hr). We then resurveyed plants for survival three days (72hrs) after resaturation. Survival was measure as a discrete variable (yes/no) with any revived green tissue or new growth counting as survival.

Manipulative Experiment Statistics

All statistics were conducted in R v4.2.2 (R Foundation for Statistical Computing, Vienna, Austria) with univariate linear models (LM) and generalized linear models (GLM) implemented with the lm() or glm() functions, respectively. First, to examine whether plant size influenced either dehydration avoidance or tolerance, we examined associations between either wilt VWC and wilt survival against leaf number at the beginning of the experiment. Neither wilt VWC or survival was significantly associated with leaf number (Fig. S3). Models below produce qualitatively similar results whether or not leaf number at day 1 is included as a covariate.

We examined whether variation in dehydration avoidance and tolerance was associated with range (native/introduced), latitude (low/high) or an interaction using generalized linear models. Specifically, we modeled wilt VWC and wilt survival as response variables within a GLM with range, latitude, and range:latitude interaction as factors. Wilt VWC was modeled with a gaussian distribution with an identity link and wilt survival was modeled with a binomial distribution with a logit link. Statistical significance was determined through ANOVA using a type II sum of squares implemented with the *Anova()* function (*car* library; Fox *et al.*, 2013)

Latitude across continental ranges may not be strongly related to water availability or aridity of populations and thus may not be the main factor driving variation in drought strategies. To examine whether variation in drought strategies was related to differences in precipitation or aridity, we examined associations between wilt VWC and wilt survival with annual aridity index, potential evapotranspiration, and coefficient of variation in annual precipitation (bio15). Distributions and links of models as well as assessment of statistical significance were the same as the above models. Leaf number at day 1 of the experiment was included as covariate in each analysis as there were weak associations between each climatic variable and initial plant size (Fig. S4).

To explore potential tradeoffs between dehydration avoidance and tolerance, we examined associations between wilt VWC and wilt survival using a GLM (binomial distribution, logit link). Ability to recover from wilting was the response variable and volumetric water content at wilting the predictor variable. Significance was tested with an ANOVA as above and model fit was assessed using the *adjR2()* function (glmToolbox package; Vanegas *et al.*, 2024) .

226

227 **RNAseq Analysis**

228 We used an RNAseq analysis to examine differential expression underlying variation in drought
229 strategies. During the manipulative experiment, leaf tissue from two healthy adult leaves was
230 flash frozen in liquid nitrogen ten days after the dry down treatment began from plants in both
231 the well-watered and dry-down treatment. Tissue sampling was done during a two-hour period in
232 the afternoon with order of collection randomized within each treatment. Total RNA was
233 extracted with Direct-zol RNA Miniprep (Zymo Research; Irvine, CA, USA). RNAseq libraries
234 were constructed with QuantSeq 3' mRNA-Seq FWD prep kit (Lexogen; Vienna, Austria) using
235 an input of ~415ng total RNA/sample following manufacturers protocols. Two samples had low
236 RNA concentrations and we followed Lexogen's modified procedure for low input samples.
237 Four samples from the manipulative experiment did not have RNAseq libraries constructed due
238 to loss of labels during shipping or near-zero read counts (RNAseq N = 47; Table S2). All
239 individuals were barcoded and multiplexed in a single tube. We sequenced this library on a
240 single HiSeqX lane (PE 150bp reads) through Novogene (Sacramento, CA, USA). A second
241 round of multiplexing and sequencing was conducted for a subset of samples that had low data
242 yield the first round, and files from both rounds were subsequently merged. Raw sequenced
243 reads per individual averaged 37,241,613 reads (SD 16,534,847; Table S2)

244

245 Fastp v.023.4; (Chen *et al.* 2018) was used to trim adapters and poly-a tails. Microbial RNA
246 contamination was removed by aligning each sample to all fungal and bacterial assembly
247 genomes within the NCBI database using bowtie2 v2.5.1(Langmead and Salzberg 2012). The
248 bacterial and fungal genome database was indexed with the 2.2.4 release of bowtie2. The

249 remaining reads that did not align to the contamination database were presumed to be white
250 clover RNA reads. A transcriptome was created from the *T. repens* reference genome
251 (Santangelo *et al.* 2023) using gffread v0.12.7 (Pertea and Pertea 2020). A full decoy-aware
252 transcriptome was constructed to mitigate specious mapping of reads that occur from
253 unannotated genomic loci that are similar to the annotated transcriptome, and a mapping-based
254 index was then constructed using a k-mer hash over k-mers of length 31 using Salmon v1.10.2
255 (Patro *et al.* 2017). Read mapping to the transcriptome and quantification was then performed
256 with Salmon v1.10.2 in mapping-based mode using the *T. repens* transcriptome and RNA
257 sequences. There was a clear issue with DNA contamination as only 3.83% of reads per
258 individual aligned to the transcriptome (SD 1.0%; Table S2); however, this should not bias our
259 comparative analysis (differential expression or transcript abundance-phenotype associations)
260 that rely on read counts of each transcript.
261
262 **Quantifying expression across treatment, range, and latitude**
263 We used DESeq2 (Love *et al.* 2014) to test for differences in transcript abundance between dry-
264 down and well-watered treatment groups, between the North American and European range, and
265 between high and low latitude populations. We controlled for the VWC at the time of tissue
266 collection by treating it as a covariate in the DESeq2 model (i.e., Transcript ~
267 Treatment*Latitude*Range + SoilVWC). False discovery rate (FDR) for each gene was
268 calculated and a transcript was then categorized as differentially expressed if FDR was < 0.1.
269 Concordance of gene expression responses to drought between ranges and latitudes was
270 determined by comparing the coefficient estimates of protected one-way ANOVAs. That is, we
271 conducted ANOVAs for each gene found to be differentially expressed between the well-watered

272 and dry-down treatments with relative expression as the response variable and either range or
273 latitude as the predictor variable.
274
275 All transcripts were used for weighted correlation network analysis (WGCNA) to determine gene
276 modules and co-expression networks within the WGCNA v1.72-5 library (Langfelder and
277 Horvath 2008). To achieve homoskedasticity amongst transcript abundance, the variance
278 stabilization transformation from the fitted dispersion-mean relations was calculated with
279 DESeq2, which was then used to transform transcript count data by dividing by the size factor.
280 Normalized transcript counts were then analyzed with WGCNA using a soft-thresholding power
281 of 9 and assuming that biological networks follow a scale-free structure (Barabási and Albert
282 1999). The sets of genes within each resulting module were then cross referenced with the
283 'biological process' gene ontology terms (GO; Ashburner *et al.*, 2000) of *Arabidopsis thaliana*
284 and used for gene enrichment analysis with an FDR cutoff of 0.1. Fold enrichment, $-\log_{10}$(FDR),
285 and number of genes for each GO term was then calculated. Genes belonging to the same
286 module were then compared to the Kyoto Encyclopedia of Genes and Genomes (KEGG;
287 Kanehisa & Goto, 2000) to visualize expression pathways.
288
289 **Identifying genes associated with wilt VWC and wilt survival**
290 GLMs were constructed to quantify the correlation of transcript abundance with either wilt VWC
291 or wilt survival. First, transcripts of drought treatment samples were standardized to acquire
292 relative expression for each gene. Univariate GLMs included relative expression as the response
293 variable and either wilt VWC (gaussian distribution, identify link) or wilt survival (binomial
294 distribution, logit link) as predictor variables with VWC at the start of the experiment as a

295 covariate. Significance was then tested using ANOVA as above. To determine the set of
296 phenotypically significant genes that are co-expressed, genes significantly associated with each
297 phenotype were compared to the previously calculated WGCNA network. We also determined
298 the location of these genes within the white clover genome (Santangelo *et al.* 2023) via *blastn*
299 (Altschul *et al.* 1990).
300
301 Finally, we then determined which of the genes associated with phenotype also were
302 differentially expressed between ranges or across latitudes to identify genes likely to be involved
303 in adaptation following introduction or to divergent climatic conditions. We conducted GLMs
304 modeling relative expression as a function of traits and either range or latitude. Specifically
305 relative expression was the response variable while trait value (wilt VWC or wilt survival),
306 spatial group (range or latitude), and the interaction between trait:group were included as
307 predictors. Genes with relative expression that is explained by trait:group interaction are good
308 candidates for producing the phenotypic variation observed in nature.
309
310 RESULTS
311 There was substantial variation in dehydration avoidance and tolerance throughout the native and
312 introduced ranges of white clover. Wilt VWC was lower in North American populations than in
313 European populations ($F_{1,23} = 4.8$, $p = 0.03$; Fig. 1A) indicating greater drought avoidance in
314 introduced populations. However, the effect size of this difference was low as the difference in
315 mean wilt VWC was only 1.3% lower water content at wilting in the North American
316 populations. There were no differences among low and high latitude populations or any
317 interaction between introduction and latitude impacting dehydration avoidance (Table S3).

Dehydration tolerance also differed among native and introduced ranges with native regions able to better survive wilting ($X^2 = 7.09$, $p = 0.012$; Fig. 1B). All but one of the plants from the native range were able to recover following wilting (11/12 plants), but only 46.7% (7/15) plants recovered from the introduced North American range. There were no differences among low and high latitude populations or any interaction between introduction and latitude impacting dehydration tolerance (Table S2).

There are strong associations between the abiotic environment of site where each line originated and wilt survival, but little correlation between abiotic variables and wilt VWC. Dehydration tolerance was associated with aridity index, with plants from areas with greater annual aridity index being more likely to survive wilting ($X^2 = 6.2$, $p = 0.013$; Fig. S5). This pattern was driven by the low latitude populations from the native range that all recovered following wilting. These low latitude populations, largely from Spain, had relatively high evapotranspiration, low annual precipitation, and higher variation in precipitation. Dehydration avoidance was not significantly associated with any abiotic variables (Fig S5.; Table S4).

There was a strong tradeoff between dehydration avoidance and tolerance. Plants that wilted at lower VWC were less likely to recover following wilting (Adj. $r^2 = 0.53$, $p = 0.005$; Fig. 2). The four plants wilting at the lowest volumetric water contents all did not survive following wilting. Moreover, six of the seven plants with the lowest wilt VWC also did not survive wilting.

Differential expression across the native and introduced ranges

341 Many genes were differentially expressed between dry down and well-watered treatments, high
342 and low latitude populations, and native and introduced ranges (Table S5). Across predictor
343 variables, there were 19,344 differentially expressed transcripts out of 86,723 sequenced
344 transcripts corresponding to 2,253 of 5,001 unique genes. Most differentially expressed
345 transcripts were differentially expressed within all three contrasts (Treatment, Range, Latitude;
346 35.3%; Fig. S6). An additional 29.4% of transcripts were differentially expressed both in Range
347 and Latitude contrasts, not in the Treatment contrast. A far lower percentage of differentially
348 expressed transcripts were differentially expressed in only one contrast (Latitude: 13.4%;
349 Treatment: 5.6%; Range: 10.0%). The large union between the three contrasts in differentially
350 expressed transcripts indicates different drought responses are likely occurring across latitudinal
351 gradients and ranges.
352
353 We examined co-expression between differentially expressed genes to determine how gene
354 expression networks are influenced by drought treatment and vary across range and latitude.
355 Eight modules were discovered from WGCNA including 5.77% of transcripts (Table 1, Table
356 S5). Expression profiles of normalized transcript counts show the "turquoise" module as having
357 elevated transcript counts in samples within the dry-down treatment in contrast to all other
358 modules that exhibit relative uniformity across sample types (Table 1; Fig. S7). Gene enrichment
359 analysis of the "turquoise" module reveals marginal or significant enrichment for many GO
360 terms typically associated with drought resistance including: hyperosmotic stress, proline
361 metabolic process, response to oxygen-containing compound, and response to water deprivation
362 (Table S6). Within the "turquoise" module, 41 of the 53 genes (77.4%) exhibit a significant
363 latitude by range interaction (24 homologues exhibit significantly different range responses

364 between high and low latitudes) – substantially higher than the genes not in modules (18.2%;
365 Table 1). These results suggest that genes within the turquoise module may underlie adaptation
366 to climate across the native and introduce ranges.
367
368 **Differentially expressed genes are associated with dehydration avoidance and tolerance.**
369 Expression of a large number of genes was associated with wilt VWC and wilt survival (635 and
370 392, respectively; 198 shared; Table S6). The overlap between this group and genes that are
371 differentially expressed within the dry-down vs. well-watered contrast represent the best
372 candidate genes for impacting dehydration avoidance and tolerance strategies. A promising
373 number of genes are found in this overlapping group – 47 differentially expressed genes were
374 associated with wilt VWC and 17 differentially expressed genes with wilt survival (7 shared
375 between phenotypes). These genes disproportionately were found in the 'turquoise' module: 80%
376 of gene homologs for wilt VWC and 50% of gene homologs for wilt survival.
377
378 From this initial group, we identified several strong candidate genes by examining genes with
379 extreme effect sizes on each phenotype and are also known drought-associated genes in other
380 species. We first examined genes which were more highly expressed in plants with low wilt
381 VWCs as genes potentially involved in dehydration avoidance. Several genes from the turquoise
382 co-expression network had the greatest negative beta estimates for wilt VWC. These included
383 multiple homologs of *NAC4* and *BCAT2* (Fig. 3AB). Notably, expression of these genes was also
384 strongly associated with death following wilting, i.e. the most negative beta estimates for
385 dehydration tolerance. Several other strong candidate genes had this same strong tradeoff
386 between phenotypes including homologs of *AlaAT2*, *GOLS2*, *SDIR1* and *P5SC1* (Fig. 3CDEF).

387 In fact, all transcripts that had significant associations with both wilt VWC or wilt survival had
388 either negative beta for both wilt VWC and wilt survival or positive betas for both traits (α
389 =0.05, 582 transcripts & 170 homologues; Fig. 4). We discuss gene functions in more depth in
390 the discussion, but wilt VWC-associated genes are strongly related to stress signaling via both
391 ABA-dependent and independent pathways, stomatal responses, and producing various
392 osmoprotectants.
393
394 Genes with expression strongly associated with wilt survival had functions more explicitly
395 associated with detoxifying reactive oxygen species and negative products of photorespiration as
396 well as continued light harvesting. The gene with the strongest association with wilt survival was
397 a homolog of *DegP5* – a gene that dampens Ca^{+2} signaling associated with stress responses and
398 is associated with reduced ROS (Fig. 5A). This gene also had the strongest positive association
399 with wilt VWC of any gene, that is, high expression of this gene is linked with wilting at a high
400 VWC. Expression of a *CUT1* (*Cutinase 1*) homolog, a gene that impacts cuticle wax
401 biosynthesis, was also strongly associated with greater survival following wilting. One additional
402 candidate gene, a homolog of *SAPK2*, also had among the highest associations with increased
403 wilt survival and higher wilt VWC (Fig. 5C). Other genes, including homologs of *PGLP2*,
404 *CAB7*, and *LHCA6*, with expression strongly associated with wilt survival are more weakly
405 associated with wilt VWC. These genes have less clear associations with known drought
406 responses. Together, these candidates suggest that genes associated with lower wilt VWC
407 include those typically associated with dehydration avoidance, while genes associated with wilt
408 survival are associated with increasing photosynthesis and protecting tissues against the harmful
409 bioproducts of stress responses.

410

411 **Expression patterns associated with phenotypic differences between ranges.**

412 While many genes are associated with dehydration avoidance and tolerance phenotypes, a
413 limited subset of these candidates have the potential to drive the differences in dehydration
414 avoidance or tolerance between ranges. We examined which of the candidate genes linked to
415 dehydration avoidance or tolerance also had differential expression across ranges or latitudes. As
416 is the case with many genes in the turquoise network, relative expression of *BCAT2* in the
417 drought treatment is greater in high latitude populations, exhibits variable expression in low
418 latitude North American populations, and has very limited expression in the low latitude
419 European populations (Range:Latitude: $\log_2$FoldChange = 30, P = 4.83e-9; Fig. 3, Fig. S8).
420 Similar patterns exist for both *P5CS* (Range:Latitude: $\log_2$FoldChange = 29.9, P = 8.28e-11) and
421 *GOLS2* (Range:Latitude: $\log_2$FoldChange = 18.5, P = 0.002). To determine other genes that may
422 create phenotypic differences between ranges or across latitudes, we identified genes whose
423 relative expression is best explained by an interaction between wilt VWC or wilt survival and
424 range. Many genes (22.8% of genes surveyed; Table S8) exhibited such interactions including 46
425 of the 52 genes in the turquoise network. These results suggest the molecular variation
426 underlying phenotypic differences between ranges either involves many loci of varying effect
427 sizes or are large effect variants that impact substantial hormonal stress responses and many gene
428 expression networks.

429

430 DISCUSSION

431 During invasions, plants encounter novel environments and may need to acclimate or adapt to
432 succeed. Here we identify differences in drought resistance strategies that have evolved

following the introduction of white clover to North America from its native range in Europe. North American populations have stronger dehydration avoidance responses while European populations have stronger dehydration tolerance responses. These differences appear to be shaped by the abiotic conditions in each region as well as a strong tradeoff between dehydration avoidance and tolerance. We also identify a number of strong candidate genes underlying each of these phenotypes with a significant proportion falling into a single co-expression network. Most of these genes have expression profiles where higher expression improves either dehydration avoidance or tolerance while decreasing the other phenotype. Additionally, the limited subset of genes that are associated with both a drought resistant phenotype and exhibit differential expression between native and introduced ranges represent the best candidates for identifying a molecular basis of drought adaptation following introduction. We discuss these results below in the context of invasion biology and the evolutionary genetics of adaptation to plant abiotic stresses: two fields that overlap but are rarely synthesized.

Evolution of Drought Resistance During Invasions

The roles of selection and adaptation during invasions is now widely documented with many species, including white clover, rapidly evolving climate-associated clines following introduction (e.g. Kooyers & Olsen, 2012; Colautti & Barrett, 2013; Battlay *et al.*, 2023). However, generalization of which traits are favored during invasions is less clear (Kolar and Lodge 2001). Different trait-based hypotheses suggest that rapid growth (EICA; Blossey & Notzold, 1995) or stress tolerance may be favored in the introduced regions (Gewing *et al.* 2019). Our results are partially consistent with both hypotheses, but are far more nuanced as drought resistance is conferred through many different mechanisms. Introduced North American populations wilt at

456 lower volumetric water contents than populations from the native European range, but also are
457 less able to tolerate wilting than native populations (Fig. 1). Thus, North American populations
458 are likely more dehydration avoidant than European populations. Greater dehydration avoidance
459 may allow plants to maintain growth during periods of low water and high evapotranspiration.
460 This would be consistent with devoting more resources to rapid growth within the introduced
461 range as well as being more resistant to initial water stress. This nuance may account for some of
462 the mixed evidence for the EICA within the literature (Rotter and Holeski 2018).
463
464 We suggest that the evolution of greater stress avoidance to facilitate rapid growth may be a
465 pattern frequently observed across invasions of perennial plants. Introduced species are likely to
466 encounter different abiotic regimes in their new range. Invasive populations of annual plants
467 often evolve more rapid life histories (Colautti and Barrett 2013; Battlay *et al.* 2023), but
468 perennial plants must survive stresses in order to establish and reproduce. There are few plant
469 systems where the evolution of drought avoidance or tolerance have been assessed across native
470 and introduced regions. These examples largely consist of species where it is clear that they have
471 a very different drought resistance strategy in the introduced range. For instance, camphorweed
472 (*Heteroteca subaxcillaris*) has drastically greater root growth in its invasive range in Israel than
473 in its native range, North America, that likely is a benefit both for low water availability and
474 structural integrity in extremely arid dune environments in Israel (Sternberg 2016). Jerusalem
475 artichokes (*Helianthus tuberous*) have evolved extreme clonality phenotypes in their introduced
476 range (Europe) to accommodate a shift to a more aquatic environment (Bock *et al.* 2018).
477 Outside of plants, a greater tolerance or plasticity to stressful environments is often observed –
478 for instance, anoles (*Anolis cristatellus)* from the introduced range survive colder temperatures

479 than from the native range (Leal and Gunderson 2012). We suggest that invasive species should
480 be more widely used for identifying natural phenotypic and genomic variation in drought
481 resistance.
482
483 While our results are consistent with classic invasion biology theory, they also make sense in the
484 context of environmental characteristics of the populations. The populations involved in this
485 experiment have very different growing seasons and historical climatic features (Table S1).
486 Southern European populations come from a Mediterranean climate with cool and temperate
487 winters and hot dry summers. Relative to other locations, there is little precipitation throughout
488 the year and almost no rainfall during summer months. A long summer with little rain is the ideal
489 conditions for the evolution of dehydration tolerance in a perennial plant that likely needs to
490 survive across multiple growing seasons (Kooyers 2015). Alternatively, eastern North America
491 has more substantial rainfall throughout the growing season with drought stress likely coming
492 during shorter stretches without rain (Table S1). These conditions may favor the evolution of an
493 avoidance strategy as additional rainfall is likely coming in the near future. Interestingly, we
494 observe very little difference in drought resistance strategy between high and low latitude
495 populations of white clover in the native or introduced ranges. In both ranges, on average, lower
496 latitude populations are less dehydration avoidant but more dehydration tolerant. This is
497 surprising as previous work documents differentiation in life history strategy evolving across this
498 gradient with warmer southern populations employing a more annualized life history (Wright *et*
499 *al.* 2021). We suggest our small sample size only has the power to detect major effects and future
500 larger studies may be able to parse differences in stress responses across latitudinal gradients.
501

502 **Molecular basis of drought tolerance and avoidance variation**

503 The molecular basis of drought resistance has been examined extensively in crop plants and
504 model systems, but there has been limited success in determining the genes that underlie
505 variation in natural populations (Des Marais *et al.* 2013; VanWallendael *et al.* 2019). Here we
506 find several candidate genes that likely influence dehydration avoidance and tolerance responses
507 across both invasive and native ranges despite relatively few mapped reads from only a single
508 tissue type (Fig. 3). Notably the turquoise co-expression network is strongly enriched for gene
509 ontologies related to drought responses (i.e. response to abiotic stress, response to water and
510 proline synthesis). A high proportion of the genes within this network are also associated with
511 wilt VWC including some of the strongest candidate genes. Interestingly, these genes represent a
512 diverse set of drought-related functions in other organisms.

513

514 *NAC4* and *SDIR* homologs are likely involved in stress signaling pathways. NAC4 (nascent
515 polypeptide-associated complex) is a transcription factor that is strongly upregulated in response
516 to drought, salt, and water stress in many species (Xia *et al.* 2010; Shao *et al.* 2015; Yu *et al.*
517 2016) and confers drought tolerance when transgenically expressed in *Arabidopsis* (Mei *et al.*
518 2021). More generally, the NAC gene family is involved in stomatal closure and in lateral root
519 development in rice (Hu *et al.* 2006; Zheng *et al.* 2009). SDIR1 (Salt and Drought-Induced Ring
520 Finger1) is a RING-finger E3 ligase that is upregulated in *Arabidopsis* in response to drought
521 stress and modulates both the ABA response and stomatal closure (Zhang *et al.* 2007). While
522 upregulation of both genes in response to drought stress in our system is consistent with findings
523 in other species and expected for dehydration avoidant plants, *sdir1* mutants in *Arabidopsis* had

524 greater mortality following wilting than wild type plants. This suggests an additional dehydration
525 tolerance role for SDIR1 that we do not observe.
526
527 Expression of multiple genes that promote the synthesis of osmoprotectants (i.e. branched chain
528 amino acids or BCAAs, proline, and raffinose family oligosaccharides) are also strongly
529 associated with lower wilt VWC. *BCAT2* (BCAA transaminase 2) catalyzes the final
530 transamination step in production of BCAAs as well as the first degradation step. Under stress,
531 BCAAs accumulate as osmoprotectants in an ABA-dependent manner, with BCAT serving as
532 the main regulator in *Arabidopsis*. P5CS, delta 1-pyrroline-5-carboxylate synthase, controls the
533 rate-limiting step of proline biosynthesis from glutamate during osmotic and salt stress (Strizhov
534 *et al.* 1997). Transgenic overexpression of P5CS consistently increases tolerance to drought and
535 salt stress across several plant species by increasing proline content (Su and Wu 2004; Yamchi *et*
536 *al.* 2007; Vendruscolo *et al.* 2007). *GOLS2* (galactinol synthase 2) encodes galactinol synthase 2,
537 a key enzyme that regulates the first step in biosynthesis of raffinose family oligosaccharides
538 resulting in the creation of galactinol (Panikulangara *et al.* 2004). While the oligosaccharides act
539 as osmoprotectants and as antioxidants, both galactinol and the oligosaccharides may also act as
540 signaling molecules (Song *et al.* 2016). In other species, GOLS activity is triggered by abiotic
541 and biotic stress (*Arabidopsis*, Coffee, Rice) and confers drought tolerance (Dos Santos and
542 Vieira 2020). Unexpectedly, our results indicate that expression of *GOLS2* is linked with lower
543 wilt VWC (greater dehydration avoidance) and with lower survival following wilting (lower
544 dehydration tolerance). Increased expression of *GOLS2* in *Arabidopsis* does reduce transpiration
545 rate (Taji *et al.* 2002).
546

547 We also note a third process that may enhance dehydration avoidance and tolerance responses:
548 the recycling and efficient use of nitrogen. *AlaAT2* (alanine aminotransferase 2) is strongly
549 upregulated in plants that wilt at low VWC. We propose that this upregulation links drought
550 response to nitrogen metabolism. Specifically, AlaAT2 catalyzes the reversible conversion of
551 alanine and α-oxoglutarate to pyruvate and glutamate, facilitating the synthesis of other amino
552 acids as needed (Miyashita *et al.* 2007). *AlaAT* homologs are upregulated during hypoxia in
553 several species, including *Arabidopsis*, Poplar, Medicago, and Corn, and are regulated by ABA
554 in other stressful contexts (Ricoult *et al.* 2005; Miyashita *et al.* 2007; Xu *et al.* 2017). Drought
555 stress typically decreases the activity of nitrate reductase and nitrile reductase (Foyer *et al.*
556 1998), impairing the plant's ability to accumulate amino acids due to limited nitrogen resources.
557 Greater AlaAT2 activity could convert alanine into glutamate, which may then be used to
558 produce BCAAs or proline as osmoprotectants. In sum, we hypothesize that the production of
559 osmoprotectants requires carbon and nitrogen resources that may be most easily procured
560 through recycling of existing metabolites.
561
562 The candidate genes associated with wilt survival generally do not have strong functional ties to
563 drought responses in other systems with two exceptions. *DegP5* (degradation of periplasmic
564 proteins 5) belongs to a family of proteases that is typically upregulated in response to abiotic
565 stress. Overexpression of *DegP5* has been linked to increased dehydration tolerance in *Nicotiana*
566 *tabacum* via the suppression of Ca^{+2} and flagellin signaling leading to a reduced accumulation of
567 reactive oxygen species (Chen *et al.* 2023). In our study, increased expression of *DegP5* is also
568 associated with wilting at higher soil moistures. This suggests that the increase wilt survival
569 comes at a cost of a less active drought response. The second exception is *SAPK2*, a plant-

570 specific protein kinase in the family of SNF1-related protein kinase 2 (*SnRK2*) genes. Expression
571 of the *SAPK2* homolog is positively associated with wilt survival (Fig. 5C). This role is similar to
572 that documented in rice, where SAPK2 not only upregulates genes involved in osmoprotection
573 but also induces genes involved in enzymatic ROS detoxification (Lou *et al.* 2017). However, a
574 role in dehydration avoidance has not been documented. Other SnRK2s do regulate cation
575 channels (KAT1) and impact stomatal closure in *Arabidopsis* (Sato *et al.* 2009). Other genes
576 strongly associated with high wilt survival in our study function in other organisms in the
577 detoxification of photorespiration byproducts (*PGLP2*; Schwarte & Bauwe, 2007) and in
578 efficient light harvesting and photosynthesis (*LHCA6*, *CAB7*; Otani *et al.*, 2017). Together, these
579 results suggest that improved survival after wilting is achieved through a combination of
580 dampening responses to drought, protecting cells from harmful bioproducts of drought stress,
581 and maintaining metabolic functions.
582
583 **A tradeoff between drought avoidance and tolerance strategies?**
584 Drought resistance strategies are often presented as non-mutually exclusive with the recognition
585 that they represent different physiological mechanisms for a plant to acclimate to low water
586 availability. The relationship between drought escape and dehydration avoidance has been the
587 most extensively scrutinized as there are clear physiological tradeoffs for C3 plants – closing
588 stomata for better dehydration avoidance leads to lower carbon uptake and the inability to
589 synthesize more sugars for rapid growth and reproduction (Geber and Dawson 1990; Dudley
590 1996). Theoretical and empirical identification of tradeoffs between dehydration avoidance and
591 tolerance is rare – potentially because there is not a true physiological tradeoff that exists or
592 because these strategies may be expressed sequentially as water stress increases (Volaire 2018).

593 We document relatively strong tradeoffs between wilt VWC and wilt survival (Fig. 2) and
594 expression responses for wilt VWC and wilt survival are strongly correlated (Fig. 4). However,
595 the functions of genes associated with each of these phenotypes suggest that our experiment is
596 not truly measuring separate dehydration avoidance and tolerance responses. Rather, our wilt
597 VWC treatment is likely catching both dehydration avoidance and tolerance responses – plants
598 that survive to lower wilting *are* effectively tolerating dehydration for longer than plants that
599 wilted earlier, likely by increasing osmoprotectants as well as closing stomata to regulate gas
600 exchange. Correlated dehydration avoidance and tolerance responses make sense as both
601 functions are at least partly regulated by ABA. However, since dehydration tolerance responses
602 often involve the production of various osmoprotectants and closing stomata limits CO_2 (and
603 useable plant nitrogen) uptake, correlated physiological responses must require recycling carbon
604 and nitrogen.
605
606 The strong correlation between wilt VWC and wilt survival more likely represents a tradeoff
607 between different elements of dehydration tolerance. Wilting occurs when the turgor pressure
608 within cells falls to zero, leading to potential cellular damage. This damage is exacerbated under
609 prolonged drought stress, as increased ROS production can overwhelm the plant's antioxidant
610 defenses and, coupled with stomatal closure, can also increase photorespiration flux (Noctor *et*
611 *al.* 2002). To survive wilting, plants must be able to protect cellular function and integrity. Our
612 study found that plants with better wilting survival had higher expression of genes associated
613 with antioxidant defense (*DegP5*) and detoxification of photorespiration byproducts (*PGLP2*).
614 While expression of each of these genes was associated with high wilt VWC, the only truly
615 strong correlation was for *DegP5* – a gene linked to diminishing Ca^{+2} signaling as well as lower

616 ROS production in tobacco (Chen *et al.* 2023). Expression of this gene may represent a true
617 tradeoff between different physiological strategies. Generally, our results suggest that a
618 dehydration tolerance strategy actually represents multiple different types of tolerance – one
619 strategy that provides resistance against dehydration and another that allows longer-term survival
620 following wilting (i.e. extreme dehydration). We hypothesize that dehydration tolerance related
621 to drought survival may have overlapping physiology with desiccation tolerance strategies
622 occurring in plants like resurrection fern. In sum, connecting variation between physiological
623 processes and plant performance will provide additional insight into the evolution of different
624 ecological strategies in specific environments.
625
626 **Loci underlying drought response adaptation following introduction**
627 Identifying the causative molecular variants that underlie natural intraspecific variation in
628 drought responses has proven challenging with limited of successes (Juenger 2013;
629 VanWallendael *et al.* 2019). This objective is largely beyond the scope of our study. We do
630 identify many genes that are both linked with wilt VWC or wilt survival phenotypes and also
631 show variable expression across ranges, conditions and latitudes. This overlap includes a
632 substantial portion of the turquoise gene co-expression network, suggesting that variation in
633 dehydration avoidance and tolerance phenotypes is physiological manifested through broad shifts
634 across gene expression networks (e.g. Lovell *et al.*, 2018) rather than through simple, large effect
635 variants that directly cause lower stomatal conductance or greater osmoprotection (e.g. Kesari *et*
636 *al.*, 2012; Des Marais *et al.*, 2014).
637

638 Recent work has identified several haploblocks that underlie climate adaptation following
639 introduction to North America as well as several candidate genes within haploblocks that were
640 differentially expressed across drought treatment, range and latitude (Battlay *et al.* 2024). Within
641 the three haploblocks on Chr04_Occ (syn. Chromosome 7), 26 differentially expressed
642 transcripts were found (9 on hb7a1; 14 on hb7a2; and 3 hb7b, Table S9). On hb7a1, 4 genes
643 within the turquoise submodule were differentially expressed. Two turquoise submodule genes,
644 *P5CS1 and FER1*, were strongly correlated with wilt VWC and wilt survival. In addition,
645 *BRR2A* and *DWF5* were correlated significantly with wilt survival whereas *IAA6* and *DWF5*
646 were correlated significantly with wilt VWC. On hb7b, transcripts belonging to the gene *CCL12*
647 are significantly correlated with wilting. Multiple of these genes have allele variation associated
648 with higher fitness in North American common gardens (Albano et al. in prep; Battlay *et al.*,
649 2024). These results are a first step to tying locally adaptive genetic variation to variation in gene
650 regulatory networks, physiology and ecological strategies.
651
652 *Conclusions*
653 Our work suggests that selection acts on rapid timescales following introduction to allow more
654 precise adaptation of drought responses to divergent environmental conditions. Specifically,
655 selection favors specific drought resistance strategies, with tradeoffs occurring between
656 strategies at both phenotypic and molecular levels. We identify a co-expression network that
657 underlies both dehydration avoidance and some elements of dehydration tolerance where many
658 of these loci also exhibit differential expression patterns between the native and introduced
659 ranges of white clover. We also identify tradeoffs at the molecular level among functions
660 (osmoprotection and detoxification) typically identified within the dehydration tolerance strategy

– a result that needs to be carefully considered in breeding efforts. This work stimulates more studies examining and relating genetic variation and expression networks to phenotypic differences within natural populations to create a broader consensus on mechanisms creating variation in drought resistance and survival.

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DATA AVAILABILITY

Phenotypic data from the growth chamber experiment is available via dryad: link. RNAseq data is available via NCBI's short read archive: link.

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TABLES

Table 1: Breakdown of the genes and module associations within phenotypes and across space

Module	# Transcripts	Wilt VWC	Wilt Survival	Latitude	Range	Latitude X Range
No-Module	45916 (4360)	2066 (557)	1201 (336)	2994 (854)	1076 (309)	3895 (875)
In-Module	4337 (662)	346 (89)	240 (62)	603 (169)	170 (51)	592 (130)
brown	2298 (428)	158 (50)	110 (29)	426 (134)	97 (28)	221 (54)
red	122 (36)	8 (1)	7 (2)	20 (5)	5 (1)	15 (4)
yellow	259 (45)	3 (1)	7 (1)	16 (5)	3 (2)	50 (9)
grey	821 (120)	52 (6)	28 (5)	37 (8)	34 (11)	36 (6)
turquoise	285 (53)	86 (28)	59 (15)	70 (12)	8 (0)	196 (41)
green	138 (31)	5 (1)	5 (2)	8 (2)	8 (4)	7 (1)
blue	324 (86)	27 (5)	21 (8)	19 (8)	15 (6)	51 (20)
black	91 (20)	7 (0)	3 (0)	7 (1)	0 (0)	16 (5)

Number outside of parentheses refers to the number of transcripts that are associated with the phenotype or differentially expressed across Latitude or Range contrasts. The number within the parentheses refers to gene homologues that are associated with the phenotype or differentially expressed across Latitude or Range contrasts.

911 FIGURE LEGENDS

912 **Fig. 1** Variation in drought strategies between native and introduced populations of white clover.
913 (A). Boxplots of variation in wilt VWC within low and high latitude population in the native
914 (European) and introduced (North American) regions. Each point is an individual. Box edges
915 represent the interquartile range, the center line in the box is the median, and the whiskers
916 represent 1.5 times less or greater than the interquartile range. (B). Barplots identify the
917 proportion of individuals that survived the wilting treatment from each latitude:region contrast.
918 Numbers above the bars are the number of individuals surveyed.

919

920 **Fig. 2** Phenotypic tradeoff between dehydration avoidance and tolerance. Lower wilt VWC
921 indicates greater dehydration avoidance while higher wilt survival indicating greater dehydration
922 tolerance.

923

924 **Fig. 3** Association between candidate gene expression and dehydration avoidance and tolerance
925 phenotypes. Lower wilt VWC indicates greater dehydration avoidance while higher wilt survival
926 indicating greater dehydration tolerance. Points and bar are colored by latitude and range: low
927 latitude Europe = blue, high latitude Europe = red, low latitude North America = purple, High
928 Latitude North American = orange. Each point represents a different individual. Gene
929 Abbreviations: *NAC4* = nascent polypeptide-associated complex, *BCAT2* = branched chain
930 amino acid transaminase 2, *AlaAT2* = alanine aminotransferase 2, *GOLS2* = galactinol synthase
931 2, *SDIR1* = Salt and Drought-Induced Ring Finger1, *P5CS* = delta 1-pyrroline-5-carboxylate
932 synthase.

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935 **Fig. 4** Correlations between model beta estimates from wilt VWC and wilt survival associations
936 with RNAseq. Each point represents estimates for one transcript. Transcripts with purple dots
937 have significant association of expression with both phenotypes, blue dots have significant
938 association of expression with wilt VWC, red dots with wilt survival and black dots with neither
939 phenotype.

940

941 **Fig. 5.** Candidate genes associated with wilt survival. Lower wilt VWC indicates greater
942 dehydration avoidance while higher wilt survival indicates greater dehydration tolerance. Points
943 and boxplots are colored by latitude and range: low latitude Europe = blue, high latitude Europe
944 = red, low latitude North America = purple, High Latitude North American = orange. Each point
945 represents a different individual. Gene Abbreviations: *DeGP5* = degradation of periplasmic
946 proteins 5; *CUT1* = Cutinase 1; *SAPK2* = sucrose non-fermenting 1-related kinase 2.

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955 SUPPLEMENTAL INFORMATION

956 **Table S1**. Geographic and environmental characteristics of white clover populations.

957 **Table S2.** Mapping statistics from RNAseq experiment

958 **Table S3.** Univariate model results examining associations between drought strategy phenotypes

959 across ranges, latitudes, and treatments.

960 **Table S4.** Univariate model results examining associations between drought strategy phenotypes

961 with water-availability related variables of collection populations

962 **Table S5**. Summary of differentially expressed genes with associated modules and phenotypic

963 associations.

964 **Table S6.** Gene Ontology analysis of WGCNA modules

965 **Table S7.** Genes with variation in expression associated with wilt VWC or wilt survival

966 **Table S8.** Genes with significant range:phenotype interactions.

967 **Table S9.** Patterns of expression within haploblocks

968

969

970 **Fig. S1.** Map of sampling locations.

971 **Fig. S2.** Volumetric water content of pots within well-watered and dry down treatments

972 throughout the experiment.

973 **Fig. S3.** Relationships between drought strategy phenotypes and leaf number at the start of the

974 experiment.

975 **Fig. S4.** Associations between water-availability related variables of collection populations and

976 leaf number at the start of the experiment

977 **Fig. S5.** Associations between water-availability related variables of collection populations and

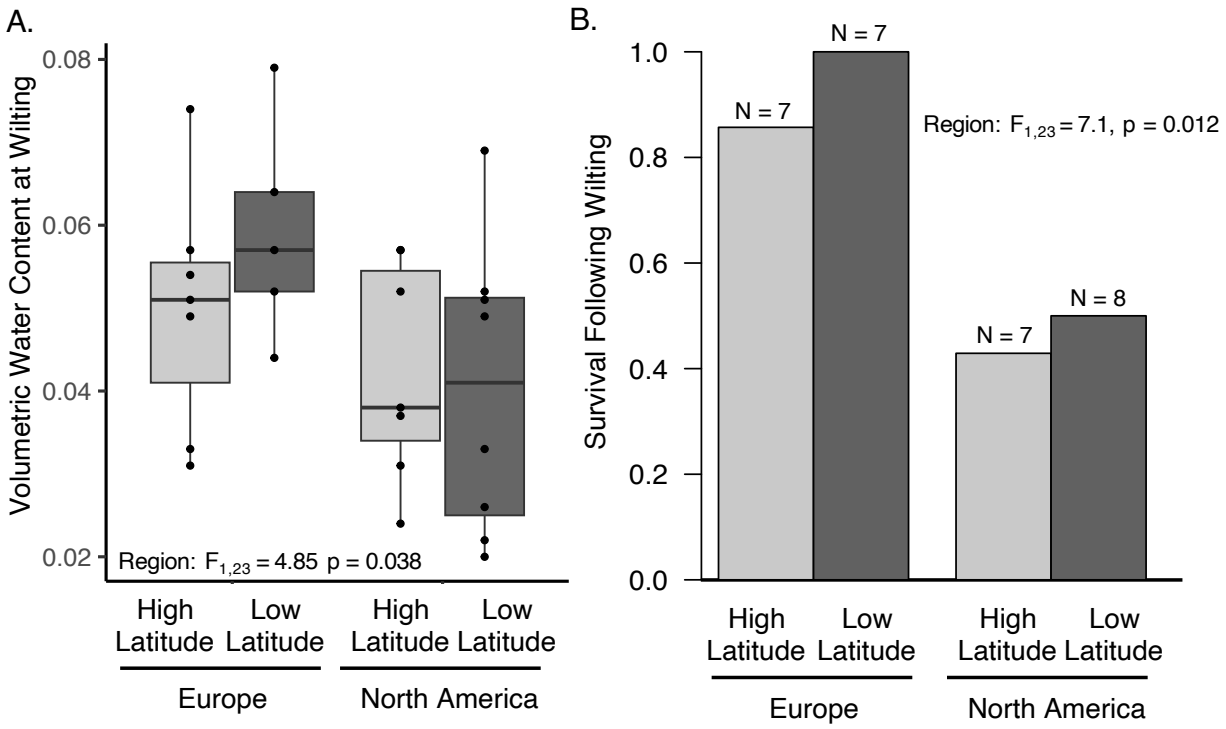
978 drought avoidance and tolerance.

979 **Fig. S6.** Venn diagram of differentially expressed genes across latitude, range and treatment

980 contrasts.

981 **Fig. S7**. Normalization expression profiles across samples.

982 **Fig. S8.** Relative expression across treatment, region, and latitude for candidate genes



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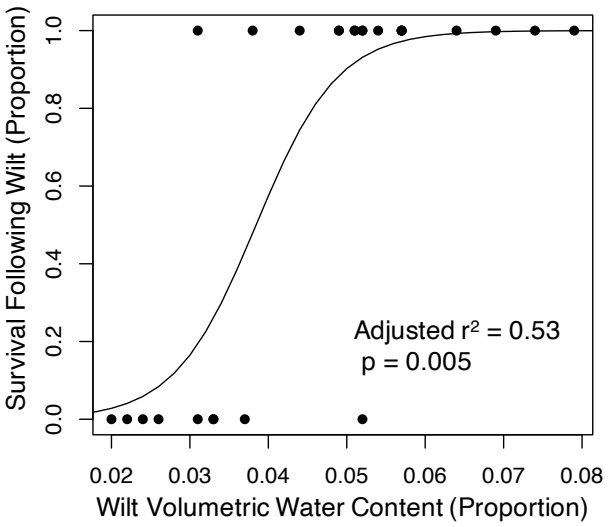

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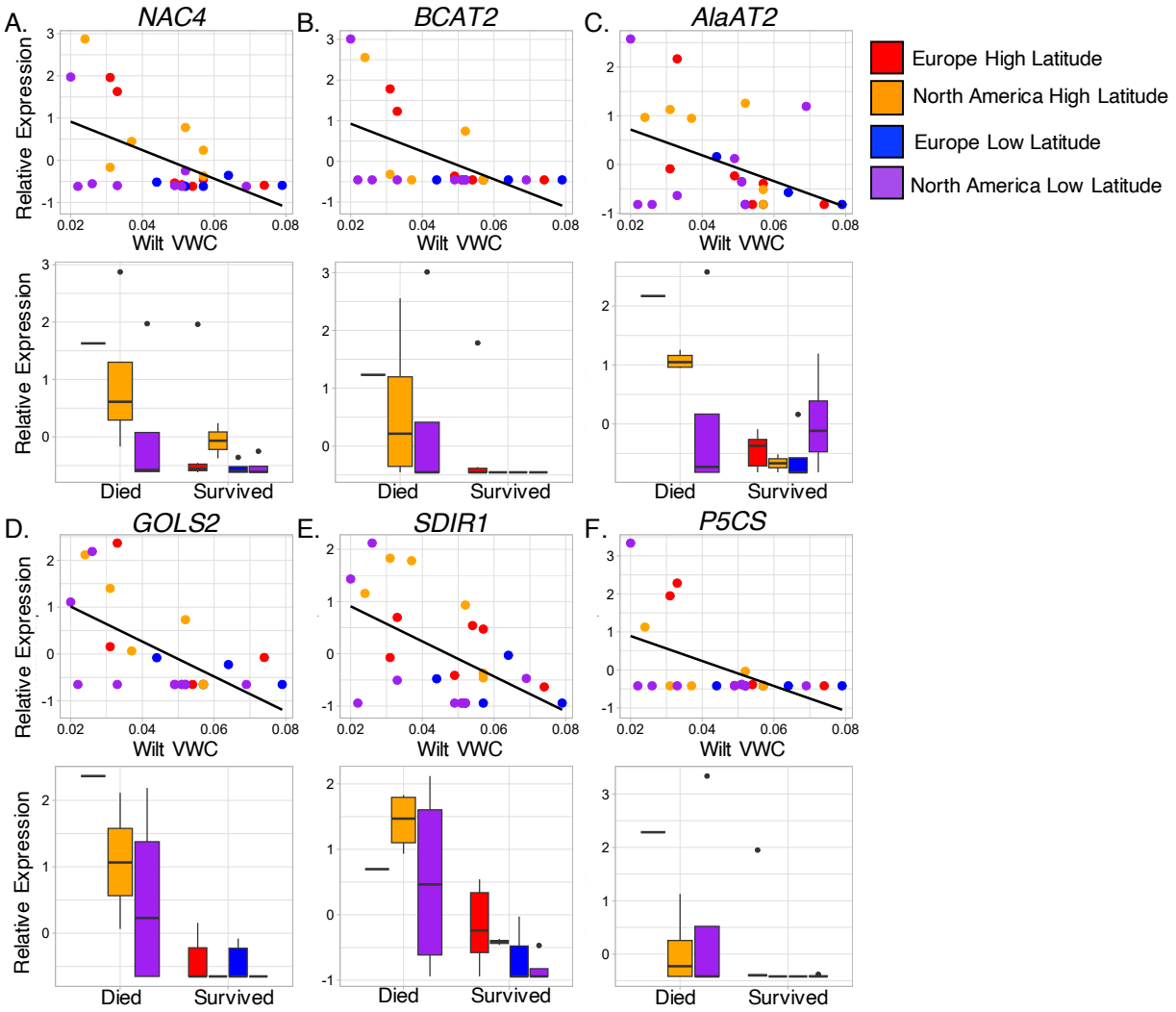
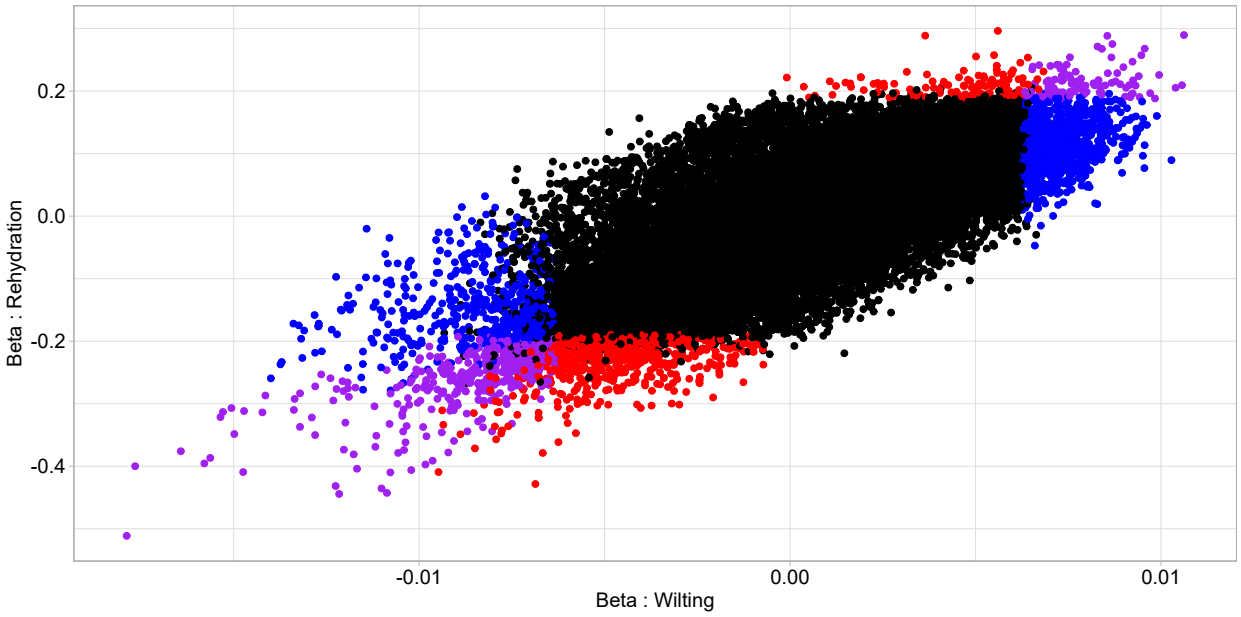


Fig. 3 Association between candidate gene expression and dehydration avoidance and tolerance phenotypes. Lower wilt VWC indicates greater dehydration avoidance while higher wilt survival indicating greater dehydration tolerance. Points and bar are colored by latitude and range: low latitude Europe = blue, high latitude Europe = red, low latitude North America = purple, High Latitude North American = orange. Each point represents a different individual. Gene Abbreviations: *NAC4* = nascent polypeptide-associated complex, *BCAT2* = branched chain amino acid transaminase 2, *AlaAT2* = alanine aminotransferase 2, *GOLS2* = galactinol synthase 2, *SDIR1* = Salt and Drought-Induced Ring Finger1, *P5CS* = delta 1-pyrroline-5-carboxylate synthase.



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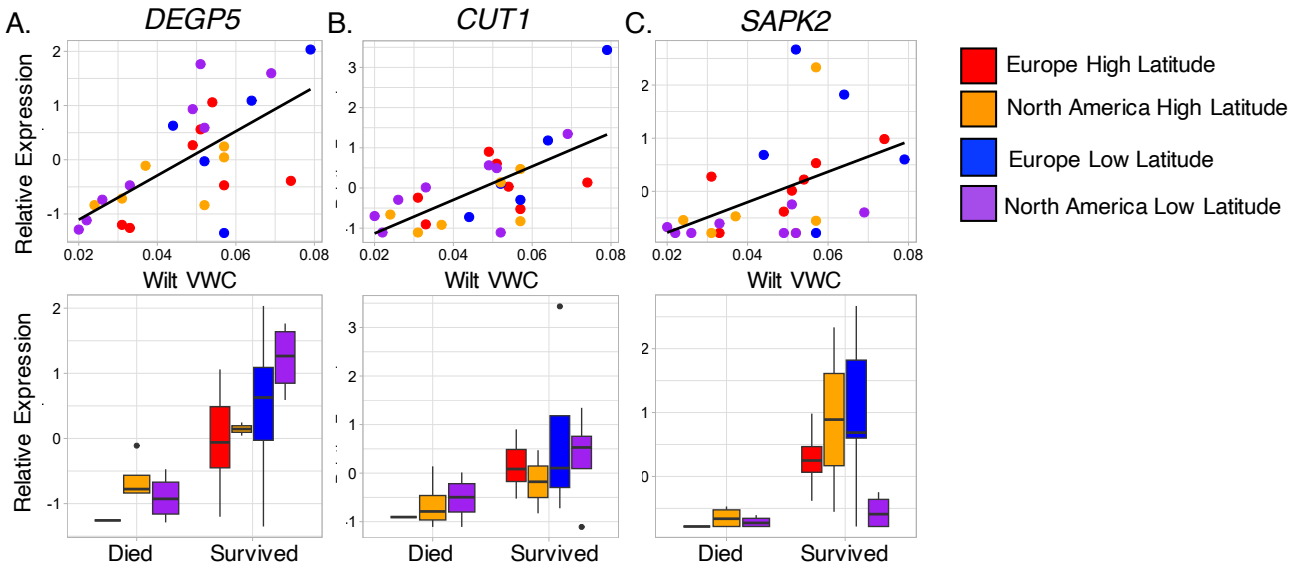

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