Telomere length and *TERT* expression are associated with age in almond (*Prunus dulcis* [Mill.] D.A.Webb)

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Abstract

While it is well known that all organisms age, our understanding of how aging occurs varies dramatically among species. The aging process in perennial plants is not well defined, yet can have implications on production and yield of valuable fruit and nut crops. Almond, a relevant nut crop, exhibits an age-related disorder known as non-infectious bud failure (BF) that affects vegetative bud development, indirectly affecting kernel-yield. This species and disorder present an opportunity to address aging in a commercially-relevant and vegetatively-propagated, perennial crop threatened by an aging-related disorder. In this study, we tested the hypothesis that telomere length and/or *TERT* expression can serve as biomarkers of aging in almond using both whole-genome sequencing data and leaf samples collected from distinct age cohorts over a two-year period. To measure telomere lengths, we employed both *in silico* and molecular approaches. We also measured expression of *TERT*, a subunit of the enzyme telomerase, which is responsible for maintaining telomere lengths. Results from this work show a marginal but significant association between both telomere length measured by monochrome multiplex quantitative PCR and *TERT* expression, and age of almond seedlings. These results suggest that as almonds age, *TERT* expression decreases and telomeres shorten. This work provides valuable information on potential biomarkers of perennial plant aging, contributing to our limited knowledge of this process. In addition, translation of this information will provide opportunities to address BF in almond breeding and nursery propagation.

Keywords

Perennial plant aging, biomarker, telomerase

Introduction

The current concept and study of aging is centered primarily around mammals with research focused on circumventing deleterious impacts on health (Kirkwood, 2005; Sanders and Newman, 2013). However, all eukaryotic organisms exhibit signals of aging, resulting in the deterioration of key biological processes and subsequent decrease in health, performance, and fitness of individuals. Perennial plants represent a unique model to address the aging process and its impact since these species undergo cycles of dormancy and growth, and maintain the ability to reproduce for multiple years. The aging process of perennial plants is relevant due to the longevity and economic importance of perennial crops such as fruit and nut trees (Munné-Bosch, 2007; Brutovská *et al.*, 2013; Thomas, 2013). Individual trees can remain productive in orchards for decades; however, aging in plants and its implications for growth and reproduction are neglected areas of research with potential consequences for production, management, conservation, and breeding.

The lack of understanding of aging in perennials is partly due to the complexity in measuring and conceptualizing age in perennial plant species since aging occurs chronologically and ontogenetically in opposite directions (Poethig, 2003). Chronologic age can be defined as the amount of time since tissue/organ formation (e.g. human skin cells replenish every few days, meaning each cell is typically a day or 2-days old), while ontogenetic age refers more to developmental time and allows for the accumulation of mutations or chromosomal alterations (e.g. 2-day old skin cells at age 6 compared to 2-day old skin cells at age 60). Agriculturally relevant perennials are often vegetatively propagated (i.e. cloned), blurring the distinction between ontogenetic and chronologic age, and tend to be grown under intensive management. The limitation in determining age in perennials creates a need to identify biomarkers in these species that enable ontogenetic age estimation.

Almond (*Prunus dulcis* [Mill.] D.A.Webb; Fig. 1) is an economically relevant, Rosaceous crop, subject to intense horticultural management to maintain maximum nut production. In California, the almond industry is estimated to contribute ~$11 billion to the state's GDP annually (Almond Board of California, 2019). Top-producing almond cultivars, some of which were first obtained more than 100 years ago, are produced for commercial orchards via vegetative propagation (Micke, 1996; Wickson, 1914). As orchards age (after 20-25

66 years), trees are replaced with "new" clones of typically the same cultivar to maintain
67 homogeneity in quality and high levels of production (Micke, 1996).

68 Almond exhibits an age-related disorder known as non-infectious bud-failure (BF)
69 affecting vegetative bud development in the spring (Micke, 1996; Kester, 1970). Genotypes
70 exhibiting this disorder show characteristic dieback at the top of the canopy, and severe levels of
71 BF can result in up to 50% yield loss (Gradziel et al. 2013). Empirical evidence shows BF is
72 associated with age (Kester *et al.*, 2004); however, as almonds are produced primarily through
73 vegetative propagation rather than by seed, their true ontogenetic age and thus susceptibility to
74 BF can be difficult to assess (Micke, 1996). Biomarkers indicative of age would be valuable to
75 growers, breeders, and producers to screen germplasm. Thus, almond represents a potential
76 model species for the study of aging in perennials due to its economic relevance, the abundance
77 of available germplasm and breeding programs, and the exhibition of an age-related disorder.

78 One biomarker of aging is telomere length measurement (Sanders and Newman, 2013;
79 Marioni *et al.*, 2016; Runov *et al.*, 2015), which has been primarily studied in animals.
80 Telomeres are nucleoproteins that cap the end of chromosomes, preventing premature instability
81 of genomic material and cellular senescence (Watson and Riha, 2011). Telomeres tend to shorten
82 over mitotic cellular divisions due to decreased levels of telomerase, an enzyme that supports
83 telomere replication during the S-phase of the cell cycle (Nelson *et al.*, 2014). Over mitotic cell
84 divisions, telomeres eventually reach a critical minimum length at which point the cell senesces
85 and dies due to genome instability resulting from single stranded DNA at the ends of
86 chromosomes (Hemann *et al.*, 2001). This progressive shortening is proposed as a marker of
87 aging in mammalian cells and is linked to physiological deterioration and some age-related
88 disorders (Sanders and Newman, 2013; Watson and Riha, 2011; Aviv and Shay, 2018). Plant
89 chromosomes also contain telomeres with similar functions. While the relationship between
90 telomeres and the aging process is not as clearly defined in plants as in animals, previous work
91 shows associations between telomere length and various stages of plant development (Zachová *et*
92 *al.*, 2013; Watson and Riha, 2011; Procházková Schrumpfová *et al.*, 2019) suggesting telomere
93 length could be a suitable biomarker of age in plants.

94 Given that telomerase activity modulates telomere length, expression of genes involved
95 in the telomerase biosynthetic pathway could also serve as biomarkers for aging (Fitzgerald *et*

96 *al.*, 1996; De la Torre-Espinosa *et al.*, 2020; Boccardi and Paolisso, 2014; Anchelin *et al.*, 2011;
97 Fossel, 1998). TERT is the catalytic subunit of the telomerase enzyme (Oguchi *et al.*, 1999) and
98 the RNA subunit (TR) functions as the template for reverse transcription (Procházková
99 Schrumpfová *et al.*, 2019). Expression of *TERT* is shown to affect telomerase activity (Sweetlove
100 and Gutierrez, 2019; Jurečková *et al.*, 2017). In Arabidopsis, increased *TERT* expression is
101 linked to proportional increases in telomerase activity and telomere length (Zangi *et al.*, 2019;
102 Fitzgerald *et al.*, 1999) which is in turn linked to age. Since *TERT* is tied to telomere length in
103 both plants and animals, its expression may also serve as an indicator of age in plants (Watson
104 and Riha, 2011).

105 This study tests the hypothesis that telomere length and *TERT* expression in almond are
106 associated with age and can thus serve as biomarkers of aging in this species. Both telomere
107 length and *TERT* expression show promise as diagnostic biomarkers since they can be measured
108 in a high-throughput manner via *in silico* and molecular approaches (Nersisyan and Arakelyan,
109 2015; Montpetit *et al.*, 2014; Cawthon, 2009). These approaches build on previous research
110 examining the relationship between telomere lengths and age in perennial plants (Flanary and
111 Kletetschka, 2005; Moriguchi *et al.*, 2007; Liang *et al.*, 2015; Liu *et al.*, 2007). The goal of this
112 work is to advance our understanding and provide a model for the study of aging and its
113 implications in perennial plant species.

114 **Materials and Methods**

115 *Whole-genome Sequencing Data*

116 To perform *in silico* mean telomere length estimation, fastq files produced from whole-genome
117 sequencing of nine almond accessions were downloaded to the Ohio Supercomputer Center
118 (Ohio Supercomputer Center, 1987) from the National Center for Biotechnology Information
119 Sequence Read Archive from bioprojects PRJNA339570 and PRJNA339144. Accessions were
120 selected for use in this study if the age of the individual was included in the metadata for the
121 biosample entry on NCBI SRA. Table 1 includes the SRA biosample number for each almond
122 accession used as well as accession name, cultivar name (if available), and age.

123 *In silico Mean Telomere Length Estimation*

124 Mean telomere lengths were estimated for nine almond accessions using files in fastq format
125 containing whole-genome sequencing data for each individual. These fastq files were used as

126 input in the program Computel v. 1.2 (Nersisyan and Arakelyan, 2015). To estimate mean
127 telomere lengths, the computel.sh script was run with the following parameters: -nchr 8 -
128 lgenome 227411381 -pattern CCCTAAA -minseed 14 using the estimated length of the peach
129 genome based on the v2 assembly (Initiative *et al.*, 2013). All work was performed using the
130 Ohio Supercomputer Center computational resources (Ohio Supercomputer Center, 1987).

131 *Plant Material*

132 Leaf samples for this study were collected in May 2018 and 2019 from almond breeding
133 selections located at the Wolfskill Experimental Orchards (Almond Breeding Program,
134 University of California – Davis, Winters, CA). Tissue was harvested from the canopy of a total
135 of 36 unique individuals representing distinct age cohorts (Table 2). Samples were immediately
136 frozen on ice and stored at -20 °C until shipment overnight on dry ice to the Ohio Agricultural
137 Research and Development Center (OARDC – Wooster, OH). Samples were stored at -20 °C
138 until processing, and all subsequent experimental procedures were conducted at the OARDC.

139 *DNA and RNA Extraction*

140 DNA was extracted from the age cohort samples using the Omega E-Z 96® Plant DNA Kit
141 (Omega Bio-tek, Norcross, GA) with slight modification. Briefly, 100 mg of leaf material was
142 weighed in 2.0 mL tubes containing two 1.6 mm steel beads and kept frozen in liquid nitrogen.
143 Samples were ground in a 2000 Geno/Grinder® (SPEX SamplePrep, Metuchen, NJ) in two 48-
144 well cryo-blocks frozen in liquid nitrogen. Following a 65 °C incubation, samples were
145 incubated on ice for 20 minutes, treated with 10 µL of RNase solution (2.5 ul RNase [Omega
146 Bio-tek, Norcross, GA] + 7.5 µl TE pH 8), equilibrated through addition of 150 µl Equilibration
147 Buffer (3 M NaOH), incubated at room temperature for four minutes, and centrifuged at 4,400
148 rpm for two minutes prior to the addition of SP3 buffer. Concentration and quality were analyzed
149 using a NanoDrop™ 1000 spectrophotometer and a Qubit 4 Fluorometer with a dsDNA HS
150 Assay Kit (ThermoFisher Scientific, Waltham, MA).

151 RNA was extracted following the protocol outline in Gambino *et al.* (2008) with slight
152 modifications. Briefly, leaf material was ground in liquid nitrogen using a mortar and pestle, and
153 150 mg of tissue was weighed into a 2.0 mL microfuge tube frozen in liquid nitrogen. To extract
154 RNA, 900 µL CTAB extraction buffer (2% CTAB, 2.5% PVP-40, 2 M NaCl, 100 mM Trish-HCl

155 pH 8.0, 25 mM EDTA pH 8.0, 2% Beta-mercaptoethanol added before use) was added to each
156 tube and samples were incubated at 65 °C for ten minutes. Following incubation, two phase
157 separations were performed using an equal volume of chloroform:isoamyl alcohol (24:1). RNA
158 was precipitated in 3 M lithium chloride and incubated on ice for 30 minutes, and samples were
159 pelleted by centrifugation at 21,000 x g for 15 minutes. Pellets were then resuspended in 500 µL
160 pre-warmed SSTE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 1% SDS, 1 M NaCl)
161 followed by a phase separation with an equal volume of chloroform:isoamyl alcohol (24:1). A
162 final precipitation was performed using 0.7 volumes chilled 100% isopropanol. RNA was
163 pelleted and washed with 70% ethanol before being resuspended in 30 µL nuclease-free water. A
164 DNase treatment was performed using DNA-*free*™ DNA Removal Kit (ThermoFisher
165 Scientific) according to the manufacturer's instructions. All materials used for extraction were
166 nuclease- free and cleaned with RNaseZap™ RNase decontamination wipes (ThermoFisher
167 Scientific) prior to use. All centrifugation steps were performed at 4°C. RNA quality and
168 concentration were assessed using a NanoDrop™ 1000 spectrophotometer and a Qubit 4
169 Fluorometer with an RNA HS Assay Kit (ThermoFisher Scientific).

170 *Monochrome Multiplex Quantitative PCR (MMQPCR) to Measure Relative Telomere Lengths*

171 MMQPCR was conducted following the protocol outlined in Vaquero-Sedas and Vega-Palas
172 (2014) with minimal modifications. Primer sequences for genes used in this study are shown in
173 Table 3, including primers for the single copy gene, *PP2A*, and for the telomere sequence (Wang
174 *et al.*, 2014; Vaquero-Sedas and Vega-Palas, 2014). Oligos were synthesized by MilliporeSigma
175 (Burlington, MA) and resuspended to a concentration of 100 µM upon arrival. Standard curves
176 were created for each primer pair by pooling six aliquots of DNA isolated from a single clone of
177 the almond cultivar 'Nonpareil', and performing successive dilutions to 20 ng/µL, 10 ng/µL, 1
178 ng/µL, 0.5 ng/µL, and 0.25 ng/µL. Reactions were carried out in triplicate for each primer by
179 concentration combination.

180 Isolated DNA from the age cohort samples was diluted to 20 ng/µL. Multiplex reactions
181 were carried out in sextuplicate for each replicate within the age cohorts in a 10 µL volume using
182 QuantaBio PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, Beverly, MA) (2X),
183 forward and reverse primers (100 nM each), and 20 ng template DNA according to the
184 manufacturer's instructions. Reactions were performed in a Bio Rad C1000 Touch Thermal

185 Cycler (Bio Rad Laboratories, Hercules, CA) using the following program: initial denaturation at
186 95 °C for 3 minutes followed by 2 cycles of incubation at 94 °C for 15 seconds and annealing at
187 49 °C for 15 seconds; telomere and *PP2A* amplicons were generated following 35 cycles at 95
188 °C for 30 seconds, 59 °C for 1 minute, 72 °C for 30 seconds, 84 °C for 15 seconds and 85 °C for
189 15 seconds; final incubation at 72 °C for 1 minute. Melting curve analysis was performed at a
190 temperature range of 74-85 °C for both primer pairs to ensure no non-specific amplification.

191 *cDNA Synthesis and Quantitative Reverse Transcriptase PCR (qRT-PCR) to Measure Relative*
192 *Expression of TERT*

193 Reactions were carried out in a 20 µL volume using the Verso™ cDNA synthesis Kit
194 (ThermoFisher Scientific). One reaction was prepared for each age cohort sample according to
195 the manufacturer's instructions. Reactions were performed in a MJ Research PTC-200 thermal
196 cycler using the following program: 42 °C for 30 minutes followed by 95 °C for 2 minutes.

197 To quantify expression of *TERT* in age cohort individuals, qRT-PCR was performed in
198 triplicate for each sample. The gene *RPII* from peach was used as a reference (Bastias *et al.*,
199 2020; Tong *et al.*, 2009), and the sequence for the *TERT* gene was derived from the 'Texas'
200 genome (https://www.rosaceae.org/analysis/295) using the homologous peach gene sequence as
201 a reference (Alioto *et al.*, 2020). Primer sequences are shown in Table 2, and all oligos were
202 synthesized by MilliporeSigma (Burlington, MA) and resuspended to a concentration of 100 µM
203 upon arrival.

204 To generate cDNA from the age cohort samples, 100 ng of RNA was used as input in the
205 Verso cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's
206 instructions. To test for relative expression of *TERT*, reactions were carried out in triplicate for
207 each biological replicate within the age cohorts in a 10 µL volume using QuantaBio PerfeCTa
208 SYBR® Green SuperMix (Quanta Biosciences) (1X), forward and reverse primers (100 nM),
209 and cDNA (1 µL) according to the manufacturer's instructions. Reactions were performed in Bio
210 Rad C1000 Touch Thermal Cycler (Bio Rad Laboratories) using the following program: initial
211 denaturation at 95 °C for 3 minutes followed by 40 cycles at 95 °C for 15 seconds and 55 °C for
212 45 seconds. Melt curves were generated at a temperature range of 74-85 °C for both primer pairs
213 to ensure no non-specific amplification.

214 *Statistical Analysis*

215 Mean telomere lengths generated *in silico* using Computel v. 1.2 for each almond accession were
216 square root transformed prior to analysis. Mean telomere length was regressed on chronological
217 age to test for a linear relationship based on *in silico* predictions. Normality was confirmed using
218 a Shapiro-Wilks test. Mean telomere lengths generated for each individual almond accession are
219 listed in Supplementary File S1.

220 Using the standard curve generated with *PP2A* (S) and telomere (T) primers for a
221 reference almond sample, relative T/S ratios were calculated for each individual sample based on
222 Cq values for the telomere and *PP2A* products (Vaquero-Sedas and Vega-Palas, 2014). Z-scores
223 were calculated from the T/S ratios as recommend in Verhulst (2020) for each replicate within
224 the age cohorts. Normality and homogeneity of variance were confirmed using Shapiro-Wilks
225 and Bartlett tests. Analysis of variance (ANOVA) was performed for each age cohort followed
226 by *post hoc* Fisher's LSD and pairwise t-tests. Gene expression data were analyzed according to
227 guidelines in Bustin et. al (2009), first by normalizing *TERT* expression to that of the reference
228 gene, *RPII*. Following normalization, data were log-transformed, and normality and homogeneity
229 of variance were confirmed using Shapiro-Wilks and Bartlett tests. ANOVA was performed for
230 each age cohort followed by *post hoc* analysis with Tukey's HSD. All analyses were performed
231 using R v. 3.6.1 and plots were generated using ggplot2 v. 3.3.0. Calculated T/S ratios, relative
232 telomere lengths, relative *TERT* expression and log-transformed *TERT* expression as well as raw
233 Cq values for each individual are listed in Supplementary File S1. All R code used to perform
234 analyses is reported in Supplementary File S2.

235 **Results**

236 *Associations of telomere length and age in almond*

237 Mean telomere lengths were estimated *in silico* using whole-genome sequencing data for nine
238 select almond accessions and regressed against accession age following a square root
239 transformation. Normality of residuals was confirmed using a Shapiro-Wilks test (p-value =
240 0.318). Linear regression suggests a negative relationship between mean telomere length and age
241 as depicted in Fig. 2.

242 Relative telomere lengths were generated for the almond individuals within each of the
243 age cohorts collected in 2018 (1, 5, 9, and 14 years) and 2019 (2, 7, and 11 years old) using the
244 MMQPCR approach. Normality of residuals and homogeneity of variance of relative telomere

245 lengths were confirmed using Shapiro-Wilks (**2018:** p-value = 0.2578, n = 4-6; **2019:** p-value =
246 0.4682, n = 3) and Bartlett (**2018:** p-value = 0.1408; **2019:** p-value = 0.4613) tests. ANOVA
247 results for the linear model, z-score ~ age, were marginally significant in both 2018 and 2019,
248 and subsequent *post hoc* Fisher's LSD and pairwise t-tests revealed significant differences
249 between ages 1 and 14 years and 5 and 14 years (Fig. 3a) in the 2018 cohorts, and between ages
250 2 and 11 years old (Fig. 3b) in the 2019 cohorts.

251 *TERT gene expression patterns associated with age in almond*

252 Normalized expression of *TERT* was measured for almond samples among the age cohorts
253 collected in 2018 and 2019 for this study using *RPII* as the reference gene. Normality of
254 residuals and homogeneity of variance were confirmed using Shapiro-Wilks (**2018:** p-value =
255 0.694, n = 2-3; **2019:** p-value = 0.09456, n = 4) and Bartlett (**2018:** p-value = 0.6976; **2019:** p-
256 value = 0.3579) tests. ANOVA results comparing the average log(expression) values for each
257 age cohort revealed significant differences between cohorts in both 2018 and 2019. *Post hoc*
258 analysis with Tukey's HSD revealed significant differences in *TERT* expression between ages 1
259 and 14 years old in the 2018 cohorts (Fig. 4a) and between ages 2 and 11 years old in the 2019
260 age cohorts (Fig. 4b).

261 **Discussion**

262 Almond, an economically-valuable nut crop, exhibits an aging-related disorder known as non-
263 infectious bud failure that negatively impacts vegetative development and ultimately, yield. As a
264 clonally propagated crop, tracking age and thus susceptibility to bud failure is difficult, making
265 biomarkers of age a valuable resource to circumvent the impacts of aging-related disorders in
266 almond germplasm. Telomere length is used as a biomarker of age and development of age-
267 related disorders in mammals, but the association between telomere length and age in plants is
268 not well-defined (Watson and Riha, 2011; Procházková Schrumpfová *et al.*, 2019). The present
269 study tests the hypothesis that telomere length and/or *TERT* expression are associated with age in
270 almond. To test this, both *in silico* and qPCR approaches were utilized to measure telomere
271 length and estimate *TERT* expression in sets of almond accessions of known chronological age.
272 *In silico* analysis was performed using whole-genome sequencing data from nine almond
273 accessions of known age. Samples were collected from three and four sets of age cohorts over

274 two years to test for an association between relative telomere length and individual age using the
275 MMQPCR method as well as between *TERT* expression and age using qRT-PCR.

276 *In silico and quantitative PCR approaches suggest a marginal association between average or*
277 *relative telomere length and age in almond*

278 Average telomere length estimated *in silico* using the program Computel (Nersisyan and
279 Arakelyan, 2015) revealed a non-significant, negative association with age in the almond
280 accessions tested. This same pattern was shown utilizing MMQPCR and almond leaf samples
281 collected from different almond age cohorts in 2018 and 2019 where telomere length decreases
282 with increasing age. The association demonstrated in this study adds to the growing body of
283 knowledge regarding the complex relationship between telomere length and plant aging.

284 Previous studies in both *Ginkgo biloba* and *Panax ginseng* showed a pattern of increased
285 telomere length with increased age, suggesting plants do not follow the same patterns of
286 telomere shortening as seen in mammals (Liang *et al.*, 2015; Liu *et al.*, 2007). Work in apple
287 (*Malus domestica*) and *Prunus yedoensis*, both members of Rosaceae like almond, show no
288 change in telomere lengths with increased plant age over a five year timespan (Moriguchi *et al.*,
289 2007). In bristlecone pine (*Pinus longaeva*), a long-lived perennial gymnosperm, telomere
290 lengths measured in needle and root tissues between 0 – 3,500 years old showed a cyclical
291 pattern of lengthening and shortening with age (Flanary and Kletetschka, 2005). Further, when
292 analyzing telomere length in relation to tissue differentiation, studies in both barley (*Hordeum*
293 *vulgare*) and Scots pine (*Pinus sylvestris*) showed telomere shortening through embryo
294 development to leaf or needle formation (Aronen and Ryynänen, 2012; Kilian *et al.*, 1995).
295 Similarly, in silver birch (*Betula pendula*), telomeres shorten when plant are grown in tissue
296 culture conditions compared to those grown outdoors, suggesting abiotic stressors may induce
297 telomere shortening (Aronen and Ryynänen, 2014).

298 The results in almond suggest a pattern closest to what was observed in bristlecone pine
299 where telomere lengths shorten and lengthen throughout an individual's lifetime. This pattern
300 could be unique to gymnosperms, however, and needs to be further characterized in angiosperms
301 including Rosaceous species. While the commercial lifespan of almond is typically less than 30
302 years, almond can live more than 150 years (Micke, 1996). In this study, the maximum age
303 tested via the *in silico* approach was 32 years-old and via qPCR was 14 years-old, suggesting

304 that a wider age-range of trees and a larger sample size could produce a more refined model of
305 telomere length patterns over time.

306 Current almond cultivars may also be ontogenetically old, such as 'Nonpareil', the most
307 relevant US cultivar representing ~40% of acreage, which was first described almost 140 years
308 ago and has been propagated by budding since (Wickson, 1914; Almond Board of California,
309 2019). The ontogenetic age of a cultivar may be a factor to consider in the onset of aging-related
310 disorders like BF in almond. Additionally, it would be interesting to track the change in telomere
311 length following clonal propagation (through budding) in which plants experience a rejuvenation
312 process, reverting to a juvenile state for a short period of time (Bonga, 1982). It was further
313 found that propagating almond from basal epicormic buds, potentially representing
314 ontogenetically young meristematic tissue, seemed to alleviate BF in resulting clones (Gradziel
315 *et al.*, 2019). Testing telomere lengths in epicormic tissues could present another avenue to both
316 track aging in almond and develop biomarkers to predict BF potential in almond.

317 *TERT expression measured by qRT-PCR is a putatively associated with age in almond*
318 *accessions*

319 To test the hypothesis that *TERT* expression can serve as a biomarker of age in almond,
320 expression patterns were tested in cohorts representing either three or four distinct ages over two
321 years. Results from this work showed a consistent pattern of marginally significant, decreased
322 expression with increased ontogenetic age. Telomerase was shown to be a modulator of
323 longevity in humans and other mammals, but work describing telomerase patterns in plants is
324 limited (Boccardi and Paolisso, 2014; Fitzgerald *et al.*, 1996).

325 A comprehensive study examining telomerase protein activity in carrot (*Daucus carota*),
326 cauliflower (*Brassica oleracea*), soybean (*Glycine max*), *Arabidopsis thaliana*, and rice (*Oryza*
327 *sativa*) demonstrated that, like telomere lengths, protein activity tends to be highest in
328 undifferentiated tissues like meristematic tissues and is lower in differentiated tissues such as
329 leaves (Fitzgerald *et al.*, 1996). This result was supported by further work in barley and maize
330 showing little activity in differentiated tissues (Kilian *et al.*, 1998). These studies were all
331 performed in annuals or biennials, however, suggesting that telomerase activity does in fact
332 decrease with increased plant age in these crops. Work in perennials including bristlecone pine,
333 *P. ginseng*, and *G. biloba* showed an association between telomerase activity and age, suggesting

334 patterns unique to perennial plant species (Liang *et al.*, 2015; Flanary and Kletetschka, 2005;
335 Song *et al.*, 2011). A study in almond could be performed using a wider age-range and larger
336 sample size to elucidate the effect of age on telomerase activity, similar to what was referenced
337 above for telomere length measurements. Additionally, many of the studies performed in other
338 plants examining patterns of telomerase activity focused on protein activity rather than gene
339 expression. A future study will be necessary in almond to examine the telomerase protein
340 activity potentially by Western blot or other proteomics approaches to corroborate the
341 association between *TERT* expression and protein activity.

342 While a pattern was established in plants demonstrating a direct relationship between
343 telomerase activity and telomere length, regulation of telomerase is still not well understood in
344 the plant kingdom (Zachová *et al.*, 2013; Jurečková *et al.*, 2017; Fitzgerald *et al.*, 1996).
345 Interestingly, work in Arabidopsis has shown a link between DNA methylation and telomere
346 length, suggesting that this epigenetic mark likely has a role in regulating telomere lengths
347 potentially by modulating telomerase activity (Vega-Vaquero *et al.*, 2016; Lee and Cho, 2019;
348 Ogrocká *et al.*, 2014). A study is ongoing in almond to analyze DNA methylation patterns in a
349 set of almond accessions representing three distinct age cohorts to determine what, if any, impact
350 age has on methylation profiles.

351 Despite the limited age-range and small sample size used in this study, a consistent
352 pattern of both decreased telomere length and decreased *TERT* expression with increased age
353 was observed over two years of sampling. These results provide a basis for future study and
354 exploration into the utility of telomere length measurement and/or *TERT* expression or
355 telomerase activity as biomarkers of aging in almond. Developing a robust biomarker to track
356 aging in almond, a primarily clonally propagated crop, would allow growers, producers, and
357 breeders to screen germplasm to eliminate selections or clones with a high susceptibility to age-
358 related disorders due to advanced ontogenetic age.

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565 telomerase in development. *CellJ*, **22**. 10.22074/cellj.2020.6290.
566
567

568 **Tables**

569

SRA Biosample	Accession Name	Cultivar	Age (years)
SRR4045222	DPRU 1207.2	N/A	29
SRR4045223	DPRU 210	'Languedoc'	32
SRR4045224	DPRU 2331.9	N/A	20
SRR4045225	DPRU 1791.3	N/A	23
SRR4045226	DPRU 2301	'Tuono'	21
SRR4045227	DPRU 2374.12	N/A	19
SRR4045228	DPRU 1456.4	'Badam'	28
SRR4045229	DPRU 1462.2	N/A	28
SRR4036105	DPRU 2578.2	N/A	15

570 **Table 1.** NCBI SRA biosample entry for almond accessions used for *in silico* mean telomere
571 length estimation.

572

573

574

Sampling Year	Age (years)	Sample Size
2018	1	6
2018	5	6
2018	9	6
2018	14	6
2019	2	4
2019	7	4
2019	11	4

575 **Table 2.** Sampling scheme for 2018 and 2019 almond age cohort collections.

576

577

Oligo Name	Oligo Sequence (5' – 3')
PP2A Forward	**CGGCGGCGGGCGGCGCGGG**CAGGATAGACATTGGAGGGTTCGGCTCGCAA
PP2A Reverse	**CGGCGGCGGGCGGCGCGGG**ACCACTGCATGCAAAGGGACCCAAGCTTAT
Telomere Forward	CCCCGGTTTTGGGTTTTGGGTTTTGGGTTTTGGGT
Telomere Reverse	GGGGCCCTAATCCCTAATCCCTAATCCCTAATCCCT
TERT Forward	GCATCAGAGAAGGGTCAGATT
TERT Reverse	CTCTGGCTCCTTGAATCGTATAG
RPII Forward	TGAAGCATACACCTATGATGATGAAG
RPII Reverse	CTTTGACAGCACCAGTAGATTCC

578 **Table 3.** Oligos used for all MMQPCR and qRT-PCR studies.

579

580

581 **Figure Legends**

582 **Figure 1.** Image of almond cultivar 'Nonpareil' (photo taken by K. D'Amico-Willman in May
583 2018).

584

585 **Figure 2.** Linear regression showing the relationship between $\sqrt{mean\ telomere\ length}$ and
586 accession age in nine almond accessions. Dashed line represents the best-fit linear model (p-
587 value = 0.1458; R^2 = 0.2767).

588

589 **Figure 3.** Boxplots depicting the calculated z-score of the T/S ratio for almond samples within
590 the age cohorts tested. **(a)** Age cohort collected in 2018. **(b)** Age cohort collected in 2019.
591 Significant differences in z-scores between age cohorts based on ANOVA followed by *post hoc*
592 Fisher's LSD (α = 0.1) are denoted by letter groupings (ANOVA 2018 p-value = 0.1077;
593 ANOVA 2019 p-value = 0.06548).

594

595 **Figure 4.** Normalized expression of *TERT* for almond samples within the age cohorts test. **(a)**
596 Age cohort collected in 2018. **(b)** Age cohort collected in 2019. Significant differences in
597 relative expression between age cohorts based on ANOVA followed by *post hoc* Tukey's HSD
598 (alpha = 0.1) are denoted by the letter groupings (ANOVA 2018 p-value = 0.09087; ANOVA
599 2019 p-value = 0.1414).

600

601 **Supplementary Files**

602 **S1.** File containing raw data for each experiment including *in silico* telomere length estimation,
603 mean telomere length quantification, and *TERT* expression analysis.

604 **S2.** R code used to perform all statistical analyses reported in this manuscript.

605

606 **Acknowledgements**

607 We would like to acknowledge Matthew Willman for his assistance with the statistical analyses
608 and Cheri Nemes for her assistance with wet lab portions of this project. We would like to thank
609 Daniel Williams for editing later versions of this manuscript. This work is supported by the Ohio
610 State University CFAES-SEEDS program 2018113, the Translational Plant Sciences Graduate

Fellowship, the AFRI-EWD Predoctoral Fellowship 2019-67011-29558 from the USDA National Institute of Food and Agriculture, and the Ohio Supercomputer Center.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Figures

Figure 1

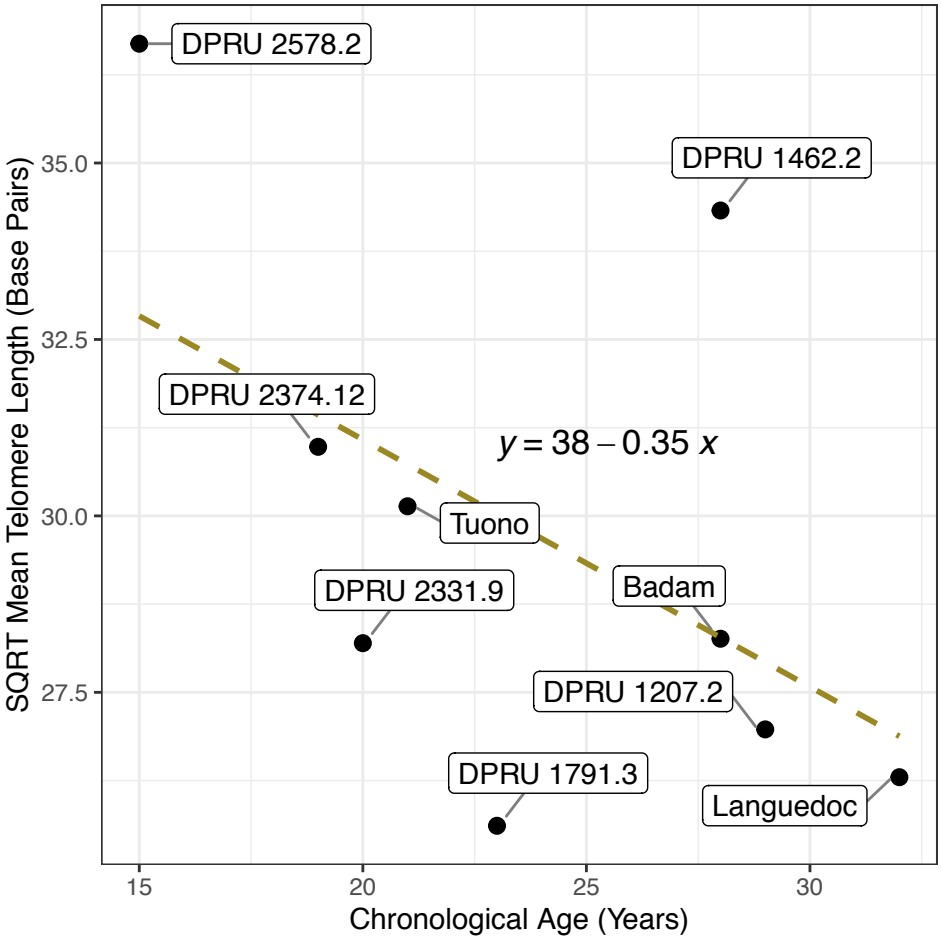
DPRU 2578.2
DPRU 1462.2
DPRU 2374.12
$y = 38 - 0.35\,x$
Tuono
DPRU 2331.9
Badam
DPRU 1207.2
DPRU 1791.3
Languedoc
35.0
32.5
30.0
27.5
15
20
25
30
SQRT Mean Telomere Length (Base Pairs)
Chronological Age (Years)

Figure 2

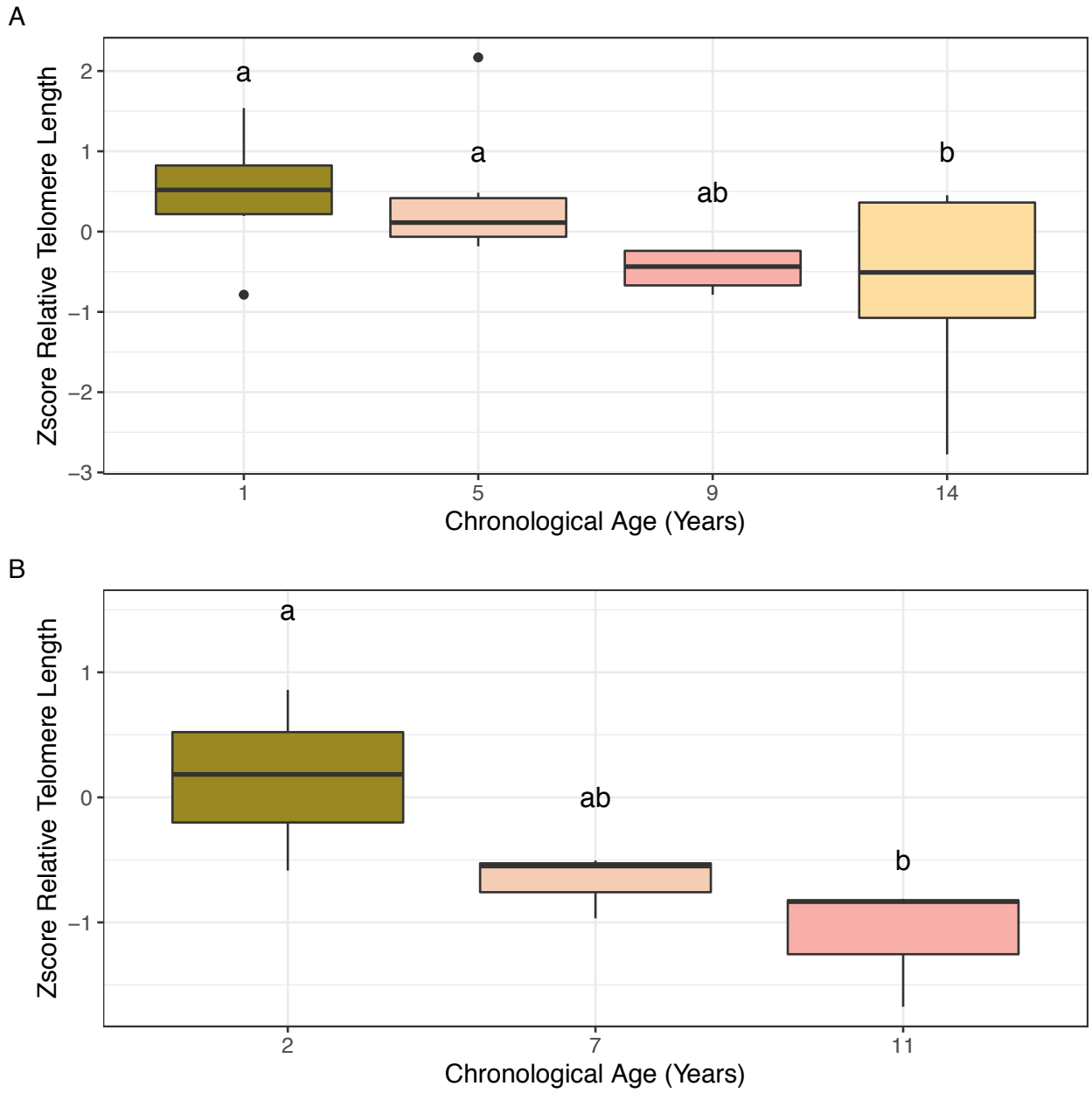
A
Zscore Relative Telomere Length
2
1
0
−1
−2
−3
a
a
ab
b
1
5
9
14
Chronological Age (Years)
B
Zscore Relative Telomere Length
1
0
−1
a
ab
b
2
7
11
Chronological Age (Years)

Figure 3

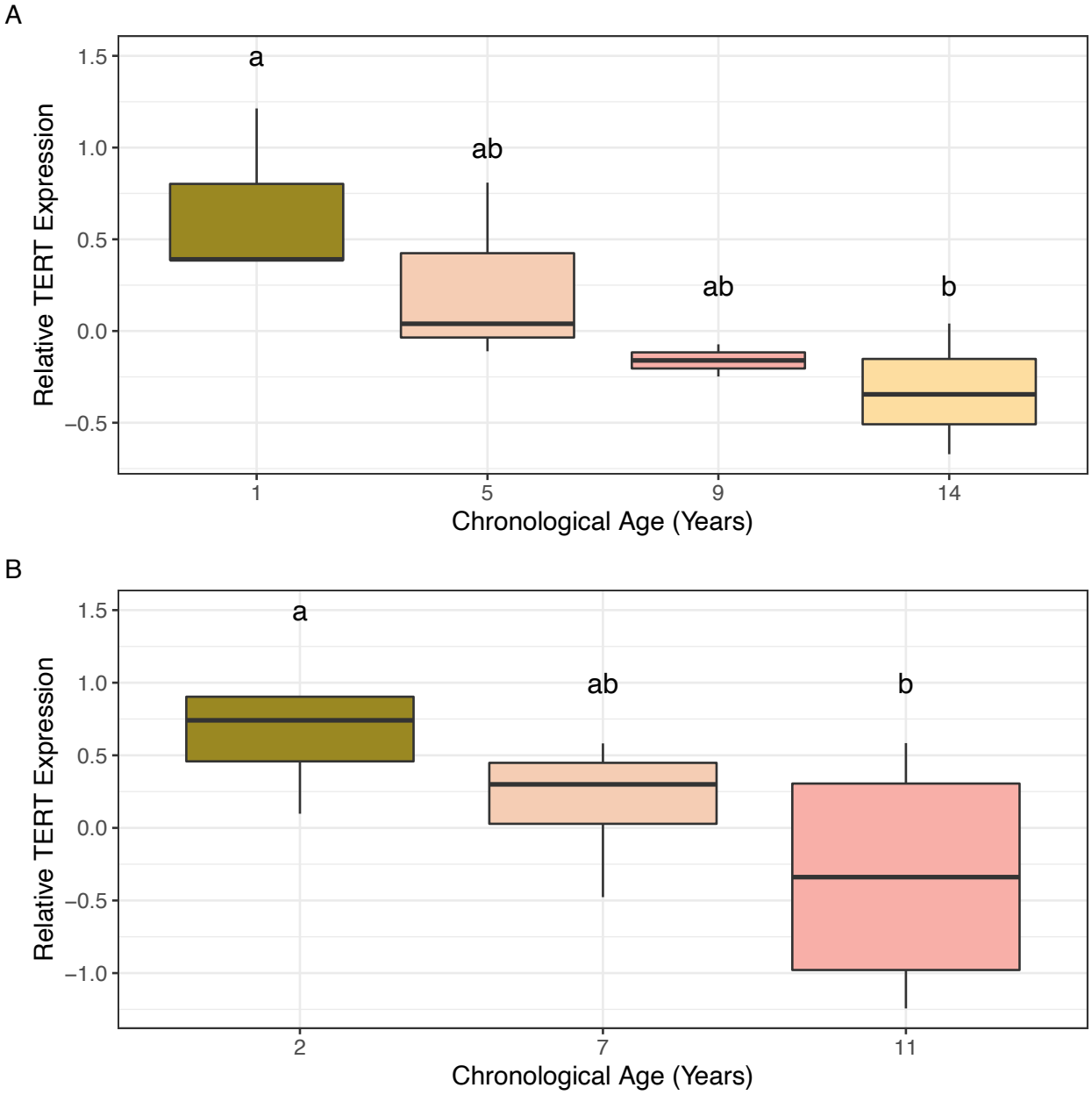
A
Relative TERT Expression
1.5
1.0
0.5
0.0
−0.5
a
ab
ab
b
1
5
9
14
Chronological Age (Years)
B
Relative TERT Expression
1.5
1.0
0.5
0.0
−0.5
−1.0
a
ab
b
2
7
11
Chronological Age (Years)

Figure 4