# 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 dehydration 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 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).

90 While drought strategies are not mutually exclusive, negative correlations between some
91 strategies are expected due to physiological constraints. For instance, the rapid growth expected
92 for drought escape requires opening stomata in order to increase photosynthetic rate (Geber and
93 Dawson 1990; Dudley 1996). However, open stomata increase transpiration and lower water use
94 efficiency – effectively neutralizing any dehydration avoidance strategy. Yet, the evidence for
95 this tradeoff is equivocal and seemly context dependent (McKay *et al.* 2003; Kooyers *et al.*
96 2015). Correlations between dehydration avoidance and tolerance strategies are less explored,
97 and are often viewed as compatible responses (Volaire 2018). However, resource allocation
98 tradeoffs could be hypothesized as the functions necessary for maintaining water balance in a
99 limited water environment are not the same resources necessary for surviving and recovering
100 from dehydration.
101
102 Although the molecular signals underlying the perception and integration of water stress are
103 becoming better resolved, variation in drought-related responses is rarely understood at the
104 molecular level (VanWallendael *et al.* 2019). For instance, it is largely unknown whether
105 variation in drought resistance arises through differences in sensitivity or perception of drought,
106 integration of signals, or re-wiring of connections within gene-regulatory networks. Molecular
107 responses to low water availability center around the activation of the abscisic acid (ABA)
108 dependent and ABA-independent signal transduction pathways in response to disruption in water
109 potential relative to the surrounding soil and/or atmosphere (Raghavendra *et al.* 2010; Dar *et al.*
110 2017; Aslam *et al.* 2022). Increases in ABA drive a signal cascade leading to guard cell
111 regulation and stomatal closure (Hsu *et al.* 2021). Notably, production of ABA also induces gene
112 regulatory cascades that lead to increases in antioxidants and osmoprotectants such as proline,

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

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

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

227

228 **RNAseq Analysis**

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

245

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

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

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

296 covariate. Significance was then tested using ANOVA as above. To determine the set of
297 phenotypically significant genes that are co-expressed, genes significantly associated with each
298 phenotype were compared to the previously calculated WGCNA network. We also determined
299 the location of these genes within the white clover genome (Santangelo *et al.* 2023) via *blastn*
300 (Altschul *et al.* 1990).
301
302 Finally, we then determined which of the genes associated with phenotype also were
303 differentially expressed between ranges or across latitudes to identify genes likely to be involved
304 in adaptation following introduction or to divergent climatic conditions. We conducted GLMs
305 modeling relative expression as a function of traits and either range or latitude. Specifically
306 relative expression was the response variable while trait value (wilt VWC or wilt survival),
307 spatial group (range or latitude), and the interaction between trait:group were included as
308 predictors. Genes with relative expression that is explained by trait:group interaction are good
309 candidates for producing the phenotypic variation observed in nature.
310
311 RESULTS
312 There was substantial variation in dehydration avoidance and tolerance throughout the native and
313 introduced ranges of white clover. Wilt VWC was lower in North American populations than in
314 European populations ($F_{1,23} = 4.8$, $p = 0.03$; Fig. 1A) indicating greater drought avoidance in
315 introduced populations. However, the effect size of this difference was low as the difference in
316 mean wilt VWC was only 1.3% lower water content at wilting in the North American
317 populations. There were no differences among low and high latitude populations or any
318 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***

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

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

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

411

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

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

430

431 DISCUSSION

432 During invasions, plants encounter novel environments and may need to acclimate or adapt to
433 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

lower volumetric water contents than populations from the native European range, but also are less able to tolerate wilting than native populations (Fig. 1). Thus, North American populations are likely more dehydration avoidant than European populations. Greater dehydration avoidance may allow plants to maintain growth during periods of low water and high evapotranspiration. This would be consistent with devoting more resources to rapid growth within the introduced range as well as being more resistant to initial water stress. This nuance may account for some of the mixed evidence for the EICA within the literature (Rotter and Holeski 2018).

We suggest that the evolution of greater stress avoidance to facilitate rapid growth may be a pattern frequently observed across invasions of perennial plants. Introduced species are likely to encounter different abiotic regimes in their new range. Invasive populations of annual plants often evolve more rapid life histories (Colautti and Barrett 2013; Battlay *et al.* 2023), but perennial plants must survive stresses in order to establish and reproduce. There are few plant systems where the evolution of drought avoidance or tolerance have been assessed across native and introduced regions. These examples largely consist of species where it is clear that they have a very different drought resistance strategy in the introduced range. For instance, camphorweed (*Heteroteca subaxcillaris*) has drastically greater root growth in its invasive range in Israel than in its native range, North America, that likely is a benefit both for low water availability and structural integrity in extremely arid dune environments in Israel (Sternberg 2016). Jerusalem artichokes (*Helianthus tuberous*) have evolved extreme clonality phenotypes in their introduced range (Europe) to accommodate a shift to a more aquatic environment (Bock *et al.* 2018). Outside of plants, a greater tolerance or plasticity to stressful environments is often observed – for instance, anoles (*Anolis cristatellus)* from the introduced range survive colder temperatures

480 than from the native range (Leal and Gunderson 2012). We suggest that invasive species should
481 be more widely used for identifying natural phenotypic and genomic variation in drought
482 resistance.
483
484 While our results are consistent with classic invasion biology theory, they also make sense in the
485 context of environmental characteristics of the populations. The populations involved in this
486 experiment have very different growing seasons and historical climatic features (Table S1).
487 Southern European populations come from a Mediterranean climate with cool and temperate
488 winters and hot dry summers. Relative to other locations, there is little precipitation throughout
489 the year and almost no rainfall during summer months. A long summer with little rain is the ideal
490 conditions for the evolution of dehydration tolerance in a perennial plant that likely needs to
491 survive across multiple growing seasons (Kooyers 2015). Alternatively, eastern North America
492 has more substantial rainfall throughout the growing season with drought stress likely coming
493 during shorter stretches without rain (Table S1). These conditions may favor the evolution of an
494 avoidance strategy as additional rainfall is likely coming in the near future. Interestingly, we
495 observe very little difference in drought resistance strategy between high and low latitude
496 populations of white clover in the native or introduced ranges. In both ranges, on average, lower
497 latitude populations are less dehydration avoidant but more dehydration tolerant. This is
498 surprising as previous work documents differentiation in life history strategy evolving across this
499 gradient with warmer southern populations employing a more annualized life history (Wright *et*
500 *al.* 2021). We suggest our small sample size only has the power to detect major effects and future
501 larger studies may be able to parse differences in stress responses across latitudinal gradients.
502

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

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

514

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

525 greater mortality following wilting than wild type plants. This suggests an additional dehydration
526 tolerance role for SDIR1 that we do not observe.
527
528 Expression of multiple genes that promote the synthesis of osmoprotectants (i.e. branched chain
529 amino acids or BCAAs, proline, and raffinose family oligosaccharides) are also strongly
530 associated with lower wilt VWC. *BCAT2* (BCAA transaminase 2) catalyzes the final
531 transamination step in production of BCAAs as well as the first degradation step. Under stress,
532 BCAAs accumulate as osmoprotectants in an ABA-dependent manner, with BCAT serving as
533 the main regulator in *Arabidopsis*. P5CS, delta 1-pyrroline-5-carboxylate synthase, controls the
534 rate-limiting step of proline biosynthesis from glutamate during osmotic and salt stress (Strizhov
535 *et al.* 1997). Transgenic overexpression of P5CS consistently increases tolerance to drought and
536 salt stress across several plant species by increasing proline content (Su and Wu 2004; Yamchi *et*
537 *al.* 2007; Vendruscolo *et al.* 2007). *GOLS2* (galactinol synthase 2) encodes galactinol synthase 2,
538 a key enzyme that regulates the first step in biosynthesis of raffinose family oligosaccharides
539 resulting in the creation of galactinol (Panikulangara *et al.* 2004). While the oligosaccharides act
540 as osmoprotectants and as antioxidants, both galactinol and the oligosaccharides may also act as
541 signaling molecules (Song *et al.* 2016). In other species, GOLS activity is triggered by abiotic
542 and biotic stress (*Arabidopsis*, Coffee, Rice) and confers drought tolerance (Dos Santos and
543 Vieira 2020). Unexpectedly, our results indicate that expression of *GOLS2* is linked with lower
544 wilt VWC (greater dehydration avoidance) and with lower survival following wilting (lower
545 dehydration tolerance). Increased expression of *GOLS2* in *Arabidopsis* does reduce transpiration
546 rate (Taji *et al.* 2002).
547

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

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

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

617 ROS production in tobacco (Chen *et al.* 2023). Expression of this gene may represent a true
618 tradeoff between different physiological strategies. Generally, our results suggest that a
619 dehydration tolerance strategy actually represents multiple different types of tolerance – one
620 strategy that provides resistance against dehydration and another that allows longer-term survival
621 following wilting (i.e. extreme dehydration). We hypothesize that dehydration tolerance related
622 to drought survival may have overlapping physiology with desiccation tolerance strategies
623 occurring in plants like resurrection fern. In sum, connecting variation between physiological
624 processes and plant performance will provide additional insight into the evolution of different
625 ecological strategies in specific environments.
626
627 **Loci underlying drought response adaptation following introduction**
628 Identifying the causative molecular variants that underlie natural intraspecific variation in
629 drought responses has proven challenging with limited of successes (Juenger 2013;
630 VanWallendael *et al.* 2019). This objective is largely beyond the scope of our study. We do
631 identify many genes that are both linked with wilt VWC or wilt survival phenotypes and also
632 show variable expression across ranges, conditions and latitudes. This overlap includes a
633 substantial portion of the turquoise gene co-expression network, suggesting that variation in
634 dehydration avoidance and tolerance phenotypes is physiological manifested through broad shifts
635 across gene expression networks (e.g. Lovell *et al.*, 2018) rather than through simple, large effect
636 variants that directly cause lower stomatal conductance or greater osmoprotection (e.g. Kesari *et*
637 *al.*, 2012; Des Marais *et al.*, 2014).
638

639 Recent work has identified several haploblocks that underlie climate adaptation following
640 introduction to North America as well as several candidate genes within haploblocks that were
641 differentially expressed across drought treatment, range and latitude (Battlay *et al.* 2024). Within
642 the three haploblocks on Chr04_Occ (syn. Chromosome 7), 26 differentially expressed
643 transcripts were found (9 on hb7a1; 14 on hb7a2; and 3 hb7b, Table S9). On hb7a1, 4 genes
644 within the turquoise submodule were differentially expressed. Two turquoise submodule genes,
645 *P5CS1 and FER1*, were strongly correlated with wilt VWC and wilt survival. In addition,
646 *BRR2A* and *DWF5* were correlated significantly with wilt survival whereas *IAA6* and *DWF5*
647 were correlated significantly with wilt VWC. On hb7b, transcripts belonging to the gene *CCL12*
648 are significantly correlated with wilting. Multiple of these genes have allele variation associated
649 with higher fitness in North American common gardens (Albano et al. in prep; Battlay *et al.*,
650 2024). These results are a first step to tying locally adaptive genetic variation to variation in gene
651 regulatory networks, physiology and ecological strategies.
652
653 *Conclusions*
654 Our work suggests that selection acts on rapid timescales following introduction to allow more
655 precise adaptation of drought responses to divergent environmental conditions. Specifically,
656 selection favors specific drought resistance strategies, with tradeoffs occurring between
657 strategies at both phenotypic and molecular levels. We identify a co-expression network that
658 underlies both dehydration avoidance and some elements of dehydration tolerance where many
659 of these loci also exhibit differential expression patterns between the native and introduced
660 ranges of white clover. We also identify tradeoffs at the molecular level among functions
661 (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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900 reforestation. *Agriculture, Ecosystems & Environment* **126**: 67–80.

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903 TABLES

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

905

| 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) |

906

907 Number outside of parentheses refers to the number of transcripts that are associated with the

908 phenotype or differentially expressed across Latitude or Range contrasts. The number within the

909 parentheses refers to gene homologues that are associated with the phenotype or differentially

910 expressed across Latitude or Range contrasts.

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923 FIGURE LEGENDS

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

931

932 **Fig. 2** Phenotypic tradeoff between dehydration avoidance and tolerance. Lower wilt VWC
933 indicates greater dehydration avoidance while higher wilt survival indicating greater dehydration
934 tolerance.

935

936 **Fig. 3** Association between candidate gene expression and dehydration avoidance and tolerance
937 phenotypes. Lower wilt VWC indicates greater dehydration avoidance while higher wilt survival
938 indicating greater dehydration tolerance. Points and bar are colored by latitude and range: low
939 latitude Europe = blue, high latitude Europe = red, low latitude North America = purple, High
940 Latitude North American = orange. Each point represents a different individual. Gene
941 Abbreviations: *NAC4* = nascent polypeptide-associated complex, *BCAT2* = branched chain
942 amino acid transaminase 2, *AlaAT2* = alanine aminotransferase 2, *GOLS2* = galactinol synthase
943 2, *SDIR1* = Salt and Drought-Induced Ring Finger1, *P5CS* = delta 1-pyrroline-5-carboxylate
944 synthase.

945

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

**Fig. 5.** Candidate genes associated with wilt survival. Lower wilt VWC indicates greater dehydration avoidance while higher wilt survival indicates greater dehydration tolerance. Points and boxplots 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: *DeGP5* = degradation of periplasmic proteins 5; *CUT1* = Cutinase 1; *SAPK2* = sucrose non-fermenting 1-related kinase 2.

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967 SUPPLEMENTAL INFORMATION

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

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

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

971 across ranges, latitudes, and treatments.

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

973 with water-availability related variables of collection populations

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

975 associations.

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

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

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

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

980

981

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

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

984 throughout the experiment.

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

986 experiment.

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

988 leaf number at the start of the experiment

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

990 drought avoidance and tolerance.

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

992 contrasts.

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

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

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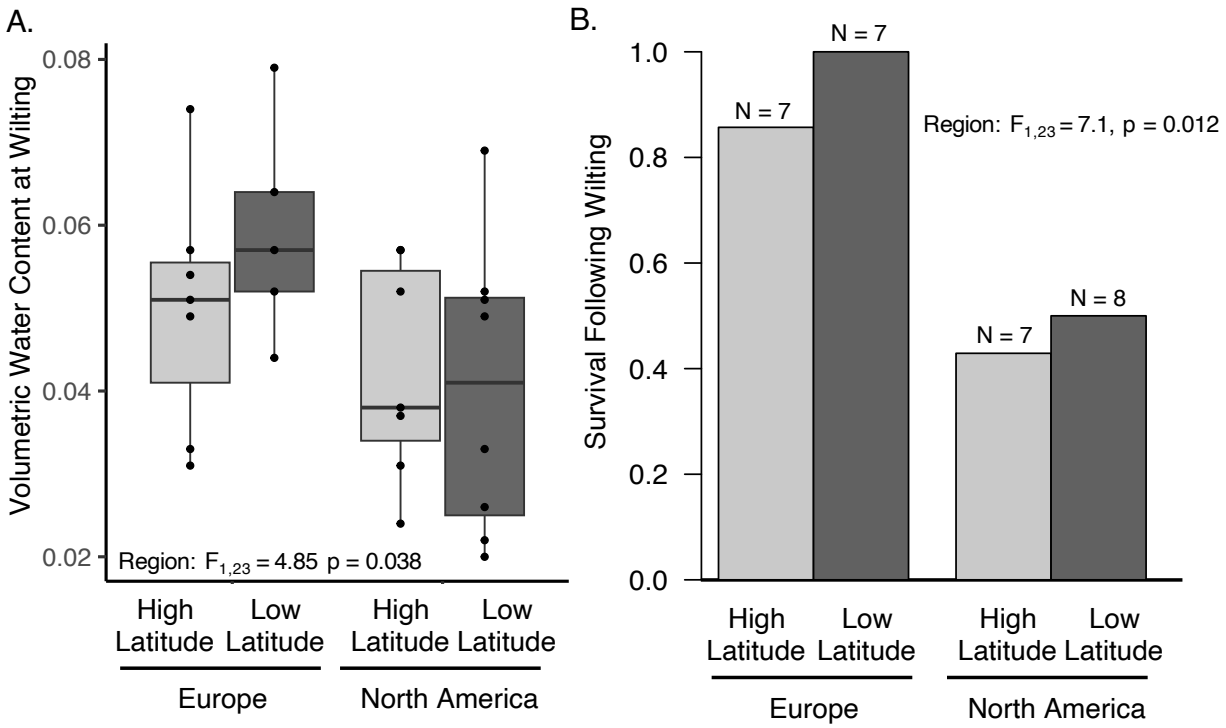


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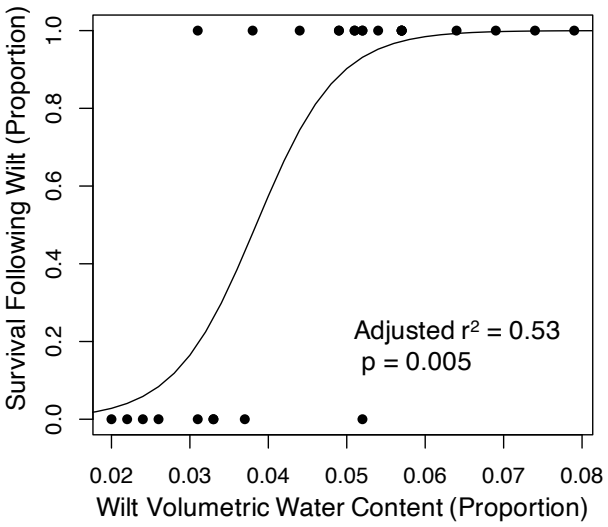


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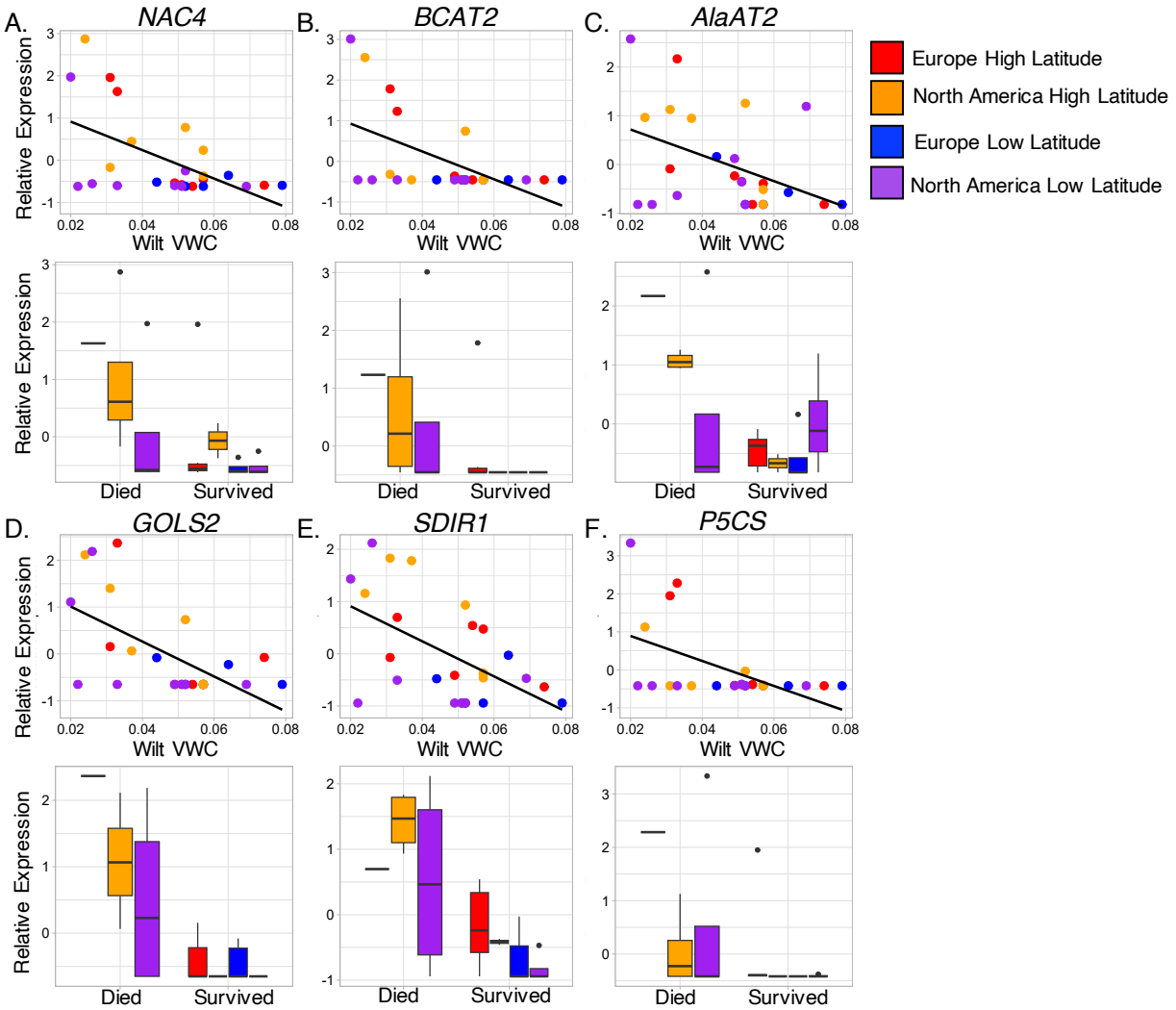


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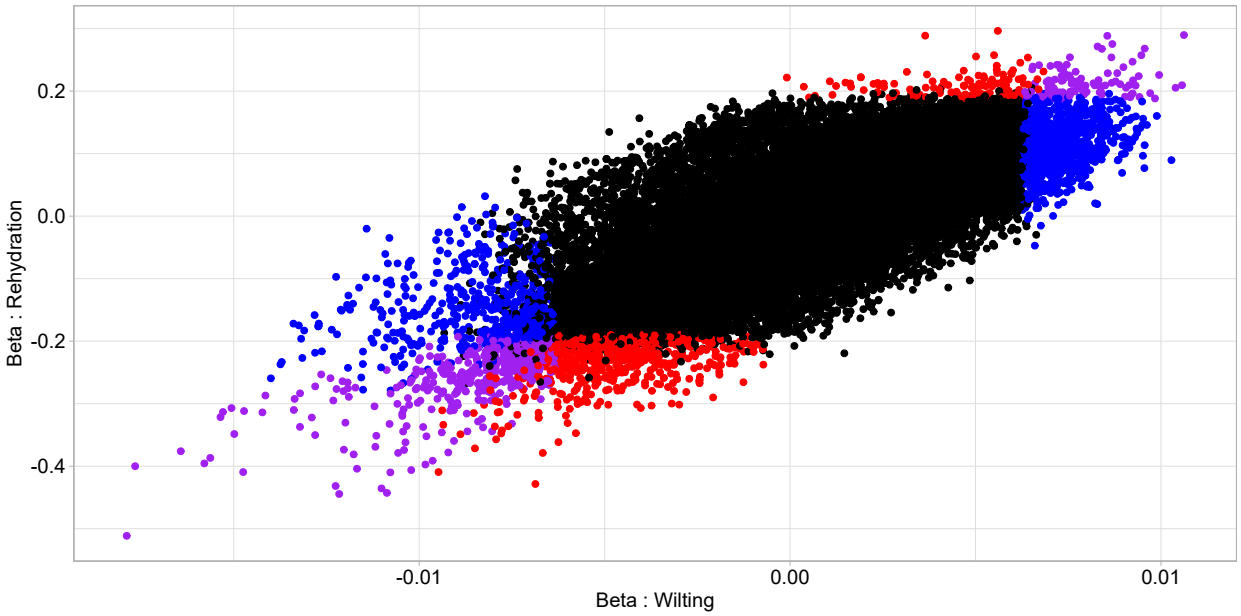


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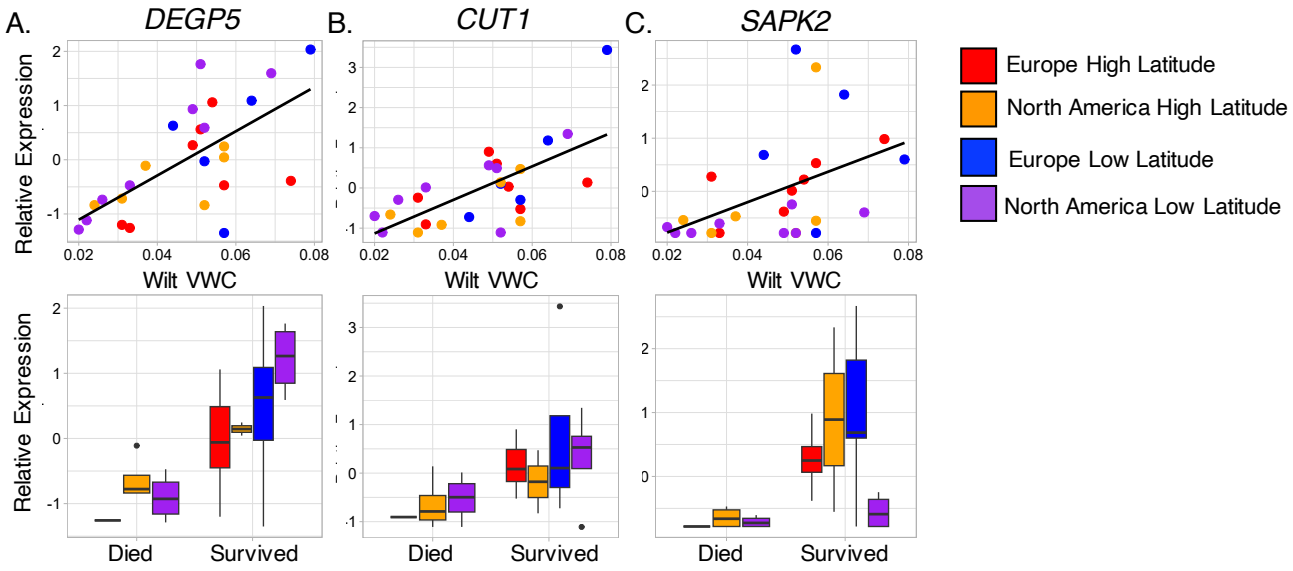


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