Allelic diversity of *NAC18.1* is a major determinant of fruit firmness and harvest date in apple (*Malus domestica*)

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Highlight:

NAC18.1 is a member of a family of conserved transcriptional regulators of ripening that underlies variation in fruit firmness and harvest date in diverse apple accessions.

Abstract:

Softening is a hallmark of ripening in fleshy fruits, and has both desirable and undesirable implications for texture and postharvest stability. Accordingly, the timing and extent of ripening and associated textural changes are key targets for improving fruit quality through breeding. Previously, we identified a large effect locus associated with harvest date and firmness in apple (*Malus domestica*) using genome-wide association studies (GWAS). Here, we present additional evidence that polymorphisms in or around a transcription factor gene, *NAC18.1*, cause variation in these traits. First, we confirmed our previous findings with new phenotype and genotype data from ~800 apple accessions. In this population, we compared *NAC18.1* to three other ripening-related markers currently used by breeders (*ACS1*, *ACO1*, and *PG1*), and found that the effect of the *NAC18.1* genotype on both traits greatly exceeded that observed for the other markers. By sequencing *NAC18.1* across 18 accessions, we revealed two predominant haplotypes containing the SNP previously identified using GWAS, as well as dozens of additional SNPs and indels in both the coding and promoter sequences. *NAC18.1* encodes a protein with high similarity to the NON-RIPENING (NOR) transcription factor, an early regulator of ripening in tomato (*Solanum lycopersicum*). To test whether these genes are functionally orthologous, we introduced *NAC18.1* transgenes into the tomato *nor* mutant and showed that both haplotypes complement the *nor* ripening deficiency. Taken together, these

results indicate that polymorphisms in *NAC18.1* underlie substantial variation in apple firmness and harvest time through modulation of a conserved ripening program.

Keywords:

Apple, fruit ripening, NAC-domain transcription factor

Abbreviations:

ABC Apple Biodiversity Collection

ACS1 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 1

ACS2 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2

ACO1 AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 1

CIELAB International Commission on Illumination 1976 color space

HRM High Resolution Melting

LD Linkage disequilibrium

PG1 POLYGALACTURONASE 1

PG2 POLYGALACTURONASE 2

PSY1 PHYTOENE SYNTHASE 1

REML Restricted Maximum Likelihood

RPL2 RIBOSOMAL PROTEIN L2

USDA United States Department of Agriculture

66 **Introduction**

67 Despite their diverse structure, ontogeny, and biochemical composition, fleshy fruits
68 from a taxonomically broad range of species undergo coordinated ripening programs that have
69 many features in common. Ripening involves numerous physiological and biochemical changes
70 that render the fruit attractive and nutritious for consumption by seed-dispersing animals, or
71 human consumers in the case of cultivated crops. These include the ripening-associated
72 accumulation of sugars, pigments, and flavor or aroma compounds, as well as a loss of flesh
73 firmness due in large part to the controlled modification and depolymerization of cell wall
74 polysaccharides (Wang *et al.*, 2018). These processes are regulated by conserved and
75 convergently evolved networks of transcription factors and hormones, such as ethylene in so-
76 called climacteric fruit (Lü *et al.*, 2018).

77 Various aspects of fruit ripening have been particularly well studied in tomato (*Solanum*
78 *lycopersicum*) and the characterization of tomato ripening mutants has revealed a regulatory
79 network, consisting of transcription factors, hormones, and epigenetic modifications (Giovannoni
80 *et al.*, 2017). Of the genes that regulate ripening, the *NON-RIPENING (NOR)* gene appears to
81 exhibit the earliest expression preceding ripening (Shinozaki *et al.*, 2018). *NOR* encodes a NAC
82 domain transcription factor (Giovannoni *et al.*, 2004). This family of transcription factors is one
83 of the largest plant-specific families of transcription factors, with specific members regulating
84 development, defense, and senescence (Mathew and Agarwal, 2018). While all members share a
85 conserved DNA-binding (NAC) domain, specific functional clades are defined in terms of their
86 more variable domains, particularly the C-terminal transcriptional regulatory region. These
87 domains can act directly as transcriptional activators, or can facilitate interaction with other
88 transcription factors in order to fine-tune transcriptional control.

89 Apple (*Malus domestica*) fruit exhibit extensive variation in the extent and timing of
90 ripening and ripening-associated softening. This is reflected in three related agronomic traits:
91 harvest date, firmness, and storability. Harvest date is typically determined by the semi-
92 quantitative measurement of starch, based on iodine-staining patterns (Blanpied and Silsby,
93 1992). Firmness can also be used as a measure of ripening, although apples soften less than many
94 other fleshy fruits, and are more noted for their characteristic and desirable "crisp" texture.
95 Importantly, postharvest storability is highly correlated with fruit firmness (McClure *et al.*, 2018;
96 Ornelas-Paz *et al.*, 2018). Thus, firmness is typically a desirable fruit quality trait in apple,
97 although it is often associated with later harvest dates that can be undesirable if the growing
98 season is short (Migicovsky *et al.*, 2016).

99 Due to a prolonged juvenile phase, it is particularly challenging to evaluate fruit quality
100 traits in the context of an apple breeding program. As a result, in recent years considerable effort
101 has been invested in developing molecular markers that can be used to select for fruit quality
102 traits at the seedling stage. In particular, three markers have been proposed for predicting
103 firmness. The first is *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 1* (*ACS1*),
104 which encodes the ripening-associated isoform of an ethylene biosynthesis gene. The *ACS1-2*
105 allele contains a retrotransposon insertion thought to confer low ethylene production and longer
106 shelf life in Fuji and other apple cultivars homozygous for this allele (Sunako *et al.*, 1999;
107 Harada *et al.*, 2000). The second is a marker for a gene corresponding to another enzyme
108 involved in ethylene biosynthesis, *AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 1*
109 (*ACO1*; Costa *et al.*, 2005). Finally, a marker has been developed for alleles of
110 *POLYGALACTURONASE 1 (PG1;* Costa *et al.*, 2010). This gene encodes an enzyme that

111 hydrolyzes pectin polysaccharides and has been implicated in apple fruit firmness by RNAi-
112 based gene silencing experiments (Atkinson *et al.*, 2012).

113 In a previous study, we conducted GWAS on a range of traits in apple using genotype
114 data and historical phenotype records for 689 accessions of the United States Department of
115 Agriculture (USDA) apple germplasm collection (Migicovsky *et al.*, 2016). Our analysis
116 revealed a single nucleotide polymorphism (SNP) on chromosome 3 within the coding sequence
117 of the *NAC18.1* gene that was significantly associated with firmness and harvest date. This SNP
118 results in an aspartate (D) to tyrosine (Y) mutation at position 5 of the NAC18.1 amino acid
119 sequence and we refer to this SNP as D5Y (Migicovsky *et al.*, 2016). Subsequently, GWAS of
120 two European apple germplasm collections confirmed the association between *NAC18.1* and
121 ripening time (Urrestarazu *et al.*, 2017; Larsen *et al.*, 2019). NAC transcription factors have also
122 been linked to harvest date in other Rosaceae species, including peach (*Prunus persica;* Pirona *et*
123 *al.*, 2013) and apricot (*P. armeniaca;* García-Gómez *et al.*, 2019).

124 Here we report an extension of our previous findings using three approaches. First, we
125 conducted additional phenotyping and genotyping of apple germplasm in order to compare the
126 predictive power of the *NAC18.1* D5Y SNP with other proposed firmness/harvest date markers.
127 Secondly, we sequenced the *NAC18.1* gene from a subset of apple cultivars in order to discover
128 additional polymorphisms in linkage disequilibrium (LD) with D5Y. Finally, we used
129 heterologous expression of the *NAC18.1* gene in the tomato *non-ripening (nor)* mutant to test
130 whether it functions as a component of a conserved fruit ripening program.

131

Materials and Methods

Apple Firmness and Harvest Date

The Apple Biodiversity Collection (ABC) is an apple orchard located in Kentville, Nova Scotia, which contains 1,113 accessions. It was first established in spring 2012, when trees were budded onto M.9 rootstocks. In fall 2012, the trees were uprooted and kept in cold storage, before planting in spring 2013. The trees were planted in an incomplete block design, which includes 1 of 3 standards per grid, allowing for correction of positional effects using a REstricted Maximum Likelihood (REML) model, described in Migicovsky *et al.* (2018).

In 2017, we evaluated harvest date for 1,348 trees and fruit firmness for 1,328 trees within the ABC orchard. Due to the diversity of apples within the collection, a variety of methods were used to determine the appropriate time to harvest. First, we observed if the tree had dropped fruit or, for red apples, if the fruit were a deep red color. Next, a sample apple was taken from each tree and touched to assess firmness, tasted to assess starch and sweetness, cut in half to check browning of seeds, and then sprayed with iodine solution to assess starch content (Blanpied and Silsby, 1992).

When fruit were determined to be mature, they were harvested and evaluated for firmness. We recorded the firmness (kg cm^{-2}) of 5 fruit per tree using a penetrometer with a 1 cm diameter (Fruit Texture Analyzer, GS-14, Güss Manufacturing). A small section of skin was removed using a vegetable peeler, and each fruit was placed on the penetrometer platform so that the piston entered the middle of the apple where the skin had been removed. Data were automatically recorded into a spreadsheet.

Due to the number of trees, harvesting the ABC orchard often lasted more than one day. Therefore, differences in harvest date within a week were simply due to the time required to

155 harvest, and the harvest date for each tree was recorded as the Monday of that week. We used the
156 "*lmer"* function in the R package *lme4* (Bates *et al.*, 2015) to fit a REML model for harvest and
157 firmness data. Next, we calculated the least squares mean using the "*lsmeans*" function in the
158 *lsmeans* R package (Lenth, 2016), resulting in one value per unique accession. We fit this model
159 for 866 unique accessions with harvest dates and 863 accessions with firmness measurements.
160

161 ***High Resolution Melting (HRM) Genotyping***

162 DNA was extracted using silica columns from fresh leaf tissue collected from the ABC
163 orchard, quantified using PicoGreen (Thermo) and normalized to a concentration of 20 ng μL^{-1}.
164 Genotyping was conducted using PCR and high resolution melting (HRM) on a LightScanner
165 HR384 (BioFire). Primers are listed in Supplemental Table S1.

166 Genotyping of the PG1 SNP marker was based on the GenBank sequence L27743.1,
167 where the G allele at position 437 is unfavorable and the T allele is favorable (Costa *et al.*,
168 2010). Three allelic combinations of the observed indel, ACS1-1/1, 1-1/2 and 1-2/2, have been
169 associated with high, medium, and low ethylene production, respectively (Sunako *et al.*, 1999;
170 Harada *et al.*, 2000; Oraguzie *et al.*, 2004; Costa *et al.*, 2005). The exact position of the indel is
171 from ACS1-2 (GenBank: AB010102.1) 1,320 to 1,483 bp (163 bp), and from ACS1-1
172 (GenBank: AY062129.1) 4,500 to 4,525 bp (25 bp). The size difference between alleles is 138
173 bp. The ACO marker involves an unfavorable 62 bp insertion in the third intron of ACO1 (Costa
174 *et al.*, 2005). The third intron spans from 1,083 bp to 1,300 bp of the GenBank sequence
175 Y14005.1 and the indel is found from position 1,297 bp to position 1,358 bp. The D5Y mutation
176 in *NAC18.1* is a nonsynonymous SNP at position 30,698,039 on chromosome 3, according to
177 reference genome version GDDH13 v1.1 (https://iris.angers.inra.fr/gddh13/) and is associated

178 with both harvest date and firmness (Migicovsky *et al.*, 2016). The desirable C allele encodes an
179 aspartic acid (D) at the fifth amino acid position of the NAC18.1 protein, while the undesirable A
180 allele encodes a tyrosine (Y).
181
182 ***Marker-phenotype associations***
183 We evaluated the ability of markers at NAC18.1, ACO1, ACS1, and PG1 to predict both
184 harvest date and firmness of accessions in the ABC orchard. The number of accessions with
185 phenotype and genotype information ranged from 754 to 852 depending on the trait/marker
186 combination. The association test was conducted using Spearman's rank correlation test. We
187 visualized results using the "*geom_boxplot*" function in *ggplot2* in R (Wickham, 2016). Lastly,
188 we tested which mode of inheritance best fit the data using SNPstats (Solé *et al.*, 2006), and
189 selected the model with the lowest Akaike Information Criterion value.
190
191 ***Sequencing of* NAC18.1**
192 DNA was isolated from leaves sampled from 18 apple accessions growing in Geneva,
193 NY. A 2.3 kb amplicon including the *NAC18.1* gene and ~800 bp of upstream sequence was
194 amplified by PCR using primers NAC18F2 and NAC18R2 (Supplemental Table S1) and
195 Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB). PCR product size and purity
196 was confirmed by agarose gel electrophoresis, and the remaining product was purified using
197 DNA Clean & Concentrator kit (Zymo Research). The resulting DNA fragment was cloned into
198 the plasmid pMiniT 2.0 and transformed into *E. coli* using the NEB® PCR Cloning Kit (NEB).
199 Individual colonies were selected for complete sequencing of the cloned amplicon using
200 the primers NAC18F2, NAC18F3, NAC18F4, NAC18R1, and NAC18R2 (Supplemental Table

201 S1). For accessions homozygous for the D5Y SNP, the NAC18.1 amplicon from a single clone
202 was sequenced. For heterozygous accessions, two clones representing each D5Y allele were
203 selected based on partial sequencing of the D5Y region, followed by complete sequencing of the
204 2.3 kb amplicon, as described above. The nucleotide sequences were aligned by MUSCLE
205 (Edgar, 2004) and used to construct a maximum-likelihood phylogenetic tree in MEGA7 (Kumar
206 *et al.*, 2016).
207
208 ***Molecular cloning and plant transformation***
209 The hypothetical coding sequences (CDSs) corresponding to the consensus sequence of
210 the "A" and "C" haplotypes of *NAC18.1* were synthesized as a double-stranded DNA (gBlock)
211 by IDT, with 20 bp of flanking sequence added to facilitate Gibson Assembly of the *NAC18.1*
212 sequences between the *Asc*I and *Pac*I restriction sites of pMDC32 (Curtis and Grossniklaus,
213 2003). Constructs were assembled using the NEBuilder HiFi DNA Assembly Cloning Kit (NEB)
214 and their integrity verified by Sanger sequencing. Plasmids were transformed into
215 *Agrobacterium tumefaciens*, which was used to transform tomato cotyledon explants (Van Eck *et*
216 *al.*, 2019) derived from the tomato *nor* mutant in the cv. Ailsa Craig background (LA3770,
217 Tomato Genetics Resource Center, https://tgrc.ucdavis.edu/). A total of 10 T0 plants were
218 recovered per construct and plants derived from two independent transformation events per
219 construct were selected for further characterization in the T1 generation.
220
221 ***Transgenic tomato characterization***
222 Fruit were harvested when visually ripe, or in the case of the *nor* mutant, at the equivalent
223 age as ripe cv. Ailsa Craig fruit (the near isogenic wild-type control), as determined by tagging

224 of flowers at anthesis. The color of the fruit surface was measured using a CR-400 Chroma
225 Meter (Konica Minolta), and fruit were weighed, photographed, and dissected. Pericarp tissue
226 was frozen in liquid nitrogen and stored at -80˚C. Frozen tissue was ground to a fine powder and
227 RNA extracted by a modified version of Chang et al. (1993). Briefly, approximately 400 mg of
228 tissue was added to a preheated (80˚C) two-phase system consisting of 500 µL of water-saturated
229 phenol and 500 µL of extraction buffer (100 mM Tris [pH 8.0], 25 mM EDTA, 2 M NaCl, 2%
230 CTAB, 2% PVP, and 2% [v/v] beta-mercaptoethanol). The mixture was vortexed and incubated
231 for 5 min at 65˚C and then cooled to room temperature before extracting and precipitating RNA,
232 as previously described (Chang *et al.*, 1993).

233 RNA was treated with RNase-free DNase (Promega) and used for cDNA synthesis with
234 RNA to cDNA EcoDry™ Premix with Oligo dT primer (Takara Bio). The cDNA was used as
235 template for quantitative PCR using Luna Universal qPCR Master Mix (NEB) and a Viia7 real-
236 time PCR instrument (Life Technologies/ABI). Gene-specific primers are listed in Supplemental
237 Table S1. Quantification used the ΔC_t method with *RIBOSOMAL PROTEIN L2* (*RPL2*) as a
238 reference gene, and statistical significance of the ΔC_t values was tested by a one-way ANOVA
239 followed by Tukey's HSD test.

240

241 **Results**

242 ***Evaluation of markers for firmness and harvest date in apple***

243 We sought to re-evaluate the ability of D5Y and other published markers to predict fruit
244 firmness and harvest date using new phenotype and genotype data: specifically, we aimed to
245 address some experimental limitations of our previous work. First, the GWAS that identified
246 D5Y, and failed to find associations for previously published markers, made use of only 8,000

247 SNPs (Migicovsky *et al.*, 2016). Given the rapid LD decay in apple, our low SNP density was
248 insufficient to conclude that we had exhaustively searched the apple genome for loci involved in
249 firmness and harvest date (Migicovsky *et al.*, 2016). Secondly, the historic phenotype data we
250 used were imprecise. For example, firmness was recorded as either "firm" or "soft" rather than
251 as a biomechanical measurement, and harvest date was recorded as simply "early", "mid", or
252 "late". These factors would have limited the detection of additional loci that underlie firmness
253 and/or harvest date.

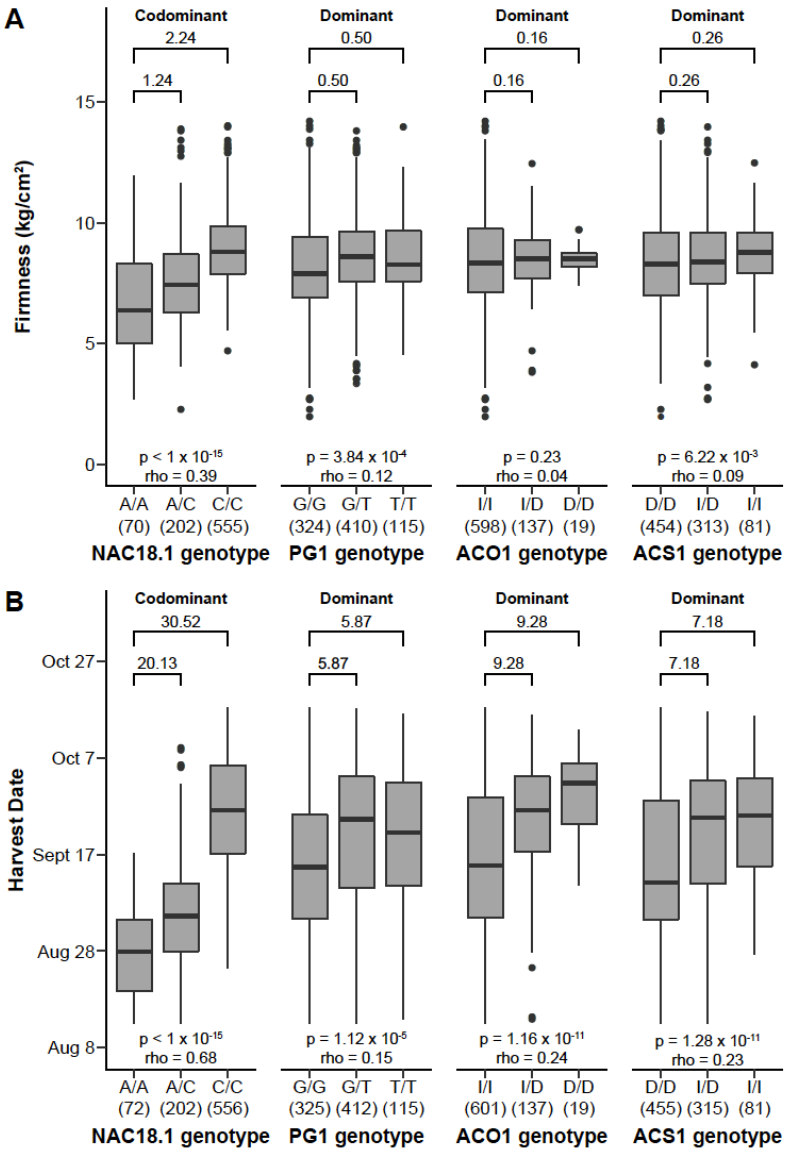
254 In order to directly compare the NAC18.1 D5Y marker that we identified with previously
255 published markers, we developed HRM genotyping assays for the D5Y SNP, as well as markers
256 at *ACS1, ACO1,* and *PG1*. We used these assays to genotype accessions from the ABC orchard
257 (Supplementary Table S2). While this orchard largely consists of clones of individuals present in
258 the population used in our previous GWAS (i.e. the USDA germplasm collection), it also
259 contains a number of additional accessions. More importantly, for the purposes of phenotyping,
260 the ABC orchard is planted in an incomplete block design that allows for modeling of location
261 effects using REML methods (Migicovsky *et al.*, 2018). Using this approach, we generated
262 firmness and harvest date measures for over 800 unique accessions for which HRM genotype
263 data were available.

264 We evaluated the mode of inheritance for each genetic marker and phenotype
265 combination and found that, while the effects of the markers at *ACS1, ACO1,* and *PG1* were all
266 dominant, the D5Y marker at *NAC18.1* had a codominant effect. As a result, accessions that
267 were heterozygous for D5Y had firmer fruit (an increase of 1.2 kg cm^{-2}) and a later harvest date
268 (20.13 days) than those that were homozygous for the 'soft' A allele. The combined effect of two
269 C alleles was notably stronger than the effect of a single C allele, increasing firmness by 2.24 kg

270 cm^{-2} and harvest date by a month (30.52 days), when compared to AA genotypes. The difference

271 between firm/late and soft/early genotypes for D5Y was at least 4 times higher for firmness and

272 3 times higher for harvest date than for the markers at *ACS1, ACO1,* and *PG1* (Figure 1).



273

274 **Figure 1. Distribution of phenotypic variation across genotypic classes for markers at**

275 ***NAC18.1*, *PG1*, *ACO1*, and *ACS1*.** A. Boxplots of variation in firmness across each marker B.

276 Boxplots of variation in harvest date across each marker. The lower and upper hinges of the

boxplots correspond to the first and third quartiles. The putative inheritance model, allele effect, correlation (rho) and *p*-value determined using a Spearman's rank correlation test, and the number of accessions within each genotypic class are listed.

To evaluate the distribution of firmness/harvest date markers across commercial cultivars, we present the genotypes for all markers genotyped here in nine of the top ten apple cultivars sold in the USA (Table 1). All nine cultivars were homozygous for the favorable (firm) C allele of *NAC18.1*, while only 2 to 4 of the cultivars had homozygous favorable genotypes for the other markers. Among the top cultivars, only 'Fuji', released in 1962, was homozygous for favorable alleles at all markers, while 'McIntosh', initially discovered in 1811 and commercially released in 1870, was homozygous for undesirable alleles at all markers except for the *NAC18.1* marker.

300 **Table 1. Genotypes of markers for 9 of the 10 most widely sold apple cultivars in the United**
301 **States.**

Release Date	Cultivar	Genotypes[a]			
		NAC18.1	*ACS1*	*ACO1*	*PG1*
1868	Granny Smith	+	±	–	+
1870	McIntosh	+	–	–	–
1895	Red Delicious	+	±	+	±
1914	Golden Delicious	+	±	-	±
1952	Braeburn	+	±	n.d.	±
1962	Fuji	+	+	+	+
1974	Gala	+	+	±	±
1990	Cripps Pink	+	±	–	+
1991	Honeycrisp	+	±	–	+

302 [a] Genotypes are coded as + for homozygous, favorable; – for homozygous, unfavorable; ± for
303 heterozygous.
304
305 ***Resequencing of* NAC18.1**
306 Since the D5Y SNP is predicted to result in an amino acid change in the NAC18.1
307 protein and LD decay is generally high in apple (r^2 decayed to < 0.2 within 100 bp), it is possible
308 that D5Y is causative for the early/firm phenotype (Migicovsky *et al.*, 2016). However, GBS
309 sequence data are too sparse to rule out additional polymorphisms in LD with D5Y contributing
310 to the phenotype. To better understand allelic diversity of *NAC18.1*, we selected 6 cultivars each
311 of the D5Y A/A, A/C, and C/C genotypes, based on our HRM genotyping data. For homozygous

312 cultivars, single alleles were sequenced, while for the heterozygous samples we resequenced
313 both alleles, resulting in *NAC18.1* sequences from 24 haplotypes. These were compared to two
314 reference sequences from the GDDH13 v1.1 apple genome (Daccord *et al.*, 2017):
315 *Md03g1222600* and *Md11g1239900*, representing *NAC18.1* and its closest paralog, respectively.
316 In addition to confirming the expected D5Y genotype in all individuals, a number of
317 additional SNPs and indels were revealed within both coding and non-coding regions of the
318 *NAC18.1* allele. Multiple sequence alignment and subsequent phylogenetic analysis indicated
319 two major clades corresponding to the D5Y A and C genotypes (Figure 2A). SNPs and small
320 indels were observed throughout the sequenced region. Notably, in addition to the D5Y amino
321 acid change, several additional amino acid changes were observed between sequences from D5Y
322 genotypes and the reference sequence of NAC18.1. For example, near the site of the D5Y
323 polymorphism, all "A" haplotypes also had a 12 nucleotide insertion that introduced the amino
324 acid sequence QPQP (Figure 2B).
325 Taken together, these results indicate that the *NAC18.1* locus exhibits a number of
326 polymorphisms that are in strong LD with each other across the 2.3 kb that we sequenced. Many
327 of these are candidates for causality, as they affect either the coding sequence or promoter of the
328 gene. On the other hand, it is possible the signal at D5Y is driven by a causal variant outside of
329 the 2.3kb region sequenced here that acts completely independently from *NAC18.1*. This led us
330 to look for further evidence of the involvement of NAC18.1 in the ripening process in apple.
331

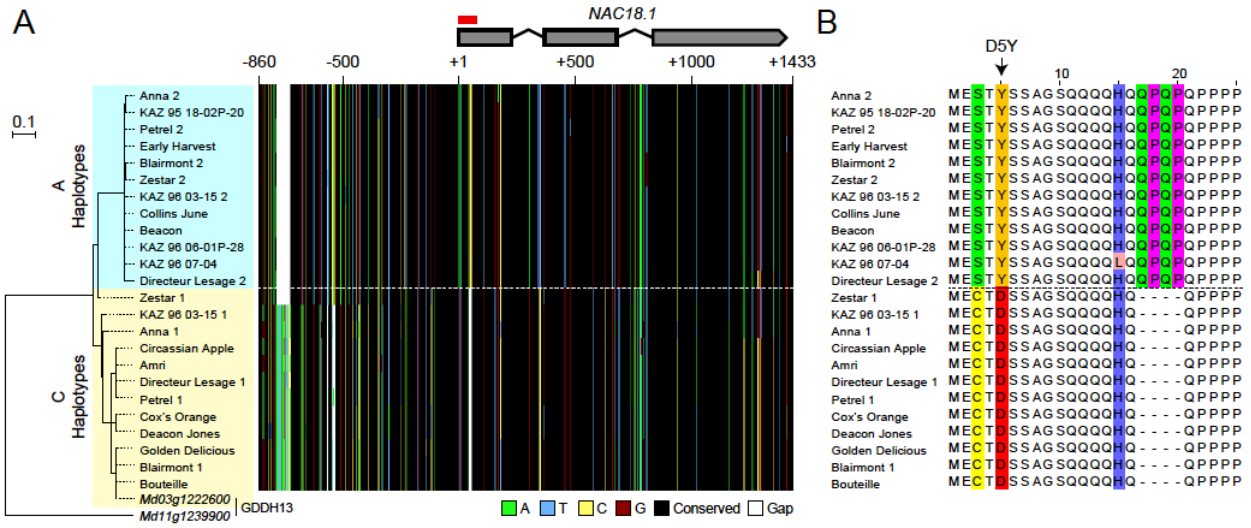


Figure 2. Polymorphisms in LD with the D5Y SNP. A. Maximum likelihood phylogenetic tree of 24 *NAC18.1* sequences from 18 apple cultivars and the reference genome (GDDH13 v1.1) sequences of *NAC18.1* (*Md03g1222600*) and its closest homolog (*Md11g1239900*). A graphical representation of the 2.3 kb multiple sequence alignment is also shown. B. Amino acid sequence alignment of the N-terminal region of NAC18.1, illustrating additional variation in the coding sequence strongly correlated with the D5Y variant. The complete amino acid sequence alignment is given in Supplemental Figure S1.

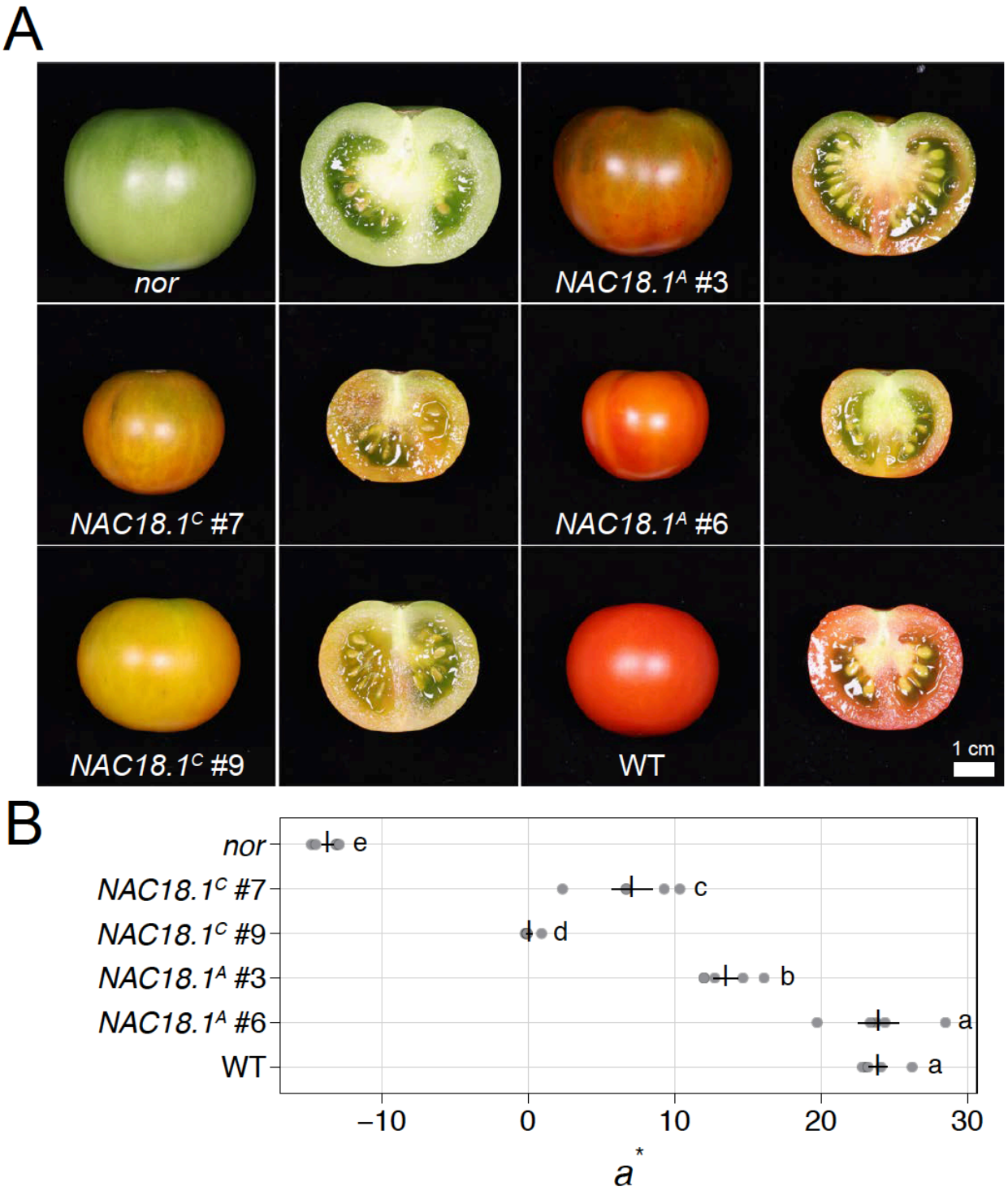
Transgenic complementation of the tomato non-ripening (nor) *mutant*

A BLAST search of predicted proteins from the ITAG 3.20 tomato genome (https://solgenomics.net/) using NAC18.1 as a query identified only two sequences matching the full length of the query: *Solyc10g006880* (*NOR*) and *Solyc07g063420* (*NOR-LIKE 1*). To test the functional conservation of *NAC18.1* and *NOR*, we introduced transgenic constructs individually conferring constitutive expression of each of the *NAC18.1* haplotype CDSs into the tomato *nor*

349 mutant. Two independent lines for each construct were characterized in the T1 generation with
350 respect to their ability to rescue the ripening deficiency of the *nor* mutant. In contrast to *nor*, all
351 four lines changed color at maturity, although internal color change did not occur to the same
352 extent as observed in a WT control (Figure 3A). To complement this qualitative phenotypic
353 assessment, we also conducted quantitative colorimetry of the surface of fruits (Figure 3B). Fruit
354 from all the transgenic lines exhibited a significant increase in the *a** (green-red) component of
355 CIELAB (International Commission on Illumination 1976) color space relative to the *nor*
356 mutant, although only *NAC18.1*^{A} *#6* achieved similar *a** levels to WT.

357 The different degrees to which ripe fruit color was restored in the transgenic plants might
358 be a consequence of either the different alleles of *NAC18.1*, or of different levels of transgene
359 expression in each line. To address this, we analyzed the expression level of *NAC18.1* using
360 qRT-PCR primers designed to target both *NAC18.1* alleles. Expression levels of the NAC18.1
361 transgene were not statistically different ($p > 0.5$) between each independent line (Figure 4A).
362 Next, we measured the expression level of several genes associated with tomato ripening
363 physiology: *PHYTOENE SYNTHASE 1 (PSY1)* encodes an early enzyme of carotenoid synthesis
364 responsible for the production of red pigments during fruit ripening (Fray and Grierson, 1993).
365 Consistent with the green color of mature *nor* fruit, expression of *PSY1* is impaired in the *nor*
366 mutant (Osorio *et al.*, 2011); *POLYGALACTURONASE 2* (*PG2*) encodes an enzyme catalyzing
367 pectin depolymerization associated with fruit softening (Biggs and Handa, 1989); and *1-*
368 *AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 2* (*ACS2*) encodes an enzyme that
369 synthesizes 1-aminocyclopropane-1-carboxylate, the immediate precursor of ethylene
370 (Nakatsuka *et al.*, 1998).

371 Expression of all three marker genes was enhanced in the *NAC18.1* transgenic lines
372 relative to the *nor* mutant control, although not to the same extent as observed in WT ripe fruit
373 (Figure 3B-D). In contrast to the consistent level of NAC18 expression observed in each line, the
374 marker genes were more variable in their expression level between lines. A similar pattern was
375 observed for all marker genes, with the $NAC18.1^{C}$ #9 line showing the smallest induction of
376 marker gene expression relative to *nor*. In the case of *PG2* and *ACS2*, the difference in
377 expression in $NAC18.1^{C}$ #9 was not statistically significant relative to *nor* (p = 0.09 and 0.18,
378 respectively). Consistent with these results, fruit from this line also exhibited the lowest amount
379 of color development (Figure 3B). Taken together, these results indicate that a canonical ripening
380 program can be induced in the tomato *nor* mutant through the heterologous expression of either
381 apple *NAC18.1* allele.
382



383

384 **Figure 3. Transgenic complementation of the tomato *nor* mutant.** A. Mature fruits of the

385 tomato *nor* mutant and four independent T1 transgenic lines constitutively expressing either of

386 two alleles of the *NAC18.1* transgene, $NAC18.1^{C}$ and $NAC18.1^{A}$ and isogenic WT control (cv.

387 Ailsa Craig). B. Quantitative colorimetry of the fruit surface of *nor,* the transgenic fruit and WT
388 fruit. The *a** component (green-red axis) is shown, with the mean ± SE superimposed in black
389 over the raw values in gray (n = 5). Genotypes not sharing a letter (a-e) are statistically distinct
390 by one-way ANOVA and Tukey's HSD test ($p < 0.05$).
391
392
393 **Discussion**
394 Genomics-assisted breeding has tremendous potential in perennial crops like apple,
395 where a lengthy juvenile phase and large plant size make phenotyping at the adult stage time-
396 consuming and expensive. However, the genetic markers used for culling progeny at the seedling
397 stage must accurately predict the trait of interest at the adult stage in order for genomics-assisted
398 breeding to be effective (Luby and Shaw, 2001). Firmness and harvest date, which are highly
399 correlated, are key breeding targets in apple, and markers in three genes (*ACS1*, *ACO1*, and *PG1*)
400 are widely used in genomics-assisted apple breeding programs to predict firmness. However,
401 recent GWAS analyses for firmness and harvest date have failed to identify significant
402 associations at any of these loci, but rather have uncovered a single major effect locus at the
403 *NAC18.1* gene (Migicovsky et al. 2016; McClure et al. 2018; Larsen et al. 2019). Based on the
404 results of this current study, we propose several possible explanations for these findings.
405 First, the markers in *ACS1*, *ACO1*, and *PG1* were identified using linkage mapping in bi-
406 parental crosses, which only captured variation segregating in the progeny of those crosses.
407 These mapping studies generally made use of commercially successful cultivars, and here we
408 show that the most commercially successful apple cultivars in the USA are all homozygous for
409 the desirable *NAC18.1* alleles (Table 1). Thus, the *NAC18.1* alleles we identified via GWAS may

410 not have segregated in the progeny of the crosses used to discover the markers at *ACS1*, *ACO1*
411 and *PG1*. This may be the primary reason *NAC18.1* was not identified using traditional linkage
412 mapping approaches. Second, the most extensive GWAS we have performed to date may have
413 failed to detect association signals at *ACS1, ACO1* and *PG1* since the SNP density used (~1 SNP
414 every 100kb) provided poor genome-wide coverage, given the rapid LD decay observed in our
415 GWAS population (r^2 decayed to < 0.2 within 100 bp). For example, the SNP closest to *ACO1* in
416 our GWAS was >100 kb away and was thus unlikely to be in LD with genetic variants within
417 *ACO1* (Migicovsky et al. 2016). Given these limitations, we examined the value of each of these
418 markers to predict firmness and harvest date in a diverse collection of over 750 unique apple
419 accessions.

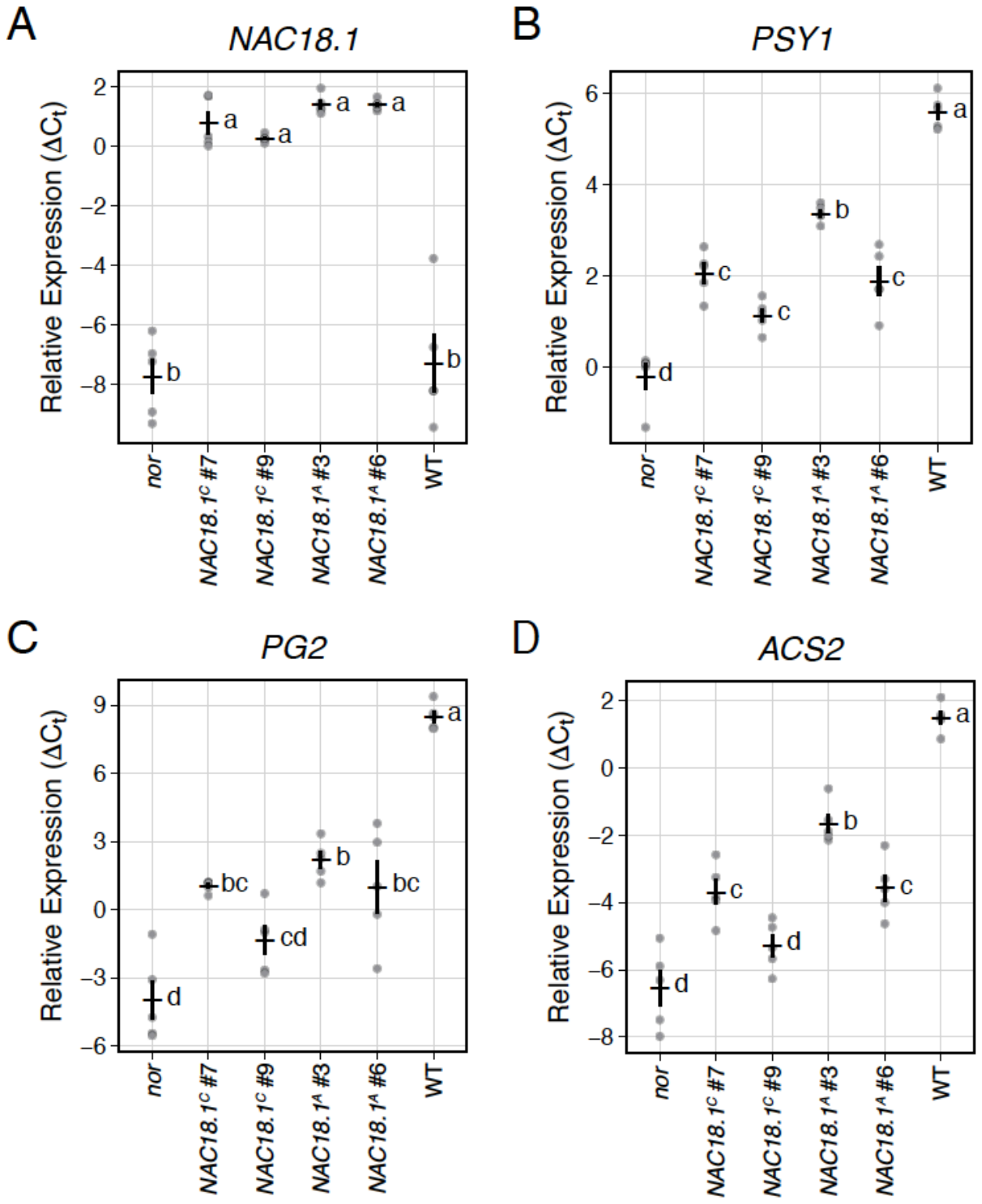
420 The correlation test between each of the markers and the two phenotypes, harvest date
421 and firmness, revealed statistically significant ($P < 0.05$) associations in every case except one
422 (Figure 1). ACO1 was not correlated with firmness in our data, which is consistent with a recent
423 study that found that a marker for ACO1 did not explain any of the variation in crispness or
424 firmness across 207 accessions from New Zealand's apple breeding program (Chagne et al.,
425 2019). Our observation that most of the marker-trait tests that we performed resulted in
426 significant associations is not surprising: many, if not most, markers genome-wide are expected
427 to be correlated with harvest date and firmness since the genetic structure of our apple population
428 is strongly correlated with these traits. For example, without correcting for the effects of
429 population structure, 39% and 17% of genome-wide SNPs were significantly associated (P <
430 0.05) with harvest date and firmness, respectively, in the USDA collection, which is genetically
431 identical to most of the population studied here (Migicovsky et al, 2016). Because we performed
432 single-marker association tests in the present study, we were unable to account for population

433 structure using genome-wide markers as is customarily done when performing GWAS. Thus, we
434 were only able to compare the relative power of each marker to predict firmness and harvest
435 date. We found that the D5Y marker in *NAC18.1* had a 3 to 14 times greater effect on firmness
436 and harvest than the markers in *ACO1*, *ACS1* and *PG1* (Figure 1). The superior predictive power
437 of the NAC18.1 marker suggests that it has more potential to advance genomics-assisted
438 breeding for firmness and harvest date compared to markers currently in use. Results from our
439 forthcoming GWAS that include all of these markers and properly account for the effects of
440 relatedness and population structure will be necessary to definitively conclude which, if any, of
441 these traditionally used markers predict firmness and/or harvest date in diverse collections of
442 apple.

443 While the D5Y mutation in *NAC18.1* is a strong functional candidate variant, we
444 discovered numerous DNA sequence variants in NAC18.1, often in perfect LD with D5Y, that
445 are also putatively functional (Figure 2). The magnitude of DNA polymorphism at this locus,
446 and the multitude of putatively causal variants, are consistent with the results of Larsen et al.
447 (2019), who found 18 SNPs and 2 indels within *NAC18.1* across 11 apple accessions. Given the
448 pattern of LD we observed across *NAC18.1* (Figure 2), we considered the possibility that the
449 GWAS signals at *NAC18.1* are in fact driven by a causal variant outside of the *NAC18.1* coding
450 sequence that acts independently of the *NAC18.1* gene. Since *NAC18.1* is a homolog of the well-
451 known ripening gene *NOR* in tomato, we used tomato as a model to explore the role of *NAC18.1*
452 in the ripening process.

453 We observed enhanced ripening in the *nor* mutant following heterologous expression of
454 either of the *NAC18.1* alleles. Notably, the synthesis of carotenoids was restored in the
455 transgenic fruit, resulting in visual color change that approached the level of WT at the fruit

456 surface (Figure 3B), although dissection of fruit revealed incomplete internal color change in all
457 lines (Figure 3A). Visual color change was consistent with the expression of the carotenoid
458 biosynthetic gene *PSY1* (Figure 4B). Gene expression analysis further confirmed the induction of
459 genes involved in ripening-associated cell wall remodeling (Figure 4C) and ethylene synthesis
460 (Figure 4D). Although the expression level of the *NAC18.1* transgene was comparable across
461 transgenic lines (Figure 4A), the level of ripening marker induction in the distinct lines was
462 significantly different (Figure 4B-D). We had hypothesized that an allele-specific trend in our
463 transgenic experiments would indicate that coding sequence variants are responsible for the
464 association of *NAC18.1* with variation in apple ripening. While our results do not indicate such a
465 trend, they do confirm that both alleles of *NAC18.1* encode functional proteins that can promote
466 ripening.


A
NAC18.1
Relative Expression (ΔCt)
2
0
−2
−4
−6
−8
a
a
a
a
b
b
nor
NAC18.1C #7
NAC18.1C #9
NAC18.1A #3
NAC18.1A #6
WT
B
PSY1
Relative Expression (ΔCt)
6
4
2
0
a
b
c
c
c
d
nor
NAC18.1C #7
NAC18.1C #9
NAC18.1A #3
NAC18.1A #6
WT
C
PG2
Relative Expression (ΔCt)
9
6
3
0
−3
−6
a
b
bc
bc
cd
d
nor
NAC18.1C #7
NAC18.1C #9
NAC18.1A #3
NAC18.1A #6
WT
D
ACS2
Relative Expression (ΔCt)
2
0
−2
−4
−6
−8
a
b
c
c
d
d
nor
NAC18.1C #7
NAC18.1C #9
NAC18.1A #3
NAC18.1A #6
WT

467

468

469 **Figure 4. Ripening marker gene expression in transgenic tomatoes.** Gene expression was
470 evaluated as the difference in threshold cycle (ΔC_t, log_2 scale) by qRT-PCR using *RPL2* as a
471 reference gene. The mean ± SE is superimposed in black over the raw values in gray (n = 5).
472 Genotypes not sharing a letter (a-d) are statistically distinct by one-way ANOVA and Tukey's
473 HSD test ($p < 0.05$). A. The *NAC18.1* transgene. B. *PSY1*, encoding the phytoene synthase
474 carotenoid biosynthetic gene. C. *PG2*, encoding the major ripening-associated polygalacturonase
475 involved in pectin hydrolysis. D. *ACS2*, encoding 1-aminocyclopropane-1-carboxylate synthase,
476 a ripening-associated isoform of the ethylene biosynthesis pathway.
477
478
479 In tomato, *nor* was first identified as a spontaneous mutation in an heirloom cultivar
480 (Tigchelaar *et al.*, 1973). While this *nor* allele exhibits recessive behavior and has been assumed
481 to be a complete loss-of-function, due to a 2 nt deletion resulting in a truncated protein
482 (Giovannoni *et al.*, 2004), it was recently demonstrated that it is actually a dominant negative
483 allele (Wang *et al.*, 2019; Gao *et al.*, 2019). These studies used CRISPR/Cas9 to generate *bona*
484 *fide* null alleles of *nor* , which showed evidence of ripening relative to the spontaneous *nor*
485 allele, although to a lesser extent than WT fruit. In light of these studies, we speculate that the
486 action of the *NAC18.1* transgenes was likely attenuated by the dominant-negative activity of the
487 spontaneous *nor* allele used in our work. Further heterologous characterization of *NAC18.1*
488 would likely benefit from the use of null *nor* mutants, or double mutants of *nor* and *nor-like 1*
489 (Gao *et al.*, 2018). This may allow for more precise quantitative comparisons between alleles in
490 order to resolve whether differences in the coding sequence of *NAC18.1* confer different degrees
491 of ripening.

492 Although we are unable to conclude whether polymorphisms in coding or regulatory
493 sequences affect the activity of *NAC18.1* in apple, an increasing body of evidence generated
494 using tomato as a model system indicates that coding sequence polymorphisms of *NOR* can
495 influence firmness and timing of ripening. For example, the Portuguese Alcobaça cultivar of
496 tomato has firm fruit, delayed ripening, and long shelf life that is conferred by the *alcobaça*
497 mutation, and a recent study has shown that *ALCOBACA* is allelic with *NOR*, and that the
498 *alcobaça* allele of *NOR* contains a valine to aspartate mutation at position 106 within the NAC
499 domain (Kumar *et al.*, 2018). An additional complete loss of function allele of *NOR* was also
500 found in another long shelf life tomato cultivar (Kumar *et al.*, 2018).

501 Our analysis of *NAC18.1* sequences revealed a number of mutations that are candidates
502 for causal association with ripening phenotypes. For example, we observed an insertion of four
503 amino acids (QPQP) 11 amino acids downstream of the D5Y mutation in all A haplotypes
504 upstream of the NAC DNA binding domain. Glutamine-rich sequences are common in
505 eukaryotic transcription factors, and polymorphisms in these motifs have been shown to alter the
506 activity of transcriptional activators (Atanesyan *et al.*, 2012). On the other hand, we also
507 observed several SNPs resulting in amino acid changes within the DNA binding domain and C-
508 terminal transcriptional activator domain of NAC18.1. Clearly, resolution of a causal
509 polymorphism within the coding sequence or promoter of *NAC18.1* will require substantial
510 additional effort. In this regard, we are resequencing the *NAC18.1* locus in our complete
511 diversity panel in order to identify intragenic recombination that is informative in combination
512 with our long-term phenotyping of the collection.

513 In conclusion, the results presented here provide evidence of the superiority of GWAS for
514 the identification of genetic markers associated with firmness and harvest date in diverse

515 germplasm. By validating NAC18.1 as a functional candidate for apple fruit ripening, we lay the
516 groundwork for future efforts to compare the effect of different NAC18.1 haplotypes and
517 determine the precise causal variant(s) underlying this agriculturally important gene. Ultimately,
518 we envision that the identification of precise causal variants for ripening in apple could be
519 exploited using gene editing for fast and efficient introgression of this desirable trait.
520

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533

534 **Supplemental Figure Legends**

535 **Supplemental Figure S1. Complete amino acid sequence alignment of 24 NAC18.1**
536 **haplotypes.**

537

538 **Supplemental Table Legends**

539 **Supplemental Table S1. PCR primers used in this work.**

540

541 **Supplemental Table S2. Genotypes of *NAC18.1* (D5Y), *ACS1*, and *ACO1* across 866 apple**
542 **accessions.**

543

544 **References**

545 **Atanesyan L, Günther V, Dichtl B, Georgiev O, Schaffner W**. 2012. Polyglutamine tracts as
546 modulators of transcriptional activation from yeast to mammals. Biological Chemistry **393**, 63–
547 70.

548 **Atkinson RG, Sutherland PW, Johnston SL, Gunaseelan K, Hallett IC, Mitra D, Brummell**
549 **DA, Schröder R, Johnston JW, Schaffer RJ**. 2012. Down-regulation of
550 POLYGALACTURONASE1 alters firmness, tensile strength and water loss in apple (Malus x
551 domestica) fruit. BMC Plant Biology **12**, 129.

552 **Bates D, Mächler M, Bolker B, Walker S**. 2015. Fitting Linear Mixed-Effects Models Using
553 lme4. Journal of Statistical Software **67**, 1–48.

554 **Biggs MS, Handa AK**. 1989. Temporal Regulation of Polygalacturonase Gene Expression in
555 Fruits of Normal, Mutant, and Heterozygous Tomato Genotypes. Plant Physiology **89**, 117–125.

556 **Blanpied GD, Silsby KJ**. 1992. Predicting Harvest Date Windows for Apples.

557 **Chang S, Puryear J, Cairney J**. 1993. A simple and efficient method for isolating RNA from
558 pine trees. Plant Molecular Biology Reporter **11**, 113–116.

559 **Costa F, Peace CP, Stella S, Serra S, Musacchi S, Bazzani M, Sansavini S, Van de Weg**
560 **WE**. 2010. QTL dynamics for fruit firmness and softening around an ethylene-dependent
561 polygalacturonase gene in apple (Malus×domestica Borkh.). Journal of Experimental Botany **61**,
562 3029–3039.

563 **Costa F, Stella S, Van de Weg WE, Guerra W, Cecchinel M, Dallavia J, Koller B, Sansavini**
564 **S**. 2005. Role of the genes Md-ACO1 and Md-ACS1 in ethylene production and shelf life of
565 apple (Malus domestica Borkh). Euphytica **141**, 181–190.

566 **Curtis MD, Grossniklaus U**. 2003. A Gateway Cloning Vector Set for High-Throughput
567 Functional Analysis of Genes in Planta. Plant Physiology **133**, 462–469.

568 **Daccord N, Celton J-M, Linsmith G, *et al.*** 2017. High-quality *de novo* assembly of the apple
569 genome and methylome dynamics of early fruit development. Nature Genetics **49**, 1099–1106.

570 **Edgar RC**. 2004. MUSCLE: multiple sequence alignment with high accuracy and high
571 throughput. Nucleic Acids Research **32**, 1792–1797.

572 **Fray RG, Grierson D**. 1993. Identification and genetic analysis of normal and mutant phytoene
573 synthase genes of tomato by sequencing, complementation and co-suppression. Plant Molecular
574 Biology **22**, 589–602.

575 **Gao Y, Wei W, Zhao X, *et al.*** 2018. A NAC transcription factor, NOR-like1, is a new positive
576 regulator of tomato fruit ripening. Horticulture Research **5**, 75.

577 **Gao Y, Zhu N, Zhu X, *et al.*** 2019. Diversity and redundancy of the ripening regulatory
578 networks revealed by the fruitENCODE and the new CRISPR/Cas9 CNR and NOR mutants.
579 Horticulture Research **6**, 39.

580 **García-Gómez BE, Salazar JA, Dondini L, Martínez-Gómez P, Ruiz D**. 2019. Identification
581 of QTLs linked to fruit quality traits in apricot (Prunus armeniaca L.) and biological validation
582 through gene expression analysis using qPCR. Molecular Breeding **39**, 28.

583 **Giovannoni J, Nguyen C, Ampofo B, Zhong S, Fei Z**. 2017. The Epigenome and
584 Transcriptional Dynamics of Fruit Ripening. Annual Review of Plant Biology **68**, 61–84.

585 **Giovannoni J, Tanksley S, Vrebalov J, Noensie F**. 2004. NOR gene compositions and
586 methods for use thereof.

587 **Harada T, Sunako T, Wakasa Y, Soejima J, Satoh T, Niizeki M**. 2000. An allele of the 1-
588 aminocyclopropane-1-carboxylate synthase gene (Md-ACS1) accounts for the low level of
589 ethylene production in climacteric fruits of some apple cultivars. Theoretical and Applied
590 Genetics **101**, 742–746.

591 **Kumar S, Stecher G, Tamura K**. 2016. MEGA7: Molecular Evolutionary Genetics Analysis
592 Version 7.0 for Bigger Datasets. Molecular Biology and Evolution **33**, 1870–1874.

593 **Kumar R, Tamboli V, Sharma R, Sreelakshmi Y**. 2018. NAC-NOR mutations in tomato
594 Penjar accessions attenuate multiple metabolic processes and prolong the fruit shelf life. Food
595 Chemistry **259**, 234–244.

596 **Larsen B, Migicovsky Z, Jappesen AA, Gardner KM, Toldam-Andersen TB, Myles S,**
597 **Ørgaard M, Petersen MA, Pedersen C**. 2019. Genome-Wide Association Studies in Apple
598 Reveal Loci for Aroma Volatiles, Sugar Composition and Harvest Date. The Plant Genome **in**
599 **press**.

600 **Lenth RV**. 2016. Least-Squares Means: The R Package lsmeans. Journal of Statistical Software
601 **69**, 1–33.

602 **Lü P, Yu S, Zhu N, *et al.*** 2018. Genome encode analyses reveal the basis of convergent
603 evolution of fleshy fruit ripening. Nature Plants **4**, 784.

604 **Luby JJ, Shaw DV**. 2001. Does Marker-assisted Selection Make Dollars and Sense in a Fruit
605 Breeding Program? HortScience **36**, 872–879.

606 **Mathew IE, Agarwal P**. 2018. May the Fittest Protein Evolve: Favoring the Plant-Specific
607 Origin and Expansion of NAC Transcription Factors. BioEssays **40**, 1800018.

608 **McClure KA, Gardner KM, Douglas GM, *et al.*** 2018. A Genome-Wide Association Study of
609 Apple Quality and Scab Resistance. The Plant Genome **11**, 0.

610 **Migicovsky Z, Gardner KM, Money D, *et al.*** 2016. Genome to Phenome Mapping in Apple
611 Using Historical Data. The Plant Genome **9**, 0.

612 **Migicovsky Z, Li M, Chitwood DH, Myles S**. 2018. Morphometrics Reveals Complex and
613 Heritable Apple Leaf Shapes. Frontiers in Plant Science **8**.

614 **Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A**. 1998.
615 Differential Expression and Internal Feedback Regulation of 1-Aminocyclopropane-1-
616 Carboxylate Synthase, 1-Aminocyclopropane-1-Carboxylate Oxidase, and Ethylene Receptor
617 Genes in Tomato Fruit during Development and Ripening. Plant Physiology **118**, 1295–1305.

618 **Oraguzie NC, Iwanami H, Soejima J, Harada T, Hall A**. 2004. Inheritance of the Md-ACS1
619 gene and its relationship to fruit softening in apple (Malus × domestica Borkh.). Theoretical and
620 Applied Genetics **108**, 1526–1533.

621 **Ornelas-Paz J de J, Quintana-Gallegos BM, Escalante-Minakata P, Reyes-Hernández J,**
622 **Pérez-Martínez JD, Rios-Velasco C, Ruiz-Cruz S**. 2018. Relationship between the firmness of
623 Golden Delicious apples and the physicochemical characteristics of the fruits and their pectin
624 during development and ripening. Journal of Food Science and Technology **55**, 33–41.

625 **Osorio S, Alba R, Damasceno CMB, *et al.*** 2011. Systems Biology of Tomato Fruit
626 Development: Combined Transcript, Protein, and Metabolite Analysis of Tomato Transcription
627 Factor (nor, rin) and Ethylene Receptor (Nr) Mutants Reveals Novel Regulatory Interactions.
628 Plant Physiology **157**, 405–425.

629 **Pirona R, Eduardo I, Pacheco I, *et al.*** 2013. Fine mapping and identification of a candidate
630 gene for a major locus controlling maturity date in peach. BMC Plant Biology **13**, 166.

631 **Shinozaki Y, Nicolas P, Fernandez-Pozo N, *et al.*** 2018. High-resolution spatiotemporal
632 transcriptome mapping of tomato fruit development and ripening. Nature Communications **9**,
633 364.

634 **Solé X, Guinó E, Valls J, Iniesta R, Moreno V**. 2006. SNPStats: a web tool for the analysis of
635 association studies. Bioinformatics **22**, 1928–1929.

636 **Sunako T, Sakuraba W, Senda M, Akada S, Ishikawa R, Niizeki M, Harada T**. 1999. An
637 Allele of the Ripening-Specific 1-Aminocyclopropane-1-Carboxylic Acid Synthase Gene
638 (ACS1) in Apple Fruit with a Long Storage Life. Plant Physiology **119**, 1297–1304.

639 **Tigchelaar EC, Tomes ML, Kerr EA, Barman RJ**. 1973. A new fruit ripening mutant, non-
640 ripening (nor). Rep. Tomato Genet. Coop **23**, 33.

641 **Urrestarazu J, Muranty H, Denancé C, *et al.*** 2017. Genome-Wide Association Mapping of
642 Flowering and Ripening Periods in Apple. Frontiers in Plant Science **8**.

643 **Van Eck J, Keen P, Tjahjadi M**. 2019. Agrobacterium tumefaciens-Mediated Transformation
644 of Tomato. In: Kumar S, Barone P, Smith M, eds. Transgenic Plants: Methods and Protocols.
645 New York, NY: Springer New York, 225–234.

646 **Wang R, Tavano EC da R, Lammers M, Martinelli AP, Angenent GC, Maagd RA de**. 2019.
647 Re-evaluation of transcription factor function in tomato fruit development and ripening with
648 CRISPR/Cas9-mutagenesis. Scientific Reports **9**, 1696.

649 **Wang D, Yeats TH, Uluisik S, Rose JKC, Seymour GB**. 2018. Fruit Softening: Revisiting the
650 Role of Pectin. Trends in Plant Science **23**, 302–310.

651 **Wickham H**. 2016. *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.