Plasticity, Pleiotropy and Fitness Tradeoffs in Arabidopsis Genotypes with Different Telomere Lengths

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PLASTICITY, PLEIOTROPY AND FITNESS TRADEOFFS IN ARABIDOPSIS GENOTYPES WITH DIFFERENT TELOMERE LENGTHS

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Running Title: Telomere length affects plant life history tradeoffs

ABSTRACT

Telomere length has been implicated in the organismal response to stress, but the underlying mechanisms are unknown. Here we examine the impact of telomere length changes on *Arabidopsis thaliana* responses to three contrasting abiotic environments, and measure 31 fitness, development, physiology and leaf-level anatomy traits. We report that telomere length in wild type and short telomere mutants is resistant to abiotic stress, while elongated telomeres in *ku70* mutants are more plastic. We also detect significant pleiotropic effects of telomere length on flowering time and key leaf physiology and anatomical traits. Furthermore, our data reveal a significant genotype by environment (GxE) interaction for reproductive fitness, with the benefits and costs to performance depending on the growth conditions. These results imply that life-history tradeoffs between flowering time and reproductive fitness are impacted by telomere length variation. We postulate that telomere length in plants is subject to natural selection imposed by different environments.

INTRODUCTION

Telomeres are evolutionarily conserved protein-DNA complexes at the physical ends of linear eukaryotic chromosomes. Telomeric DNA consists of multiple copies of short G-rich repeats, TTAGGG in vertebrates and TTTAGGG in most plants. In the absence of telomerase, the end replication problem causes telomeres to shorten with each somatic cell division, eventually leading to cellular senescence or death[1]. Thus, telomere length is viewed as an accurate cellular marker of biological age, and its improper maintenance has been linked to cancer and human aging-associated diseases[2].

Telomere length is highly dynamic, but the opposing actions of telomerase and the end replication problem ultimately establish a species-specific telomere length set point, which may reflect a fine compromise between potential positive gains in organismal survival and associated negative energy costs[3]. For instance, telomerase inhibition in human somatic cells is a known defense mechanism against tumorigenesis, but as a consequence it eventually leads to cell senescence and death[4]. In contrast, mice do not regulate telomerase in somatic tissues, and develop cancer more often[5]. These findings suggest that telomere length is under stabilizing selection with intermediate lengths favored. Average telomere length also differs considerably between genotypes of the same species[6-9]. Hence, telomere length is a quantitative trait, controlled by many genes[10-13] and possibly influenced by the environment. Intra-species variation in telomere length is consistent with ongoing evolution, but the degree to which this evolution is neutral or adaptive is unknown.

Telomere ecology, the study of telomere dynamics in an ecological context, is an emerging field that seeks to elucidate evolution of fitness trade-offs as they relate to telomere length[14-16]. Some of the initial work was conducted in birds. Such studies indicated that 3-year survival of *Tachycineta bicolor* increased in individuals with longer telomeres compared to those with shorter telomeres[17]. In *Sternia hirundo,* telomere length serves as a biomarker of reproductive success[18], while in *Acrocephalus arundinaceus* malaria infection correlates with a long-term degradation of telomere length, shortened lifespan and decreased reproductive success[19]. Despite these intriguing observations, little is known about the interplay between telomere length control and organismal fitness under environmental change.

Studies manipulating environmental conditions simultaneously with telomere length could provide new insight into the adaptive role of telomeres. The model plant *Arabidopsis thaliana*

provides a valuable system for examining the influence of environmental conditions on telomere biology, organismal fitness and plant physiology. Several *A. thaliana* mutants with well-defined positive or negative changes in telomere length have been described. For example, in plants deficient in the Ku heterodimer (Ku70/Ku80) blunt-end telomeres are deprotected, allowing telomerase unfettered access[20]. As a consequence, telomeres expand several fold[21,22]. In contrast, plants lacking functional TERT, the catalytic subunit of telomerase, undergo progressive shortening at a rate of up to 500 bp per plant generation, ultimately triggering massive genome instability and developmental arrest[23]. Mutations in two other genes cause telomeres to reach a shorter than in wild type, but stable set point length. One is NOP2A, a putative ribosomal RNA maturation and ribosome assembly factor, which was recently identified through QTL mapping as a positive regulator of telomere length[10]. A second gene, *TAD3*, encodes a tRNA adenosine deaminase and promotes telomerase-independent telomere length maintenance[24].

We previously reported that natural variation in telomere length set point in rice, maize and *A. thaliana* genotypes affects flowering time, a key aspect of life-history strategy[25]. Here we explore the interplay between the genetic and environmental factors driving telomere biology in Arabidopsis by evaluating telomere length plasticity and pleiotropy in five genotypes grown under varying environmental conditions. We provide evidence that genotypes with distinct telomere lengths exhibit differential phenotypic responses under changing abiotic conditions, implying that mutations affecting telomere length homeostasis also impact other plant traits and, thus, show pleiotropic effects. Our experiments also reveal interesting GxE interactions for telomere length and several major plant phenotypes, suggesting that natural telomere length variation in plants could be under environment-driven selection pressures.

RESULTS

Experimental setup

To explore the relationship between telomere length and life history strategies, we experimentally manipulated telomere length in *A. thaliana* genotypes and measured the impact of these changes on fitness, development, physiology and leaf-level anatomy under varying environmental conditions. We analyzed five *Arabidopsis thaliana* wild type and mutant genotypes with different telomere lengths: short (< 2 kb; *tert* (generation 5, G5) and *nop2a* mutants), medium (2-3 kb; wild type Col-0 accession and *tad3-2* mutant) and long (> 3 kb; *ku70* mutant) (Figure 1; Supplementary

Table 1). Telomeres in G5 *tert* mutants are not critically shortened[23], but since changes in plant physiology or gene expression can occur before telomere failure[24], we selected two genotypes each for short and medium telomere length categories to detect both genotype-specific and telomere length-specific patterns in plant traits. Ku mutants represent the only stable, long-telomere mutants described for Arabidopsis Col-0 background that do not have major genome abnormalities[21,22]. All selected single-copy homozygous T-DNA mutant lines with no background contamination were previously generated in the same reference Col-0 background and well-characterized[10,24,26,27].

We manipulated soil moisture content (wet and moderate dry) and ambient temperature (22ºC and 30ºC) in an experimental design consisting of three contrasting abiotic environments: wet|22C, mod|22C and wet|30C. We selected soil moisture levels and ambient temperatures that represent increasing levels of environmental pressure (from control wet|22C to moderate dry mod|22C to high temperature wet|30C) expected to generate detectable and differential changes in fitness and physiological response[28,29] without irreversibly impairing the plants (Supplementary Figure 1).

Global patterns suggest strong genotypic, treatment and genotype by environment (GxE) effects on many, but not all, trait groups

We measured 24 *raw* traits in all three environments. After removing eight raw traits that were used to generate additional 15 *composite* traits, a total of 31 traits covering six major phenotype groups defined as telomere length, phenology, morphology, fitness, leaf anatomy, and physiology were used for our statistical analyses (Supplemental Table 2). We performed a principal component analysis (PCA) on all traits to identify unique patterns and structures of individual genotypes and treatments in multivariate space. PC1 clearly partitioned the treatment effects separating the harsher wet|30C environment from the other two treatments and partially separated *ku70* and *tert* (genotypes with the longest and the shortest telomere lengths, respectively) from the other three genotypes (Supplemental Figure 2). Overall, the first two PC axes explain 42.5% of phenotypic variation, demonstrating that telomere length genotypes and the applied treatments are strong drivers of observed variation.

To explore the global responses of traits within each group, we constructed a series of eight separate multivariate analyses of variation (MANOVAs)[30], each targeting a specific group of traits (Supplemental Table 2). Our results indicate strong genotypic effects across all trait groups except

morphological features and final change in average telomere length, very strong treatment effects (plasticity) across all trait groups, notably except telomere length, and evidence for genotype by environment interactions among many trait groups (Table 1). To disentangle observed global patterns, we further analyzed individual traits within these groupings by exploring how telomere length variation in the Arabidopsis mutants pleiotropically impacts various trait sets.

Telomere length plasticity in response to environment is only observed in the long-telomere *ku70* mutants

Telomere lengths for three individual *Arabidopsis* chromosome arms (1L, 2R and 5L) were assayed before experimental treatments were initiated (To) and after target treatments were achieved (Tf) using the Primer Extension Telomere Repeat Amplification (PETRA) assay[31]. Similar to the STELA approach used for human cells[32], this PCR-based assay measures telomere length on individual chromosome arms and produces distinct bands (Supplemental Figure 3), as opposed to broad smears observed in Terminal Restriction Fragment (TRF) assays. As expected, significant differences in telomere length were observed between genotypes before (To; $F = 19.02$, $P < 0.0001$) and after (Tf; $F = 23.32$, $P < 0.0001$) treatments (Table 1). We estimated the change in telomere length over time for individual chromosome arms (Tf - To) and in aggregate across all three arms (AvgTf - AvgTo). Telomere length tended to slightly shorten over time irrespective of treatment or genotype when considered in aggregate (AvgTf - AvgTo; Figure 2A), despite some stochastic variation for individual chromosome arms (Supplemental Figure 4). Hence, regardless of the environment, telomere length in somatic tissues (leaves) generally shortens over the plant life span.

We next analyzed all genotypes for telomere length plasticity (changes in telomere length in response to the environment) over time (Tf - To) in different treatments. We also explored interactions between genotypes and treatment (GxE) using MANOVAs that consider all tested chromosome arms simultaneously (Table 1). Not surprisingly, we found no evidence for plasticity prior to experimental treatments (To; no significant Treatment and Genotype x Treatment effects). Interestingly, while we did not observe a direct treatment effect on telomere length after treatments were achieved (Tf), we did detect significant GxE for Tf ($F = 2.37$, $P < 0.05$) (Table 1). This effect appears to be driven almost exclusively by *ku70* mutants experiencing a relatively large telomere length decrease of ~340 (±70) bp in the mod|22C environment (Figure 2A), suggesting that dryer

soils may accelerate telomere shortening for genotypes with initially longer telomeres. However, this GxE pattern of length plasticity for aggregate telomeres in *ku70* mutants disappears when considering all genotypes and chromosome arms separately using ANOVAs (Table 2), and results in non-significant treatment (F = 0.31) and GxE (F = 1.63) effects when assessing the average change in telomere length over time (AvgTf - AvgTo) using MANOVAs (Table 1). Overall, our data provide some evidence for telomere length plasticity in the *ku70* genotype with overly long telomeres, but not in genotypes with medium or short telomeres.

Telomere length pleiotropically affects plant development, phenology, leaf physiology and anatomy.

To more fully characterize the response of Arabidopsis genotypes with different telomere lengths to changing abiotic environments, we assessed several phenological traits. We observed indications of genotypic variation in Flower day ($F = 13.62$, $P < 0.0001$), Primary ($F = 4.51$, $P < 0.01$) and Secondary inflorescence bolts ($F = 10.37$, $P < 0.0001$), Height ($F = 3.41$, $P < 0.05$) and Bolt thickness ($F = 4.83$, $P < 0.01$) (Table 3). Flowering time phenotypes were particularly striking, with the long telomere *ku70* mutants being the most divergent from all other genotypes. On average, *ku70* mutants began flowering 3-4 days later than wild type across treatments (Figure 2B). Additionally, flowering was delayed in all other mutant genotypes by 1-2 days in the wet|30C environment compared to the Col-0 wild type. These data complement our recent findings on the impact of natural telomere length variation on flowering time in three different plant species[25].

Environmental conditions associated with drought and high heat stress are known to induce substantial changes in plant physiology, including shifts in leaf osmotic potential, proline concentration, chlorophyll and relative water content[29,33]. MANOVA analyses detected strong genotypic ($F = 9.02$, $P < 0.0001$), treatment ($F = 27.21$, $P < 0.0001$) and GxE ($F = 4.01$, $P < 0.01$) responses across these physiology traits (Table 1). As expected for plants grown in more stressful environments, the decrease in chlorophyll content was significant in mod|22C and wet|30C conditions for all genotypes, as compared to the control wet|22C environment (Supplemental Figure 5A and Table 3). Similarly, proline concentrations increased in all genotypes in response to mod|22C and wet|30C environments (Supplemental Figure 5B and Table 3).

Unexpectedly, plants showed a genotype-specific response for the two leaf-level water balance traits, relative water content (RWC) and osmotic potential (OP). In the control wet|22C conditions,

RWC was reduced in mutant lines with either short (*tert, nop2a*) or long (*ku70*) telomeres, but not in Col-0 and *tad3-2* genotypes with medium telomere length set point (Figure 2C). This decrease in RWC is largely driven by *tert* mutants, which have the shortest telomeres among the genotypes tested. A more diverse response was observed for osmotic potential (OP) values. In the control wet|22C environment, *tert* plants showed the lowest OP values and *ku70* mutants displayed the highest (Figure 2D).

Finally, we looked at several anatomical leaf traits. Analysis of treatment effect indicated that the environment had a substantial impact on five out of seven anatomical leaf traits (Table 3), confirmed by a MANOVA ($F = 5.22$, $P < 0.01$) (Table 1). For two of these traits we detected significant GxE interactions (Stomatal Density, Epidermal Cell Area) (Table 3), which was confirmed by a MANOVA ($F = 4.22$, $P < 0.01$). These GxE effects appear to result from nearly all genotypes exhibiting radically different phenotypes in the control wet|22C environments compared to mod|22C and wet|30C treatments. Taken together, these data indicate that changes in telomere length homeostasis lead to significant pleiotropic effects (*sensu lato*, the number of phenotypes impacted by a single gene knockout) in key plant development, phenology and leaf-level anatomy and physiology traits,

Telomere length impacts reproductive but not vegetative fitness parameters.

We next tested whether telomere length changes can impact above ground plant biomass and an estimate of total seed production, which we defined as parameters of vegetative and reproductive fitness, respectively. MANOVA results revealed a significant effect of genotype ($F = 5.46$, $P < 0.01$) and treatment ($F = 69.45$, $P < 0.0001$) on overall fitness, and a significant genotype by environment (GxE) interaction ($F = 9.08$, $P < 0.0001$) (Table 1). Interestingly, an examination of vegetative fitness (biomass) and reproductive fitness (total Seeds) separately revealed differential contributions of these two traits to overall fitness. While Biomass showed some variation across genotypes and treatments, an ANOVA analysis did not detect a significant biomass difference between genotypes across all 3 growth conditions ($F = 0.64$, $P = 0.79$) (Table 3). These findings suggest that telomere length, or the genes governing telomere length homeostasis, do not significantly affect overall vegetative fitness.

In contrast, analysis of the reproductive fitness parameter, total Seeds, revealed a markedly different profile. Under normal growth conditions (wet|22C), *ku70* and especially *tert* mutants

appeared as clear outliers on the opposite sides of the seed quantity spectrum (Figure 2E), with *tert* (shortest telomeres) producing the most seeds and *ku70* mutants (longest telomeres) producing the fewest. Additionally, seed numbers for most genotypes plummeted in the wet|30C environment, while *ku70* produced slightly more seeds relative to wet|22C (Figure 2E), resulting in a rank change and a significant GxE interaction for reproductive fitness ($F = 3.82$, $P < 0.0001$) (Tables 1, 3). Collectively, these findings indicate that plant genotypes with telomeres on the opposite ends of the length spectrum show significant variation in reproductive fitness.

As an additional measure of reproductive success, we explored seed quality by comparing germination efficiency of seeds produced by all genotypes grown in wet|22C and mod|22C. Because most seeds produced in wet|30C did not separate well from siliques, seeds from this treatment were omitted from the analysis. Overall, greater than 79% of all seeds germinated irrespective of the genotype or treatment they originated from (Supplemental Figure 6), and we did not detect significant genotypic ($F = 0.99$, $P = 0.44$), treatment ($F = 0$, $P = 0.99$) or GxE ($F = 0.49$, $P = 0.75$) effects. These results confirm that total seed count is an accurate and consistent proxy for reproductive fitness, and that telomere length mutants tested here do not have major defects in seed quality.

Evidence of GxE interactions leading to life history trade-offs

We discovered several instances of interactions among genotypes and treatment (GxE), implying that telomere length may differentially impact plant phenotypes depending on the environment (Table 1, Table 3). Of particular note is a GxE interaction for reproductive fitness (Table 3), driven by clear rank changes in seed set for genotypes with the most extreme telomere sizes, *tert* and *ku70* (Figure 2E). Overall, these GxE interactions indicate that telomere length variation can exact environment-dependent fitness costs. Thus, the natural genetic variation for telomere length previously observed in plants[25] may be maintained through varying selection pressures imposed by different environmental conditions.

Scanning across all measured phenotypes revealed interesting patterns that may partially account for the observed fitness GxE interactions. For example, in line with higher reproductive fitness, *ku70* and *tert* individuals had a higher stomatal index (SI) than all other genotypes in the wet|30C environment (Figure 2F), indicating that these genotypes develop a higher ratio of stomatal to epidermal cell density on their leaf surfaces. Given that stomata regulate transpiration

and by extension leaf cooling[34,35], higher SI in *ku70* and *tert* may enable plants to maintain slightly cooler leaves, conferring a physiological advantage in warmer temperatures. In support of SI as a driver of fitness differences, *Col-0* individuals exhibited lower SI in cooler and dryer mod|22C treatment (Figure 2F), where it is presumably beneficial to conserve water, potentially explaining why wild type outperformed most other genotypes in this environment (Figure 2E). In addition, in the wet|30C environment *ku70* individuals develop fewer epidermal cells (Supplemental Figure 5C), further contributing to a high SI, while *tert* individuals develop smaller epidermal cells overall (Supplemental Figure 5D), suggesting that other leaf anatomical features may also be contributing to an elevated fitness in this environment. Further experiments that explore transpiration, gas exchange and leaf temperature regulation may provide insight into how cell size and structure influence the physiological and fitness tradeoffs of these genotypes in varying environments.

Phenotypic response strength is proportional to telomere length divergence from wild type.

To further address correlations between telomere length and measured traits, phenotypic "response strength" was defined as the absolute (positive or negative) divergence from the *Col-0* wild type trait values and plotted for all genotypes arranged from the shortest (*tert*) to the longest (*ku70*) average telomere length (Figure 3; Supplemental Figure 7). For several traits, we detected a U-shape profile, with the shortest and the longest telomere genotypes having the most variation from the *Col-0* norm. For reproductive fitness (Seeds), this effect was most apparent and observed in all three environmental growth conditions (Figure 3A). For flowering time, relative water content RWC1 and osmotic potential OP (Figure 3 B-D) and for several other plant phenological traits (Supplemental Figure 7), the U-shape response was also observed in one or more conditions. Overall, the "divergence from the wild type" analysis demonstrated a strong correlation between the extent of telomere length change and the level of measured phenotypic response, with trait variation generally greater in genotypes that differ the most from the *Col-0* wild type norm (*tert* and *ku70*). Furthermore, these phenomena span multiple biological levels, from plant morphology and physiology to development to fitness, and can be detected in one, two or all three environments. We conclude that genotypes with the highest degree of deviation from Col-0 telomere length set point in either direction show the largest effect on plant biology traits.

DISCUSSION

In humans, telomere length dynamics and plasticity in response to environmental or lifestyle factors has received substantial attention due to compelling connections to aging and disease[36]. Consistent with these findings, our data show that telomere length in plant somatic tissues (leaves) decreases slightly over time in all environmental conditions tested. Interestingly, most cells in mature leaves are non-cycling, and hence telomere attrition in leaves is not replication dependent and, possibly, stochastic in nature. In marked contrast to several animal studies[16,37], we found that Arabidopsis genotypes with short and medium telomere length displayed little evidence for telomere length plasticity in response to environmental conditions. However, the long-telomere *ku70* mutant emerged as an outlier, exhibiting some evidence of additional telomere shortening in response to the dry mod|22C environment. Abnormally long telomeres in Arabidopsis *ku70* mutants are inherently unstable and prone to trimming through a telomere rapid deletion-like mechanism[38]. Notably, abrupt telomere shortening in *A. thaliana* has also been reported for mutants of a nucleosome remodeler DDM1 and a chromosome end protection factor TEN1 in response to environmental and genome-wide stressors[39,40], suggesting that the plastic response observed in *ku70* mutants may be specific for genes involved in genome maintenance.

In humans and animals, short telomere length is indicative of poor biological state, higher disease risk, poor survival and low reproductive success[15,17,41,42]. Similarly, we observe major changes in plant physiology and fitness associated with short telomeres. Under the control wet|22C conditions, short telomere *tert* mutants showed significant changes in leaf physiology parameters, such as reduced leaf water content and osmotic potential, and yet surprisingly also displayed higher reproductive fitness. Since the *tert* mutants used in our study do not yet show major developmental or reproductive defects[23], our findings imply that telomere length status has a pleiotropic effect on many physiological and fitness parameters.

Less is known about fitness effects of abnormally long telomeres. Elongated telomeres in humans are rare and typically associated with cancer[43-45]. Studies in yeast revealed no fitness impacts of longer telomeres[46]. Similarly, longer telomeres in wild *Caenorhabditis elegans* isolates do not correlate with alterations in offspring production or longevity[9]. While we observed no substantial vegetative fitness (biomass) effects in long telomere Arabidopsis *ku70* mutants, we did detect significant and complex reproductive fitness associations for seed counts in these plants. *ku70* mutants set fewer seeds under the control wet|22C growth conditions than wild type or

293 mutants with shorter or medium telomeres, but unlike most other genotypes remained relatively
294 fertile in the most extreme wet|30C environment. These fitness effects were accompanied by
295 significant changes in plant physiology (higher osmotic potential), development (later flowering)
296 and other traits in all growth conditions. Thus, our analysis of *ku70* mutants represents the first
297 demonstration of significant effects of very long telomeres on fitness and physiology, indicating
298 that improper telomere elongation can indeed have both positive and negative consequences for
299 plant biology, depending on the environment.

300 Our analyses detected widespread pleiotropy and provided clear evidence that mutations
301 affecting telomere length genes also impact many other phenotypes. Signatures of functional
302 pleiotropy for telomere length genes have been described in different eukaryotes[8,10,47], and thus
303 appear to be evolutionarily conserved. The *TERT* gene is a classic example. This gene is essential
304 for telomere elongation, but it is also implicated in mitochondrial biology[48]. Similarly, Ku
305 functions in both DNA damage repair and telomere protection[20,49], while *NOP2A* impacts both cell
306 size and telomere length[10,50].

307 Despite evidence of multiple pleiotropic actions of telomere-related genes, it is unknown
308 whether such outcomes occur directly or indirectly, and function in linear or parallel fashions[51,52].
309 Several mechanisms can be envisioned. For example, telomere length impact on flowering time,
310 now detected in three plant species (this study; 25) could occur in a linear sequential fashion (gene
311 mutations > telomere length > flowering time) or in parallel modes (i.e. gene mutations > telomere
312 length, and independently gene mutations > flowering time). Both mechanisms are supported by
313 available literature. Human NOP2 and TERT have non-telomere functions in cell cycle control
314 through direct transcriptional activation of G1 phase cyclin D1 expression[53]. Similarly, the direct
315 pleiotropic effects of mutations in Dyskerin and NOP10 genes, which function in telomere biology
316 and ribosomal RNA maturation, could account for the remarkable similarities in human telomere
317 disorders and diseases of ribosome biogenesis[54]. Conversely, it is unlikely that hundreds of yeast
318 genes previously discovered in genetic telomere length screens all have a direct role in telomere
319 metabolism[8,47,55]. Hence, the question of causation needs to be carefully considered. While
320 telomere length could causally drive differences in performance, an alternative indirect mechanism
321 through pleiotropy is also plausible. A better understanding of the molecular mechanisms of
322 telomere length control is needed to disentangle the many different aspects of pleiotropy observed
323 in our study.

Our previous analysis of telomere length variation in 653 natural *A. thaliana* accessions indicated that genotypes with longer telomeres flower earlier[25]. These experiments provided preliminary evidence that telomere length may be an adaptive trait in plants and suggested an intriguing link between life history strategies and chromosome structure. Curiously, the data we present here are consistent with the opposite outcome – mutants with longer telomeres flower later. This contrast may reflect differences in mutant plants (this study) versus natural Arabidopsis accessions[25]. Analyzing a panel of flowering time mutants for variation in telomere length set point, essentially reversing this study, might shed light on the conundrum. Nevertheless, our new findings confirm the major role of telomere length homeostasis in plant life history strategies, including the developmental decision to flower earlier or later.

Our data are also consistent with a recent "pace-of-life" theory addressing the evolution of life history strategies as they relate to telomere length[56]. According to this hypothesis, shorter telomeres should lead to a faster pace-of-life strategy with less investment in self-maintenance as a means of conserving energy for reproduction. Inversely, longer telomeres would favor an increased investment in soma maintenance and late reproduction. Our findings support this model. Under optimal wet|22C conditions, the medium and short telomere genotypes flowered faster than the long-telomere *ku70* mutants, while producing significantly more seeds (especially the shortest telomere *tert* mutant). In contrast, long telomere *ku70* mutants favor a long-term strategy with a reduced investment in reproduction (less seeds under control wet|22C conditions), but an increased allocation of resources toward self-maintenance processes (late flowering).

Genotype by environment (GxE) interactions are hallmarks of species adaptations to specific environmental conditions and have been proposed to play a role in telomere biology[57]. We found significant telomere length GxE effects on many plant traits, including reproductive fitness. We postulate that the reproductive fitness GxE, and the relatively higher performance of *ku70* and *tert* in wet|30C, at least partially reflects changes in leaf stomatal index (SI) and other anatomical traits. Our data further suggest that the telomere length benefits in one environment through impact on specific phenotypes (e.g, SI) may come at a cost to performance in alternative environments resulting from their impact on other phenotypes. For example, the *ku70* mutants in the more stressful environment (i.e. wet|30C) produce more seeds than other genotypes in our study, suggesting that in this environment longer telomeres provide selective advantages. However, early

354 flowering is often favored by natural selection[58,59], which may explain why late-flowering *ku70*
355 plants exhibit lower fitness in the cooler mod|22C and wet|22C conditions.
356 Overall, our experimental findings provide intriguing evidence for the existence of life history
357 tradeoffs associated with a differential impact of telomere length variation (or the genetic variants
358 underlying telomere length) on leaf anatomy, physiology and reproductive fitness in different
359 environmental conditions. We identify heavy competitive costs between one such trade-off pair,
360 flowering time and the number of seeds, providing the first evidence that the maintenance of overly
361 long telomeres can indeed be advantageous or unfavorable, depending on the environment, and
362 substantially impact life history strategies.
363
364 **METHODS**
365 **Plant genotypes**
366 Seeds for *A. thaliana* accession Col-0 (CS6673) were obtained from ABRC. Arabidopsis mutant
367 lines *tad3-2* (SALK_121147), *tert-1* (5th generation), *oli2-2/nop2a-2* (SALK_129648; 3rd
368 generation) and *ku70-2* (SALK_123114, 2nd generation) were described previously[24,26,27,50]. All
369 mutant lines are in the Col-0 genetic background, and all seeds were bulked at the same time.
370
371 **Plant growth and Experimental conditions**
372 Seeds were cold-stratified in 1.5 mL microcentrifuge tubes at 4°C for 3 days, and
373 subsequently sowed on 1/2 MS medium plates and grown in a culture room at 22°C with 16 h
374 light/8 h dark photoperiod. On experimental day 00 (Supplemental Figure 1), 7-day old seedlings
375 were transplanted to soil (3:1 ratio of Pro-Mix BioFungicide with Profile Field and Fairway
376 Calcined Clay) in 9 cm^2 plastic pots (2 plants of the same genotype per pot) and grown in a plant
377 growth chamber (Conviron model #CO2-BP). Six biological replicates (individual pots with
378 plants) of all genotypes were used for each experimental condition. Pots with seedlings were
379 labeled with randomly assigned numbers and their positions in trays were randomized at the
380 beginning of the experiment. Pot positions in trays were re-randomized weekly to minimize spatial
381 effects within growth chambers. We manipulated both water availability and temperature for the
382 following three treatment conditions: Well-watered/ Normal Temperature (wet|22C), Water
383 limited/ Normal Temperature (mod|22C), and Well-watered/ High Temperature (wet|30C).
384 Conditions were as following: wet|22C (95% SWC, 22 °C); mod|22C (35% SWC, 22 °C); wet|30C

(95% SWC, 30°C).

Mod|22C *and* wet|22C *treatments*

Prior to the start of the experiment, each pot was filled with dry soil and weighed to obtain 0% soil water content (SWC) values, then left in standing water overnight to soak up 100% soil water content and weighed again. Based on the known weight values for 0% and 100% soil water content, 95% (wet|22C treatment) and 35% (mod|22C treatment) soil water content values were calculated, as described earlier[28]. After transferring plants to soil, pots were weighed daily to monitor evaporation and soil water content. All pots with plants in wet|22C and mod|22C conditions were allowed to reach 95% and 35% soil water content values, respectively, by evaporation, after which individual pots were watered daily as needed to maintain target weight throughout the rest of the experiment. All plants in dry conditions reached target 35% SWC in 23-28 days after they were moved from plates to soil. To independently confirm SWC values, we measured soil moisture twice throughout the experiment using a time-domain reflectometry (TDR) soil moisture probe (Campbell Scientific Hydrosense, Logan, USA).

Wet|30C *treatment*

It is known that growth at 30ºC does not kill Arabidopsis plants, but substantially stresses them, often leading to seed abortion or poor seed set[29]. The high temperature wet|30C treatment started at day 17 after seedlings were transferred to soil and was conducted in a dedicated Conviron CO2-BP growth chamber set at 30ºC but with otherwise identical settings as for the wet|22C experiment. To make sure that plants are stressed specifically by high temperature and not by substantial water loss due to increased transpiration at 30ºC, soil water content was kept at >80% by bottom-watering the plants daily. Under these conditions, many plants grown at 30ºC flowered and subsequently senesced earlier than their counterparts grown at 22ºC, thus the experiment was terminated one week earlier for this treatment.

Sample collection

Initially, each pot contained two plants of the same genotype. Leaves from the first plant were used throughout the experiment to obtain data for telomere length dynamics and to analyze physiological, biochemical and morphological parameters before and after treatment started (0-15

days after reaching target SWC values, depending on a trait). The second plant was kept until the end of the experiment (when plants showed clear characteristics of physiological death, such as leaf yellowing and death, flowering cessation, seed maturation in siliques). These plants were then used to evaluate morphology, development and fitness parameters, i.e. flowering time, dry weight (biomass), plant height, number of primary and secondary stems, number of leaves, number and length of siliques, number of seeds, etc.

Traits measured

Analysis of vegetative, anatomical and reproductive parameters of plant fitness.

Raw data for all measured traits are presented in Supplementary Data 1 file. We collected *Leaf count* as the total number of rosette leaves prior to bolting, as a measure of vegetative productivity. During the experiment we recorded the day of the first inflorescence bolt emergence (a minimum of 1 cm) as a measure of when plants shift into a reproductive phase, defined as *Flowering time*. Upon physiological aging of plants (leaf senescence, flowering cessation, seed drying), the experiments were terminated and the following parameters were recorded: *Height* of the tallest inflorescence branch, the *Number of primary bolts* and *Number of secondary bolts* as the number of inflorescence branches originating at the rosette, *Fruit number, Fruit length* as the mean of three randomly selected fully developed fruits per plant (taken from separate inflorescences, where possible). We collected 3 mature fruits from all plants in each treatment, and counted the *Number of seeds per fruit*. To estimate *Total seeds* as a reproductive Fitness proxy, we multiplied *Fruit number x Fruit length x Number of seeds per fruit* for every plant, as described previously[28]. To measure vegetative fitness, at the end of the experiment all above ground material was harvested, dried at 55°C for 4 days, and *DryWeight* was measured. *LeafThickness* and *StemThickness* measurements were performed following bolting time using digital calipers (99MAG011B measuring apparatus, Mitutoyo, Japan).

Leaf anatomy traits.

One randomly selected fully grown leaf of the same developmental stage was sampled to prepare slides for epidermis phenotyping. Clear nail polish was applied to the leaf surface and allowed to dry for 10 mins at room temperature. The dried nail polish area was peeled off with a clear tape and slides were then prepared for microscopy. Images to evaluate the number and size of stomata

and epidermal cells were captured with Nikon Eclipse Ni microscope equipped with a Nikon DS-Ri2 color camera at 20x magnification, and ImageJ software was used to analyze the images. We measured the length and width of one randomly selected stomata cell to estimate stomatal cell area. We also traced the largest epidermal cell from each leaf and used the FreeHandLine tool in ImageJ software to estimate epidermal cell area. We then counted every stomata and epidermal cell observed in the microscope field of view (FOV) and also measured the area of the total view of the microscope field. We calculated total stomatal cell and epidermal cell area by multiplying the total number of cells under a microscopic field by the single cell area. The ratio between stomatal to epidermal cell area was calculated by dividing total stomatal area by total epidermal cell area. The following measurements were used to calculate other leaf anatomy traits:

Stomatal density = number of stomata in entire FOV / area of total microscope field (μm^2);

Epidermal cell density = number of epidermal cells in entire FOV / area of total microscope field (μm^2)

Stomatal Index (%) = (Stomatal density)/(Stomatal density + Epidermal cell density) × 100.

Biochemical and physiological markers of stress response.

Leaf samples for chlorophyll, osmotic potential, proline and leaf water content were collected after plants reached their target SWC values on day 28. Relative Leaf Water content (*RWC)* analysis was performed as previously described[28]. One mature leaf was carefully removed from each plant, and its fresh weight was measured and recorded. The leaf was then placed in an Eppendorf tube filled with water in the dark and after 3 h turgid weight of the leaf was measured. The leaf was then dried in 55ºC for 3 days and dry weight was also recorded. Leaf water content is calculated according to the formula: (fresh weight - dry weight)/(turgid weight - dry weight).

Analysis of leaf osmotic potential was performed with an osmometer as described before[28,60]. Chlorophyll content and proline concentration analyses were performed as described[61,62]. In brief, we collected fully grown leaves (~50 mg) for both chlorophyll and proline assay. For chlorophyll assay, leaves were cut into small pieces, mixed with 3 ml of 80% acetone and kept in dark for 48 hrs. 1 ml of sap was then collected and placed in 96 well plate. Absorbances at 645 nm and 663 nm were measured for total chlorophyll estimation. Absorbance of 80% acetone was used as the blank control. For proline, ~50 mg of leaf tissue was homogenized in 1 mL of sulfosalicylic acid (3%) and centrifuged at 13,000 rpm for 15 min at 4ºC. 200 µl of solution was

transferred to a new tube with 200 µl of acid ninhydrin and 200 µl of acetic acid. The mixture was boiled for 30 min and cooled down at 4ºC for 30 min. 400 µl of toluene was added to the solution and thoroughly mixed by vortexing for 30 sec. Finally, 200 µl of the toluene phase was collected in a glass plated 96 well plate to record absorbance at 520 nm in a spectrophotometer (Beckman). Toluene was used for blank control.

Seed germination tests

Germination efficiency was tested using seeds collected from plant genotypes grown in mod|22C and wet|22C environmental conditions. The third treatment, wet|30C, resulted in seeds not separating well from siliques, and thus, these seeds were not evaluated for germination. Seeds were surface-sterilized by immersing in 70% ethanol for three minutes and 10% (v/v) sodium hypochlorite for 7 min, and then rinsing five times with sterile distilled water. Seeds were spread on standard MS plates and counted immediately. Germinated seedlings were counted on the 14th day after plating. Germination efficiency was calculated using the equation:

Germination Rate = (number of seedlings/number of seeds) x 100.

Telomere length measurements.

To initially evaluate bulk telomere length in each genotype, TRF assay was performed with genomic DNA digested with Tru1I (Fermentas, Hanover, MD) restriction enzyme. ^{32}P 5'-end-labeled $(T_3AG_3)_4$ oligonucleotide was used as a probe (Fitzgerald et al. 1999). Radioactive signals were scanned by a Pharos FX Plus Molecular Imager (Bio-Rad Laboratories), and the data were analyzed by Quantity One v.4.6.5 software (Bio-Rad). Mean telomere length (mean TRF) was calculated using the TeloTool program[63]. To evaluate telomere length values for leaf DNA samples before and after each experimental treatment, we performed the PCR-based PETRA (primer extension telomere repeat amplification) assay, which allows precise telomere length measurement on individual chromosome arms in *A. thaliana*[31]. Telomere length for each chromosome arm tested (5R, 1L and 2R) was calculated using the TeloTool program[63] and used to evaluate the effects of abiotic environments on telomere dynamics.

Missing Data and Imputation

Given the large number of traits measured in this experiment (24 *raw* and 15 *composite* traits with up to six biological replicates), missing data became problematic, especially for multivariate analyses such as PCA and MANOVA; these methods drop individuals from the analysis if there is a single missing trait value, decreasing overall power. In our experiment, many phenotypes (primary and secondary bolts, height, total seeds, number of leaves, dry weight, leaf and bolt thickness) had little to zero missing data (1-1.8%). Telomere length on individual chromosome arms is notoriously challenging to measure, but we still had data for 3-6 biological replicates for all conditions, except for 2R for which we had lower numbers but still at least 2-3 biological replicates. Missing data in physiological traits (proline, chlorophyll, OP and RWC) varied 13-42%. To account for missing data, we used the "impute missing data" function in JMP genomics (v. 9.1) which utilizes the mean and covariance matrix, estimated by the least squares, to impute missing values prior to any analyses. We note that missing values were randomly and evenly spread across all genotypes in all environments, and that an initial comparison of running the analyses below on raw and imputed data sets revealed that (1) they were very similar, and (2) our statistical findings using the imputed data set were more conservative in that fewer significant results were discovered. Thus, we chose to use the imputed data set for all analyses performed here.

PCA, Multivariate Analyses of Variance (MANOVA) and Analyses of Variance (ANOVA)

Principal component analysis (PCA) was performed to acquire a multidimensional overview of phenotypic trait variations and integration using PROC PRINCOMP function in SAS. To further explore global patterns of trait variation while controlling for the likely correlation between traits within a trait grouping (identified in Supplemental Table 2), we constructed a series of eight MANOVAs in SAS version 9.4 (SAS Institute, Cary, NC) that took the following form:

$$y_i + y_j + \ldots = \text{intercept} + \text{Treatment}|\text{Genotype} + \text{error}$$

In this model, '$y_i + y_j + \ldots$' represents the phenotypes within a trait grouping, 'Treatment' represents the three environments, 'Genotype' represents the five genotypes included in this study, and '|' indicates that all terms were considered both separately and as interaction combinations.

We then evaluated how all phenotypes individually responded to the environmental treatments by statistically comparing least-squared means (lsmeans) using general linear mixed

model ANOVAs[64] (proc mixed) in SAS version 9.4 (SAS Institute, Cary, NC): y_i = intercept + Treatment|Genotype + error

In this model, 'y_i' represents the focal phenotype, and all other terms are the same as in the MANOVA model above. To compare lsmeans we used pairwise contrasts (pdiff statement in SAS) and employed Tukey's honestly significant difference (Tukey's HSD; adjust=tukey in SAS) test to control for multiple comparisons.

Response strength and difference from the wild type

To assess how any deviation away from the wild type telomere length impacted the phenotypes, we determined the absolute value of average difference of phenotypes for all genotypes relative to the Col-0 wild type. We applied the following transformation to all individuals: [1] We first calculated the mean trait value for Col-0 for each phenotype in each of the three treatments to generate a matrix of wild type phenotypes. [2] We then estimated the difference from the wild type by subtracting the observed phenotypic value for each individual from the mean value for Col-0 in the appropriate treatment. [3] We then estimated the absolute value by transforming all of the difference from wild type estimates to positive values. [4] We performed linear mixed model ANOVAs (as stated in the section above) on these transformed values to obtain a least-squared mean difference from the wild type for each genotype in each treatment. Note that because we performed an absolute value transformation (step 3) prior to estimating least-squared means, it is possible for Col-0 to have a non-zero estimate.

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experiments. B.B. measured telomere length. B.E.C. and S.R. analyzed treatment data. S.R. and M.H.H. analyzed seed germination. B.E.C., D.E.S., T.E.J. and E.V.S. wrote the paper with contributions from all other authors.

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Competing interests

The authors declare no competing financial interests.

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Table 1. MANOVA analysis results for trait groups.

	Trait group							
Effect	**Morphology**	**Phenology**	**Leaf anatomy**	**Physiology**	**Fitness**	**Initial telomere length To**	**Final telomere length Tf**	**Change in telomere length AvgTf-AvgTo**
Genotype	1.31 (4)	23.19 (5)***	4.15 (6)**	9.02 (4)***	5.46 (4)**	19.02 (4)***	23.32 (4)***	2.47 (4)
Treatment	3.78 (2)*	105.67 (5)***	5.22 (6)**	27.21 (4)***	69.45 (2)***	2.63 (3)	2.54 (3)	0.31 (3)
Genotype x Treatment	1.10 (8)	7.63 (8)***	4.22 (8)**	4.01 (8)**	9.08 (8)***	1.82 (8)	2.37 (8)*	1.63 (8)

Model: traitA + traitB etc = genotype + treatment + (genotype x treatment). Genotypes: Col-0; *ku70; nop2a; tad3-2; tert*. Numbers represent F-values with degrees of freedom in parentheses. Significance: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.0001$.

Table 2. ANOVA analysis results of telomere length.

TELOMERES											
Effect	**To_1L**	**Tf_1L**	**Tf_To_1L**	**To_2R**	**Tf_2R**	**Tf_To_2R**	**To_5R**	**Tf_5R**	**Tf_To_5R**	**AvgTo**	**AvgTf**	**AvgTf-AvgTo**
Genotype	9.24 (4)***	11.26 (4)***	0.72 (4)	6.31 (4)***	6.78 (4)***	0.83 (4)	11.08 (4)***	13.51 (4)***	0.46 (4)	15.67 (4)***	18.74 (4)***	1.94 (4)
Treatment	1.99 (2)	2.33 (2)	0.04 (2)	3.75 (2)*	3.60 (2)*	0.06 (2)	0.10 (2)	0.07 (2)	0.41 (2)	2.98 (2)	2.93 (2)	0.34 (2)
Genotype x Treatment	1.08 (8)	1.15 (8)	0.38 (8)	1.23 (8)	1.11 (8)	0.56 (8)	1.51 (8)	1.70 (8)	0.96 (8)	1.08 (8)	0.95 (8)	1.43 (8)

Model: trait = genotype + treatment + (genotype x treatment). Genotypes: Col-0; *ku70; nop2a; tad3-2; tert*. Numbers represent F-values with degrees of freedom in parentheses. Significance: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.0001$. To, telomere length at the start of the experiment; Tf, telomere length at the end of the experiment. Tf_To, change in telomere length over time. AvgTo, average telomere length at the start of the experiment; AvgTf, average telomere length at the end of the experiment. AvgTf – AvgTfo, average change in telomere length over time. 1L, left arm of chromosome 1; 2R, right arm of chromosome 2; 5R, right arm of chromosome 5.

Table 3. ANOVA analysis results of plant traits.

PHENOLOGY					
Effect	**Flower Day**	**Primary Bolts**	**Secondary Bolts**	**Height**	**Bolt Thickness**
Genotype	13.62 (4)***	4.51 (4)**	10.37 (4)***	3.41 (4)*	4.83 (4)**
Treatment	5.70 (2)**	74.11 (2)***	244.17 (2)***	47.32 (2)***	1.48 (2)
Genotype x Treatment	0.82 (8)	1.81 (8)	6.09 (8)***	1.31 (8)	1.03 (8)

PHYSIOLOGY				
Effect	**Relative Water Content**	**Osmotic Potential**	**Total Chlorophyll**	**Proline**
Genotype	3.74 (4)**	7.36 (4)***	0.64 (4)	2.94 (4)*
Treatment	7.72 (2)***	13.66 (2)***	39.15 (2)***	16.90 (2)***
Genotype x Treatment	1.19 (8)	2.17 (8)*	0.58 (8)	3.59 (8)**

Table 3. ANOVA analysis results of plant traits (continued)

LEAF ANATOMY

Effect	Stomatal Area	Stomatal Density	Stomatal Area Per Leaf Area	Epidermal Cell Number	Epidermal Cell Area	Stomatal Index	Stomatal to Epidermal Area Ratio
Genotype	0.11 (4)	0.56 (4)	1.11 (4)	1.31 (4)	1.10 (4)	2.34 (4)	1.05 (4)
Treatment	1.68 (2)	9.03 (2)***	8.39 (2)***	1.64 (2)	5.28 (2)**	6.76 (2)**	4.12 (2)*
Genotype x Treatment	1.32 (8)	2.43 (8)*	1.53 (8)	0.95 (8)	2.79 (8)**	1.87 (8)	1.42 (8)

FITNESS

Effect	Total Seeds	Biomass
Genotype	2.23 (4)	0.64 (4)
Treatment	16.44 (2)***	2.11 (2)
Genotype x Treatment	3.82 (8)***	1.24 (8)

MORPHOLOGY

Effect	Leaf Count	Leaf Thickness
Genotype	0.57 (4)	0.90 (4)
Treatment	0.40 (2)	3.15 (2)*
Genotype x Treatment	1.05 (8)	0.71 (8)

Model: trait = genotype + treatment + (genotype x treatment). Genotypes: Col-0; *ku70; nop2a; tad3-2; tert*. Numbers represent F-values with degrees of freedom in parentheses. Significance: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.0001$.

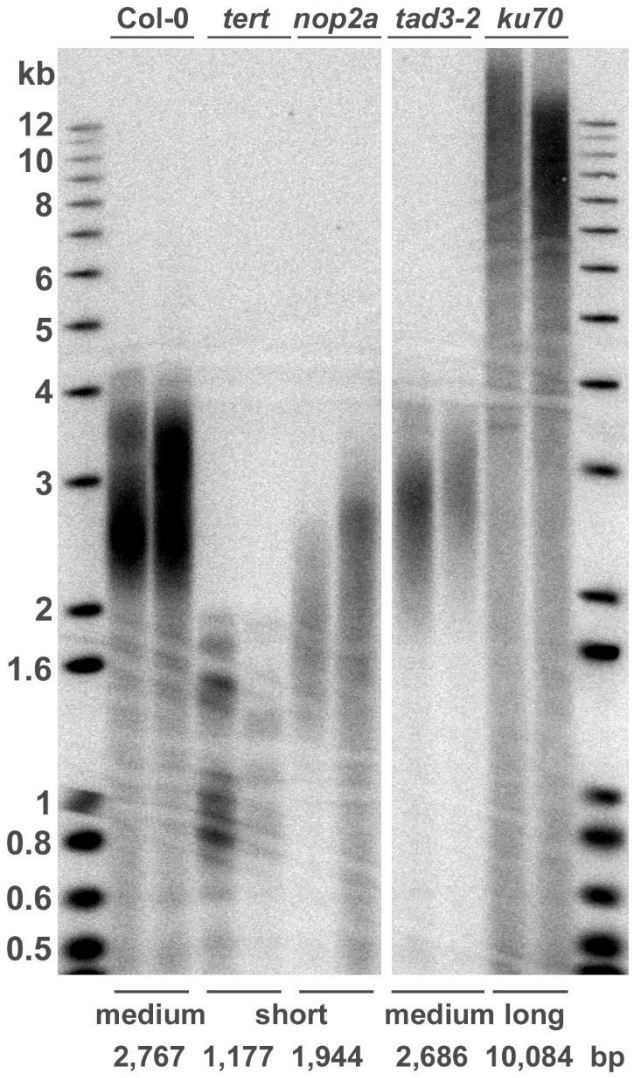

Figure 1. Telomere length in Arabidopsis genotypes used in the study. A representative TRF Southern blot is shown. Each lane represents genomic DNA from one individual plant of the corresponding genotype. All Arabidopsis genotypes were analyzed individually and also grouped as medium (Col-0, *tad3-2*), short (*tert, nop2a*) and long (*ku*70) telomere genotypes. The mean telomere length for each analyzed genotype is indicated at the bottom (in base pairs). DNA molecular weight markers and their corresponding sizes in kb are shown.

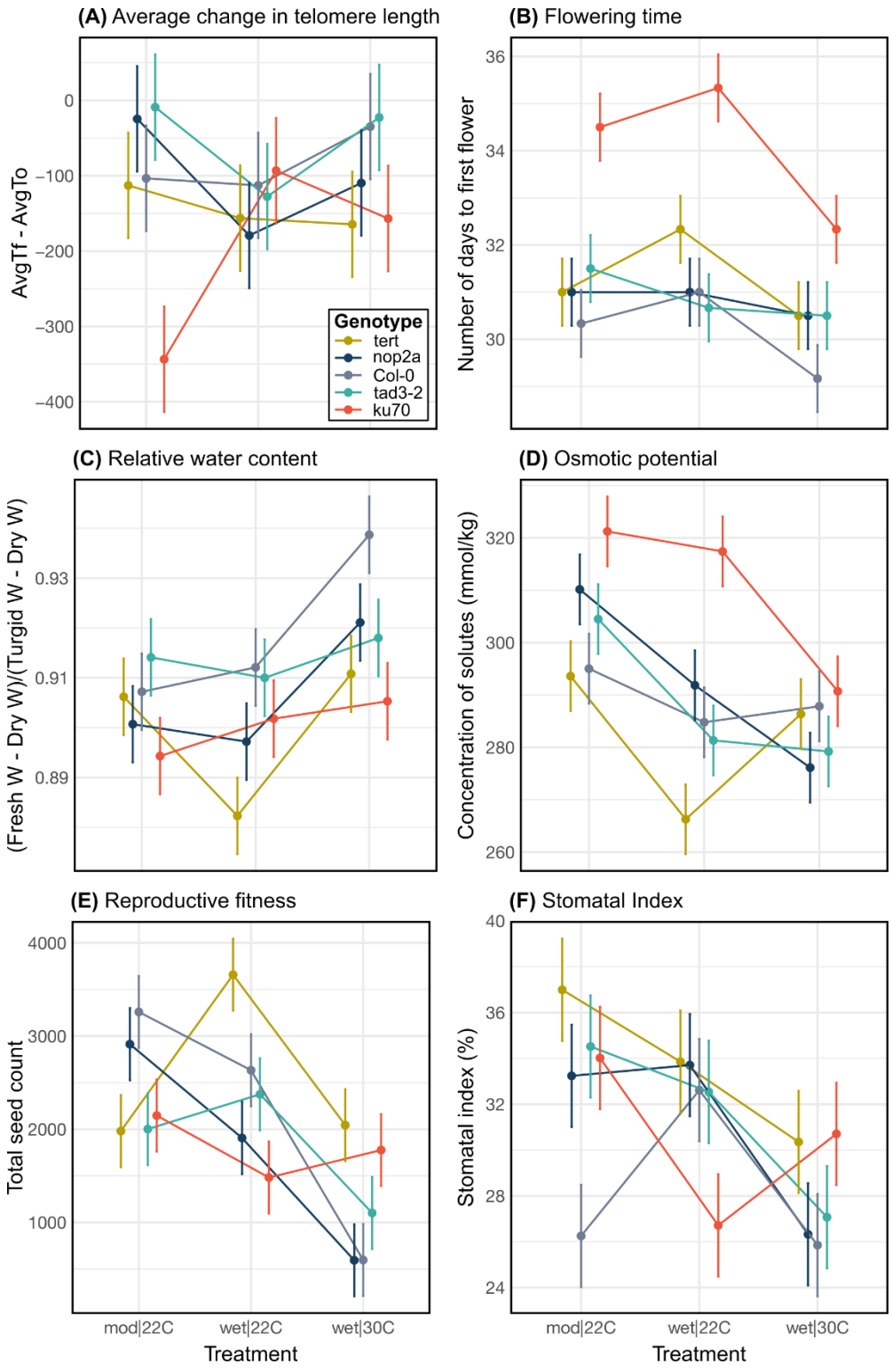

Figure 2. Changes in telomere length, developmental, physiological, fitness, and anatomical leaf parameters in Arabidopsis genotypes exposed to contrasting abiotic environments. The following parameters were analyzed for all genotypes: (A) Telomere length changes (AvgTf_AvgTo) for the average of three chromosome arms; (B) Flower day was analyzed as a developmental parameter; Relative water content RWC (C) and osmotic potential OP (D) were analyzed as physiological leaf parameters; (E) Total seed count was selected as reproductive fitness proxy; (E) Stomatal Index was analyzed as an anatomical leaf parameter.

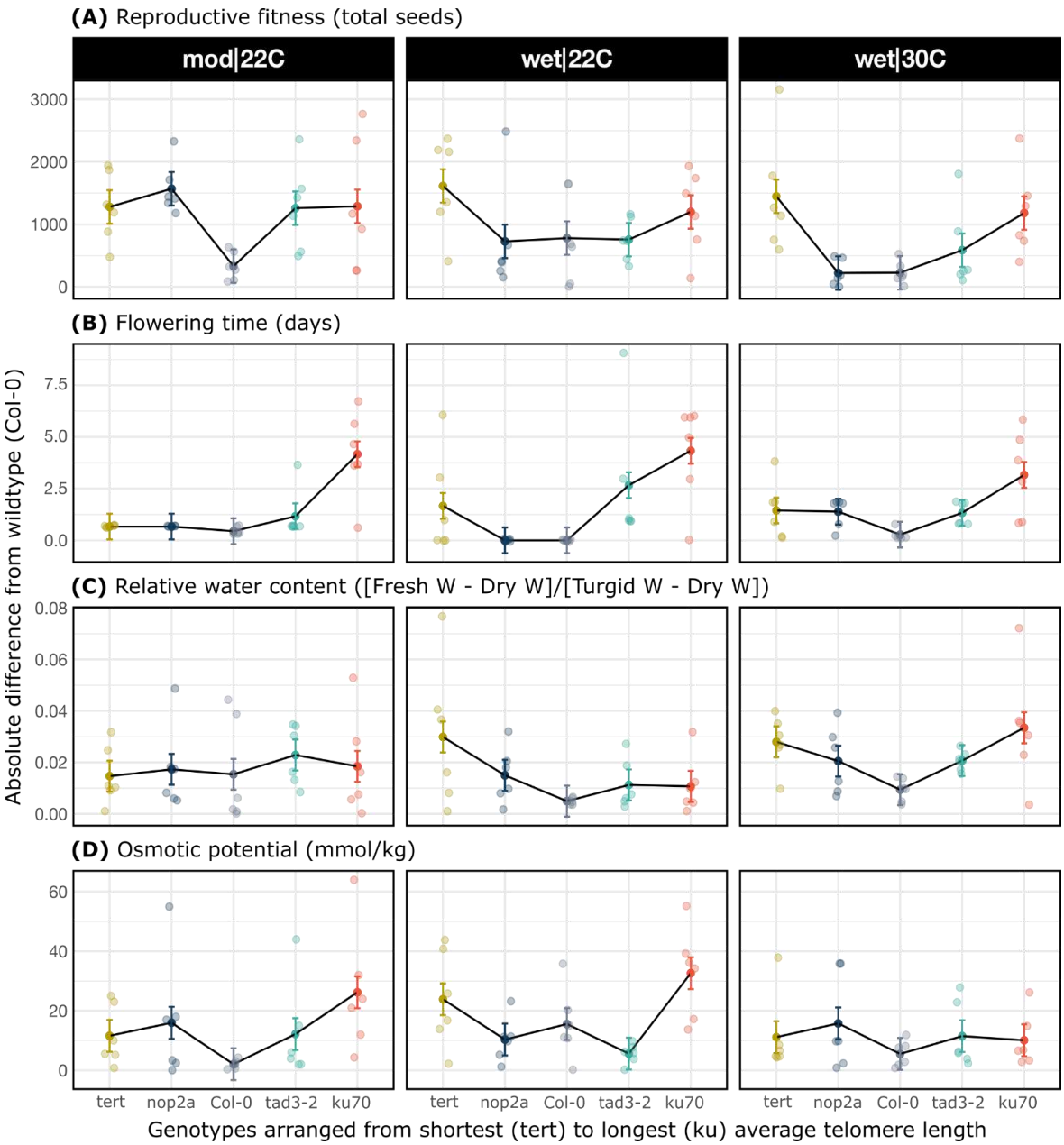

Figure 3. Analysis of strength response in plant traits. Response strength is defined as the absolute (positive or negative) variation from the Col-0 wild type trait values. Response strength was analyzed for Reproductive fitness (A), Flowering time (B), RWC (C) and OP (D). Trait values of Col-0 genotype were subtracted from corresponding values for all other genotypes, transformed to positive values and plotted, with genotypes from left to right arranged from the shortest (*tert*) to the longest (*ku70*) telomere length.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalData1.xlsx
- CampitelliSupplemental.pdf