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Biochemical and genetic implications of the slow ripening phenotype in peach fruit

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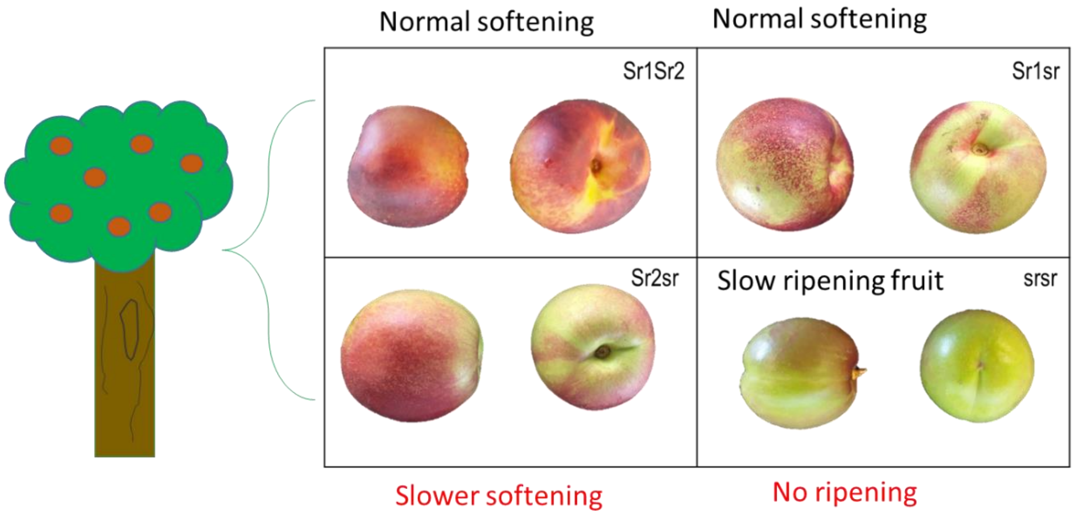
Highlights

- Different allele combinations of the *Sr* gene influence peach ripening physiology and biochemistry.
- The *Sr* gene affects ethylene synthesis, and therefore all the ethylene-dependent ripening changes.
- *Sr2sr* hybrids showed a longer harvest window and improved postharvest behaviour compared to *Sr1sr* and *Sr1Sr2*.
- Selection of *Sr2sr* hybrids could be an efficient strategy for improved shelf life in new peach cultivars.

Abstract

The peach [*Prunus persica* L. (Batsch)] slow ripening (SR) trait is a mutation preventing the normal fruit ripening process. It is determined by a single Mendelian gene (*Sr*/*sr*) located on linkage group 4, where only homozygous individuals for a recessive allele (*sr*) show the SR phenotype and are generally discarded from breeding programs. Ripening-related traits such as fruit weight, firmness loss, ethylene production, ACO activity, sugars and organic acids composition, malondialdehyde, antioxidant capacity and total phenolic content were evaluated in a segregating population for the SR trait during two consecutive harvest seasons and at different maturity stages. Although there is no commercial value for the slow ripening (*srsr*) individuals, our results demonstrate that a heterozygous combination involving *sr* and another allele at this locus (*Sr2*) showed interesting traits including a longer harvest window and improved postharvest behaviour if harvested at the appropriate maturity ($I_{AD} \geq 2$). All these traits seem to be linked to a delayed ripening behaviour mediated, in turn, by a lower ethylene production capacity and an altered sugar (mainly sucrose) and organic acid accumulation/utilisation on-tree. The selection of this allelic combination could be an easy and efficient strategy to obtain new peach cultivars with potentially improved shelf life.

Graphical abstract



Keywords

Breeding, climacteric, fruit quality, firmness, softening, texture.

Introduction

Peach is a typical climacteric fruit, with a rapid softening and short shelf life after harvest that adversely affect its market value. Fruit maturation is a complex and highly coordinated developmental process that affects colour, firmness, taste and flavour. Fruit ethylene production rate has a clear effect on the firmness loss and senescence of the fruit, and therefore in the fruit storage life (Barry and Giovannoni, 2007; Osorio et al., 2011; Giné-Bordonaba et al., 2016).

Peach fruit showing the slow ripening (SR) phenotype fail to ripen. This character was first described in some breeding populations as fruit that remained firm when mature, with a very slow rate of flesh softening, a reduced CO_2 and C_2H_4 production, poor sensory qualities and a high susceptibility to internal breakdown (Brecht et al., 1984, 1982). The same authors also reported that exogenous C_2H_4 application failed to induce ripening as normally observed in other climacteric fruit.

Some years later (Ramming, 1991; Tataranni et al., 2010), the slow-ripening trait was proposed to be controlled by a single gene (*Sr/sr*), and when the recessive *sr* allele was in homozygosis, it prevented the fruit undergoing normal ripening. More recently, the *sr* gene has been mapped on linkage group 4 (G4) and molecular markers for its selection have been developed (Eduardo et al., 2015; Meneses et al., 2016; Nuñez-Lillo et al., 2015). These markers are based on a large deletion of 26.6 kb containing two NAC transcription factors that could be the causal mutation. In fact, genes of the NAC family have been shown to be involved in the regulation of ethylene-mediated ripening in tomato (Osorio et al., 2011) and banana (Chen et al., 2012). A major gene/QTL for maturity date (Eduardo et al., 2015; Pirona et al., 2013) and a QTL for the chilling injury symptoms of fruit fresh mealiness and bleeding (Martínez-García et al., 2013) have also been identified at the *Sr* genomic region. More recently, Botton et al. (2016) proposed that the peach HEC3-like gene FLESHY may be involved in the SR phenotype, having an important role in fruit tissue patterning at early fruit development. Information also exists on the relationship between the ripening behaviour and ethylene production of different SR (*srsr*) fruit (Brecht et al., 1982), but no other studies have ever investigated and compared the physiological and biochemical differences occurring during ripening among *Srsr* and *SrSr* genotypes.

Understanding the effect of the *sr* allele on the softening of peach in combination with the above-mentioned and available molecular marker (Eduardo et al., 2015; Meneses et al., 2016; Nuñez-Lillo et al., 2015), would offer breeders the possibility to include this character in their progenies to obtain new cultivars with potentially longer shelf life. For instance, a slower softening would allow a wider harvesting window, as well as an extended postharvest life. In addition, a better understanding on the biochemical mechanisms underlying such regulatory control of ripening would open the possibility to modify certain biochemical pathways aiming to obtain peaches with extended postharvest life.

Accordingly, the objective of this work was to deep insight the effect of each *sr* allele configuration in the peach maturation/ripening physiology. To do so, we analysed a series of traits such as fruit firmness, ethylene production, I_{AD}, weight, biochemical compounds (glucose, sucrose, malic and citric acid) and antioxidant metabolism related molecules (malondialdehyde, antioxidant capacity, and total phenolic content) along ripening in a SR segregating population for two consecutive harvest seasons.

Material and Methods

Plant material

A segregating F1 progeny (BbxNl) from the cross between the white peach cultivar ‘Belbinette’ (Bb) and the yellow nectarine ‘Nectalady’ (Nl) was used in this study. Trees were planted in the fields at the IRTA Experimental Station in Gimenells (Lleida, Spain) initially on their own roots (2007) and later (2008) grafted on ‘Cadaman’ rootstock.

For fruit phenotyping, including physiological and biochemical measurements, a subset of 25 individuals plus the parents was selected. Available information about the allelic composition of the parents Belbinette (*Sr2sr*) and Nectalady (*Sr1sr*) and offspring from this population (Eduardo et al., 2015; Meneses et al., 2016), was used to select the subset of individuals analyzed. The difference between *Sr1* and *Sr2* could be established by Eduardo et al. (2015) as they were associated with an ~10-day interval in maturity dates of the parents, earlier for *Sr1* and later for *Sr2*. The selection in the progeny was made in order to have represented all the genotypic classes, and the most variability of maturity dates within each class:

- Slow ripening individuals (*srsr*, N=3)
- Individuals heterozygous for the *sr* allele: (*Sr1sr*, N=7) and (*Sr2sr*, N=7)
- Individuals not carrying the *sr* allele (*Sr1Sr2*, N=8)

Fruit growth

On-tree fruit growth (size) and non-destructive evaluation of the fruit maturity stage (DA-value) both from the parents and two hybrids from each allelic class (*srsr, Sr1sr, Sr2sr* and *Sr1Sr2)* was periodically monitored (every 7-10 days) from fruit set to harvest on 10 fruit randomly selected. Fruit size was measured with a digital calliper, as the distance between the suture and the opposite side at the equatorial zone. DA-values, also known as the index of Absorbance difference (I_{AD}), were measured with a commercial equipment

(DA-Meter, TR Turoni, Forli, Italy), which measures non-destructively Vis-spectroscopy according to the I_{AD} index (index of absorbance difference = A670–A720) (Ziosi et al., 2008).

Fruit ripening and ethylene measurements

Forty fruit were harvested from each tree at 3 different maturity stages based on fruit firmness (60-80N (M1), 40-60N (M2), <30N (M3)), I_{AD} values and historical data on their ripening pattern. In the case of sr fruit (srsr), firmness did never reach values <30N, and therefore fruit were harvested at different maturity stages based exclusively in days after full bloom (DAFB). Upon each harvest, fruit were immediately transported to the lab for quality measurements (t0). Fruit was individually weighted in a digital scale. Then, I_{AD} values and firmness were measured on both sides of each fruit using 8 fruits/tree/harvest (total of individuals = 25). I_{AD} values were measured as described above whereas flesh firmness was measured with a digital penetrometer (Model. 53205; Turoni, Forlí, Italy) equipped with an 8-mm diameter plunger tip after the removal of a 1 mm thick slice of skin. Full bloom date for each tree was recorded to calculate days after full bloom (DAFB) at the moment of harvest. In the selected individuals harvest window ranged from early August to mid September.

Ethylene production and firmness were evaluated the same day of harvest (t0) and after 1, 3 and 6 days after harvest (t1, t3, t6). Firmness at t1, t3 and t6 was measured as described for t0 using 8 fruits/tree/harvest. Remaining fruit were stored at 20ºC (70% RH) for the following evaluation times. Ethylene production (uL $kg^{-1}h^{-1}$) was measured in an acclimatized chamber at 20ºC. The fruit (2 or 3 fruit per replicate, and at least 3 replicates per tree and harvest) were placed in airtight 1.5 L flasks. After 2 h incubation, gas samples

(1 mL) were taken from the headspace of the flasks, using a 1 mL syringe, and injected into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) following the methodology described elsewhere (Giné-Bordonaba et al., 2017). Samples were taken again from the headspace of the flasks following the same methodology at t1, t3 and t6.

The fruit flesh from each sample (8 fruits/tree) at each maturity stage (M1, M2 and M3) was frozen at the time of harvest and stored at -80ºC until being used for biochemical measurements.

Biochemical measurements

The enzyme 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO) was extracted as described by Lindo-Garcia et al. (2019) with some modifications. The sample (0.5 g of frozen tissue) was homogenized in 1 mL of buffer containing 400 mM MOPS at pH 7.2, 10% glycerol, 30 mM ascorbic acid sodium salt and PVP 40000 2%. The homogenized was slightly shaken for 10 min at 1ºC and centrifuged at 17,000 x *g* for 30 min at 4°C. Subsequently, the supernatant was stored at -80°C until analysis of the enzyme activity using the methodology described by Giné-Bordonaba et al. (2017).

Sugars (glucose and sucrose) and organic acids (citric and malic acid) were extracted from frozen tissue as described by Giné-Bordonaba et al. (2019) with some modifications. For sugars determination, 2 g of sample were diluted in 5 mL of 62.5% (v/v) aqueous methanol solvent and placed in a thermostatic bath at 55°C for 15 min, mixing the solution with a vortex every 5 min to prevent layering. Then, the samples were centrifuged at 24,000 x *g* for 15 min at 20 °C. The supernatants of each sample were recovered and used for enzyme coupled spectrophotometric determination of glucose (hexokinase) and sucrose (βfructosidase) using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer instructions.

Citric and malic acid were extracted by dissolving 2 g of frozen tissue in 5 mL of distillate water. The solution was slightly shaken for 10 min at room temperature and then centrifuged at 24,000 x *g* for 7 min at 20°C. The resulting supernatant was recovered and used to determine the concentration of citric (citrate lyase/malate dehydrogenase) and malic acid (malate dehydrogenase) using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer instructions.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute Inc. Mean comparisons for the interaction genotype * maturity was evaluated using HSD test at a significance level of $p \leq 0.05$, while comparisons for specific traits between genotypes along storage time or days after full bloom was done by least significant difference values (LSD; $p \leq 0.05$) using critical values of t for two-tailed tests. Correlations between experimental variables were made using Spearman's Rank Correlations and, if required, presented as Spearman's Correlation Coefficient (r) and P value based on a two-tailed test.

Results

Fruit maturation

As described above, the different maturity stages (M1, M2 and M3) were selected based on both firmness and I_{AD} values and generally comprised fruit harvested from 140 to 188 DAFB yet depending on the genotypic class (Table 1). The fruit weight of all the genetic classes increased progressively in the tree during maturation (Fig. 2). However, fruit weight of the SR individuals *(sr/sr*) individuals increased very little, reaching very low weights if compared to the other individuals. In all genotypes, the increase in fruit size seemed to be intimately regulated by the fruit maturity since the increase in fruit size was negatively correlated (R^2 >0.85; Figure 8) to the loss of fruit firmness and the I_{AD} value.

The I_{AD}, which indicates the degree of chlorophyll degradation in the fruit, showed a progressive decrease at different maturities for the genetic classes *Sr1Sr2*, *Sr1sr* and *Sr2sr* (Table 1). In the slow ripening fruit (*sr/sr*), the I_{AD} failed, as expected, to decrease indicating that the chlorophyll levels remained very high and it was not degraded as it occurs in a normal ripening process. Based on the I_{AD} values, the selected maturity classes (M1, M2 and M3) were comparable among the different genotypic classes except for SR individuals that besides not growing (Table 1) did not show a substantial chlorophyll degradation on-tree.

Indeed, fruit growth was similar for all the genetic classes from fruit set to approximately 135 days after full bloom (DAFB) (Fig. 3). However, after approximately 145 DAFB the fruit growth of the SR (*sr/sr*) individuals started to slow down if compared to the rest of individuals, reaching smaller sizes at the end of the fruit development (*ca*. 190 DAFB). Similarly, the degradation of chlorophyll (I_{AD}) in the SR individuals started to slow down at around 155 DAFB compared to the rest of individuals. It can be also observed that the genetic classes with one copy of the *sr* allele (*Sr1sr* and *Sr2sr*) had a slower degradation of chlorophyll at these last stages of the fruit ripening than the rest of the non-slow ripening (*Sr1Sr2*) individuals.

Firmness loss and ethylene production

The flesh firmness of the fruit from genetic class *Sr1Sr2* rapidly began to decrease after 1 day of shelf life (Fig. 4), regardless the initial fruit firmness at harvest (M1, M2 and M3). On the other side, and as expected, the SR genotypes (*sr/sr*) failed to soften during the shelf life period (6d), and their firmness remained over 60N. The hybrids *Sr1sr* and *Sr2sr* showed a different behaviour to the former genetic classes, showing mixed results depending on the fruit maturity at harvest. In detail, these genotypes soften rapidly if harvested at firmness ≤ 60N yet the loss of firmness was prevented if the fruit were harvested at less mature ripening stages (firmness > 60N; $I_{AD} \geq 2$). In the case of the *Sr2sr* individuals, fruit firmness decreased slightly after one day of shelf life (generally referred as softening), but then it remained stable after 3 days of shelf life (non-melting phase) contrasting with *Sr1sr* individuals, where it kept decreasing and hence experienced both softening and melting. Differences on the on-tree flesh firmness reached by the individuals from the four genetic classes were very clear after 190 DAFB (Fig. 4). As expected, slow ripening individuals (*srsr*) showed the highest firmness, individuals without the *sr* allele (*Sr1Sr2*) showed the lowest, whereas the heterozygous classes (*Sr1sr* and *Sr2sr*) showed intermediate values. Fruit from *Sr1Sr2* and *Sr1sr* individuals showed the highest and similar firmness loss (FL) rates (-0.95 and -1.05 N/day, respectively), whereas *Sr2sr* showed a similar FL rates than *sr/sr* individuals (-0.76 and -0.62 N/day, respectively).

We observed clear differences in the ethylene production rates among the four genetic classes in the BbxNl population (Fig. 5). Ethylene production at harvest for M1 was negligible for all the individuals, independently of their genetic class. However, significant differences were observed in the fruit after some days of shelf life at 20ºC. No detectable or negligible levels were found for *srsr* and *Sr2sr* individuals during shelf life, whereas fruit from *Sr1Sr2* trees produced large amounts of ethylene after 12 days of shelf life at 20ºC. Fruit from *Sr1sr* individuals started to produce some ethylene after 13 days of shelf life. The fruit from *Sr2sr* individuals did not produce any ethylene during shelf life when harvested at flesh firmness of 60N. However, they were able to produce ethylene during shelf life when harvested in a more advanced maturity stage (M3, flesh firmness <30N) (results not shown). On the other side, the slow ripening fruit (*sr/sr*) did not produce ethylene during shelf life for any of the three maturity stages investigated herein.

ACC oxidase activity

Similarly to what happened for ethylene production, no ACC oxidase (ACO) activity was observed at harvest for any of the genetic classes when harvested at M1 (Fig. 6). Significant differences were observed at more advanced maturity stages (M2 and M3). At M2, ACO activity was detected in fruit from *Sr1sr* and *Sr1Sr2* individuals, whereas it was almost no detectable for *Sr2sr* and *srsr* individuals. The ACO activity was negligible for any of the maturity stages in the fruit from the SR individuals (*sr/sr*). However, in the case of the *Sr2sr* individuals, the ACO activity significantly increased in the more mature fruit (M3), although reaching much lower levels than *Sr1Sr2* and *Sr1sr* classes at similar maturity stages.

Sugars and organic acids

The pattern of sucrose accumulation in the fruit was significantly different among different genetic classes (Table 1). In general, sucrose increased as fruit ripened on the tree. However, accumulation in fruit from *Sr2sr* and *sr/sr* individuals was slower, reaching much lower levels than the rest of fruit, especially in the slow ripening individuals (*sr/sr*). In contrast, glucose levels decreased throughout maturation in all the genetic classes, except in the *sr/sr* individuals where it slightly increased (Table 1).

Malic acid increased during maturation on the tree for the four genetic classes (Table 1). Thus said, final malate content was significantly lower in the *sr/sr* and *Sr2sr* genotypes. Contrary, citric acid decreased over on-tree maturation in all cases. In this case, *Sr2sr* and *sr/sr* genotypes showed a slower rate of citric acid catabolism, whereas *Sr1Sr2* and *Sr1sr* genotypes showed a rapid drop in the concentration of this acid in the first stages of maturation.

Discussion

Peach ripening involves dramatic changes in the colour, firmness, texture, aroma, sugars and organic acids composition. As for other climacteric fruit, these changes are triggered by rapid changes in the rate of C_2H_4 production (Tonutti et al., 1991; Alexander, 2002; Baró-Muntel et al., unpublished). However, SR peach fruit show a failure to ripen and hence do not undergo such dramatic changes (Brecht et al., 1984; Ramming, 1991). This behaviour may be caused by the inability to synthesize ethylene as in anti-sense tomato (Picton, 1993) and melon mutants (Ayub et al., 1996), or due to problems in the ethylene perception as in the *Nr* tomato mutant (Wilkinson et al., 1995). Non-climacteric phenotypes may be also due to alterations upstream the ripening cascade as in *rin*, *nor* and *Cnr* tomato mutants (Giovannoni, 2001). SR fruit do not respond to exogenous ethylene treatment, as it occurs with *Nr* tomato and PI 161375 melon mutants (Wilkinson et al., 1995; Périn et al., 2002), which demonstrates that SR mutation blocks not only ethylene synthesis but also ethylene perception (Supplementary Fig. 1).

In this study, the effect of some alleles of the *Sr* gene in the ripening physiology and biochemistry as well as in the fruit postharvest behaviour, even when present in heterozygosis, have been investigated. Whereas *Sr1Sr2* and *Sr1sr* allelic combinations resulted in fruit undergoing a normal ripening process, both on- and off-tree, we observed changes in several ripening related parameters in offspring with the *Sr2sr* genotype. Individuals carrying the *sr* allele as *Sr1sr*, *Sr2sr* and *srsr* produced little or no ethylene when harvested at $I_{AD} \geq 2$. The results observed at the firmness loss and ethylene production rate in the individuals carrying the *sr* allele, and especially in *Sr2sr* individuals, indicate changes at the biochemical or physiological level resulting in inhibition of normal ripening. Such differences at the fruit ethylene production capacity were not explained by ACO activity since all individuals at M1 (when most of the differences on the postharvest ripening occurred among individuals) showed minimal enzyme activity (Fig. 6). Thus said, our results clearly indicate that the *Sr* gene undoubtedly affected ethylene synthesis (Fig. 5 and 6), and therefore altered all the ethylene-dependent ripening changes. Such results are in agreement with Brecht et al. (1984) who reported that SR fruit harvested and stored at 20ºC, showed delayed and reduced climacteric peaks of respiration and ethylene production if compared to non-SR 'Fantasia' fruit. As reported by other authors (Botton et al., 2016), SR fruit maintained typical immaturity traits throughout development. However, these authors suggested a disturbance at the level of fruit patterning as the cause for SR phenotype.

Previous work by Eduardo et al., (2015) mapped the *sr* gene in the same region of linkage group 4 (G4), where a NAC transcription factor candidate gene (ppa008301m) is located. This gene has been proposed as a candidate gene for the maturity date (*MD*) trait (Pirona et al., 2013). Genes from the NAC family have been shown to be involved in the regulation of ethylene mediated ripening in tomato (Osorio et al., 2011) and banana (Shan et al., 2014, 2012). Rapid softening occurring at late ripening stages of peach requires significant levels of ethylene (Hayama et al., 2006, 2003), and therefore decreases in the ethylene production rates as observed in *Sr2sr* fruit had a direct effect on the fruit softening rate. Recently, several candidate genes for MD and mealiness (M) traits have been identified in the same region of G4 (Nuñez-Lillo et al., 2015), which would explain the high susceptibility to internal breakdown found in the SR fruit. Accordingly, Giné-

Bordonaba et al. (2016) also reported that a greater capacity of the fruit to produce ethylene after cold storage was associated to lower mealiness incidence in peach fruit.

Fruit from *srsr* individuals failed to develop normal ripening traits such as fruit size, color, aroma and flavour. Whether other loci are also responsible for the altered fruit weight in SR individuals is still unknown, what is clear is that the SR locus clearly altered this fruit attribute. In the peach ripening model (Lü et al., 2018), ethylene-activated NAC transcription factor binds to the promoter of key fruit ripening genes such as those involved in pigment accumulation, volatile secondary metabolite production, cell wall softening and sugar accumulation. Therefore, the *srsr* allelic combination in the SR fruit would disrupt the activation cascade triggered by the NAC transcription factor in some way. Both the peach and melon NAC loci are located in quantitative trait loci that are associated with late ripening phenotypes (Perin, 2002; Pirona et al., 2013). However, we could not discard the contribution of other transcription factors or regulatory mechanisms such as post-translational regulations in the ripening inhibition in the SR fruit. Indeed, *Sr2sr* fruit, contrary to what occurs in *sr/sr* fruit, developed normal fruit size, normal fruit colour, aroma and flavour, which would demonstrate a normal function of the transcriptional ripening feedback circuit, although with certain delay likely attributed to a blockage of the auxin-ethylene hormonal crosstalk (Botton et al., 2016). This delay could be an interesting trait in a new peach cultivar since it would extend the shelf life of the fruit and the harvest window as detailed herein (Figure 3).

Ethylene is synthesized from S-adenosyl-Lmethionine (SAM) via the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC). The conversion of SAM to ACC is catalysed by ACC synthase, and the subsequent oxidation of ACC to ethylene is catalysed by ACC oxidase (ACO) (Ververidis and John, 1991; Yang and Hoffman, 1984). The direct effect of ethylene production in ACO activity has been proven previously by the strong depression of ACO expression by the treatment with 1-MCP, and its induction by ethephon (Zhang et al., 2012). It has been demonstrated that ACO is one of the proteins with the largest change in relative abundance during the fruit transition from the pre-climacteric ("unripe") to the climacteric ("ripe") phase (Prinsi et al., 2011). The small increase of ACO in the *Sr2sr* genotypes observed in this work demonstrated an altered pattern of ripening, althoug those individuals did not show the SR phenotype. In the peach ripening model (Lü et al., 2018), ethylene transcription factor EIN3 activates the NAC transcription factor, which binds to the ACO and ACS promoters to activate the ethylene synthesis. Therefore, the lower ACO activity observed in the *Sr2sr* individuals could also explain their lower ethylene levels. The same effect of a delayed ripening due to a reduction in the ethylene production has been reported by the expression of the tomato ACO in the antisense orientation in transgenic plants (Ayub et al., 1996; Hamilton et al., 1990). RNA gel blot analyses in normal ripening fruit demonstrated that ACO transcripts greatly increased at late stages of fruit development (Callahan et al., 2004).

Glucose and sucrose contents observed in the NlxBb progeny are similar to those reported in the flesh of other peach cultivars (Moing et al., 1998; Genard et al., 1999; Famiani et al., 2016; Baró-Montel et al., unpublished). As observed in our work, glucose content has been reported to decrease during peach ripening as a result, among others, of the large increase in the fruit volume and therefore, the dilution of the glucose content within the fruit (Famiani et al., 2016). The larger increase in the glucose concentration observed in the slow ripening individuals (*sr/sr;* Table 1) through ripening could be explained by the scarce increase in flesh volume as well as by the lower utilization of sugars as respiratory substrates since ethylene production and respiration are largely depleted in these individuals. In certain fruit species, evidence suggests that sucrose accumulation is linked to the initiation of the ripening process on-tree (i.e. pears; Lindo-García et al., 2019). The lower sucrose content and accumulation during on-tree ripening for *sr/sr* or *Sr2sr* individuals agrees with the results from Botton et al. (2016) and further suggests a putative role of this compound on triggering on-tree ripening also for peach fruit.

Malate together with citrate accounts for a large proportion of the organic acid content of peach flesh (Chapman and Horvat, 1990; Byrne et al., 1991; Moing et al., 1998; Baró-Montel et al., unpublished). As reported for other peach cultivars (Chapman and Horvat, 1990; Byrne et al., 1991; Moing et al., 1998; Famiani et al., 2016; Baró-Muntel et al., unpublished), the concentration of malate in the NlxBb population increased throughout ripening, whereas, that of citrate decreased. On the other hand, while sugars can be synthesized both in fruit and leaves, acids are exclusively synthesized in leaves and then translocated to sink (fruit). Alterations not only at the ethylene level but also in the translocation pathways of these compounds, likely mediated by auxins (Daie et al., 1986) may explain the lower levels of malic acid in *sr/sr* and *Sr2sr* individuals' fruit. Accordingly, our results also support previous findings from Famiani et al. (2016) who pointed out that malate and citrate account only for negligible amounts of the respiratory substrates during peach ripening since no association was found between these compounds and the fruit respiration pattern throughout on-tree ripening (data not shown).

Some other authors have reported an anomalous accumulation pattern of phenolic compounds at the flesh of SR fruit, more typical of lignifying endocarp (Masia et al., 1992). Our results do not support such findings and rather reveal that the fruit antioxidant capacity was similar among individuals. Although there is not commercial value for the slow ripening (*sr/sr*) individuals, the *Sr2sr* hybrids could be interesting due to their longer shelf life and slower rate of firmness loss. Postharvest losses are not only a current problem in developing countries but also in modern supply chains. Therefore, extending shelf life of peach fruit without compromising flavour and texture quality attributes is a desired feature in peach industry and could be accomplished by the selection of *Sr2sr* allelic combination. Selection of this allelic combination through the already available molecular markers (Meneses et al., 2016) could be an easy and efficient strategy to obtain new peach cultivars with potentially improved shelf life. However, our results should be confirmed in other populations with a different genetic background.

The presence of SR phenotypes in the progenies of commercial breeding programs is frequent. Based on the data of Meneses et al. (2016) on a sample of 27 peach cultivars we estimated that the frequency of the *sr* allele is 0.33. While this is probably an overestimation of its real value because the sample of cultivars chosen included some known to carry *sr* in heterozygosis, this indicates that the presence of this allele in the peach elite breeding pool is very high, particularly considering that SR individuals are systematically selected against. This scenario was already pointed out by Eduardo et al. (2015) that proposed that the favourable selection of certain heterozygous combinations of *sr* with other alleles at this locus could counteract the directional selection against *sr*, leading to the maintenance of this allele at intermediate frequencies.

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References

Alexander, L., 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J. Exp. Bot. 53 (377), 2039–2055. https://doi.org/10.1093/jxb/erf072

Ayub, R., Guis, M., Amor, M. Ben, Gillot, L., Roustan, J.P., Latché, A., Bouzayen, M., Pech, J.C., 1996. Expression of Selection of this allelic combination through the already available molecular markers acc oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nat. Biotechnol. 14, 862-866. https://doi.org/10.1038/nbt0796-862

Barry, C.S., Giovannoni, J.J., 2007. Ethylene and Fruit Ripening. J. Plant Growth Regul. 26, 143–159. https://doi.org/10.1007/s00344-007-9002-y

Botton, A., Rasori, A., Ziliotto, F., Moing, A., Maucourt, M., Bernillon, S., Deborde, C., Petterle, A., Varotto, S., Bonghi, C., 2016. The peach HECATE3-like gene FLESHY plays a double role during fruit development. Plant Mol. Biol. 91, 97–114. https://doi.org/10.1007/s11103-016-0445-z

Brecht, J., Kader, A., Ramming, C., 1982. Postharvest physiology of non-ripening nectarine selections. HortScience 17, 490.

Brecht, J., Kader, A., Ramming, D., 1984. Description and postharvest physiology of some slow-ripening nectarine genotypes. J. Amer. Soc. Hort. Sci 109, 596–600.

Callahan, A.M., Scorza, R., Bassett, C., Nickerson, M., Abeles, F.B., 2004. Deletions in an endopolygalacturonase gene cluster correlate with non-melting flesh texture in peach. Funct. Plant Biol. 31, 159–168. https://doi.org/10.1071/FP03131

Chen, J., Chen, J. ye, Wang, J. ning, Kuang, J. fei, Shan, W., Lu, W. jin, 2012. Molecular characterization and expression profiles of MaCOL1, a CONSTANS-like gene in banana fruit. Gene 496 (2), 110-117. https://doi.org/10.1016/j.gene.2012.01.008

Daie, J., Watts, M., Aloni, B., Wyse, R.E., 1986. In vitro and in vivo modification of sugar transport and translocation in celery by phytohormones. Plant Sci. 46 (1), 35-41. https://doi.org/10.1016/0168-9452(86)90129-9

Eduardo, I., Picañol, R., Rojas, E., Batlle, I., Howad, W., Aranzana, M.J., Arús, P., 2015. Mapping of a major gene for the slow ripening character in peach: co-location with the maturity date gene and development of a candidate gene-based diagnostic marker for its selection. Euphytica 205, 627–636. https://doi.org/10.1007/s10681-015-1445-9

Giné-Bordonaba, J., Cantín, C.M., Echeverría, G., Ubach, D., Larrigaudière, C., 2016. The effect of chilling injury-inducing storage conditions on quality and consumer acceptance of different Prunus persica cultivars. Postharvest Biol. Technol. 115, 38–47. https://doi.org/10.1016/j.postharvbio.2015.12.006

Giné-Bordonaba, J., Echeverria, G., Ubach, D., Aguiló-Aguayo, I., López, M.L., Larrigaudière, C., 2017. Biochemical and physiological changes during fruit development and ripening of two sweet cherry varieties with different levels of cracking tolerance. Plant Physiol. Biochem. 111, 216-225. https://doi.org/10.1016/j.plaphy.2016.12.002

Hamilton, A.J., Lycett, G.W., Grierson, D., 1990. Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants. Nature 346, 284–287. https://doi.org/10.1038/346284a0

Hayama, H., Ito, A., Moriguchi, T., Kashimura, Y., 2003. Identification of a new expansin gene closely associated with peach fruit softening. Postharvest Biol. Technol. 29(1):1-10. https://doi.org/10.1016/S0925-5214(02)00216-8

Hayama, H., Tatsuki, M., Ito, A., Kashimura, Y., 2006. Ethylene and fruit softening in the stony hard mutation in peach. Postharvest Biol. Technol. 41, 16–21. https://doi.org/10.1016/j.postharvbio.2006.03.006

Lü, P., Yu, S., Zhu, N., Chen, Y.R., Zhou, B., Pan, Y., Tzeng, D., Fabi, J.P., Argyris, J., Garcia-Mas, J., Ye, N., Zhang, J., Grierson, D., Xiang, J., Fei, Z., Giovannoni, J., Zhong, S., 2018. Genome encode analyses reveal the basis of convergent evolution of fleshy fruit ripening. Nat. Plants. https://doi.org/10.1038/s41477-018-0249-z

Martínez-García, P.J., Parfitt, D.E., Bostock, R.M., Fresnedo-Ramírez, J., Vazquez-Lobo, A., Ogundiwin, E.A., Gradziel, T.M., Crisosto, C.H., 2013. Application of genomic and quantitative genetic tools to identify candidate resistance genes for brown rot resistance in peach. PLoS One 8. https://doi.org/10.1371/journal.pone.0078634

Masia, A., Zanchin, A., Rascio, N., Ramina, A., 1992. Some Biochemical and Ultrastructural Aspects of Peach Fruit Development. J. Am. Soc. Hortic. Sci. 117: 808–815.

Meneses, C., Ulloa-Zepeda, L., Cifuentes-Esquivel, A., Infante, R., Cantin, C.M., Batlle, I., Arús, P., Eduardo, I., 2016. A codominant diagnostic marker for the slow ripening trait in peach. Mol. Breed. 36. https://doi.org/10.1007/s11032-016-0506-7

Nuñez-Lillo, G., Cifuentes-Esquivel, A., Troggio, M., Micheletti, D., Infante, R., Campos-Vargas, R., Orellana, A., Blanco-Herrera, F., Meneses, C., Blanco-Herrera mblanco, F., 2015. Identification of candidate genes associated with mealiness and maturity date in peach [Prunus persica (L.) Batsch] using QTL analysis and deep sequencing. Tree Genet. Genomes 11, 86. https://doi.org/10.1007/s11295-015-0911-9

Osorio, S., Alba, R., Damasceno, C.M.B., Lopez-Casado, G., Lohse, M., Zanor, M.I., Tohge, T., Usadel, B., Rose, J.K.C., Fei, Z., Giovannoni, J.J., Fernie, A.R., 2011. Systems Biology of Tomato Fruit Development: Combined Transcript, Protein, and Metabolite Analysis of Tomato Transcription Factor (nor, rin) and Ethylene Receptor (Nr) Mutants Reveals Novel Regulatory Interactions. Plant Physiol. 157(1):405-25. https://doi.org/10.1104/pp.111.175463

Perin, C., 2002. Molecular and Genetic Characterization of a Non-Climacteric Phenotype in Melon Reveals Two Loci Conferring Altered Ethylene Response in Fruit. Plant Physiol. 129(1): 300–309. https://doi.org/10.1104/pp.010613

Pirona, R., Eduardo, I., Pacheco, I., Da Silva Linge, C., Miculan, M., Verde, I., Tartarini, S., Dondini, L., Pea, G., Bassi, D., Rossini, L., 2013. Fine mapping and identification of a candidate gene for a major locus controlling maturity date in peach. BMC Plant Biol. 13, 1. https://doi.org/10.1186/1471-2229-13-166

Prinsi, B., Negri, A.S., Fedeli, C., Morgutti, S., Negrini, N., Cocucci, M., Espen, L., 2011. Peach fruit ripening: A proteomic comparative analysis of the mesocarp of two cultivars with different flesh firmness at two ripening stages. Phytochemistry 72, 1251–1262. https://doi.org/10.1016/J.PHYTOCHEM.2011.01.012

Ramming, D.W., 1991. Genetic control of SR fruit trait. Can. J. Plant Sci. 71, 601–603.

Shan, W., Kuang, J.F., Chen, L., Xie, H., Peng, H.H., Xiao, Y.Y., Li, X.P., Chen, W.X., He, Q.G., Chen, J.Y., Lu, W.J., 2012. Molecular characterization of banana NAC transcription factors and their interactions with ethylene signalling component EIL during fruit ripening. J. Exp. Bot. https://doi.org/10.1093/jxb/ers178

Shan, W., Kuang, J.F., Lu, W.J., Chen, J.Y., 2014. Banana fruit NAC transcription factor MaNAC1 is a direct target of MaICE1 and involved in cold stress through interacting with MaCBF1. Plant, Cell Environ. 37(9). https://doi.org/10.1111/pce.12303

Tataranni, G., Spada, A., Fuzz, C., Bassi, D., 2010. AFLP-based bulk segregant analysis for tagging the slow-ripening trait in peach [Prunus persica (L.) Batsch]. J. Hortic. Sci. Biotechnol. 85, 78–82. https://doi.org/10.1080/14620316.2010.11512634

Tonutti, P., Casson, P., Ramina, A., 1991. Ethylene Biosynthesis during Peach Fruit Development. J. Am. Soc. Hortic. Sci. 79 (4): 315–326.

Ververidis, P., John, P., 1991. Complete recovery in vitro of ethylene-forming enzyme activity. Phytochemistry 30 (3), 725-727. https://doi.org/10.1016/0031-9422(91)85241-Q

Yang, S.F., Hoffman, N.E., 1984. Ethylene Biosynthesis and its Regulation in Higher Plants. Annu. Rev. Plant Physiol. 35, 155-189. https://doi.org/10.1146/annurev.pp.35.060184.001103

Zhang, L., Jiang, L., Shi, Y., Luo, H., Kang, R., Yu, Z., 2012. Post-harvest 1-methylcyclopropene and ethephon treatments differently modify protein profiles of peach fruit during ripening. Food Res. Int. 48, 609–619. https://doi.org/10.1016/J.FOODRES.2012.05.022

Tables

Table 1. Main organic acids (malic and citric acid), sugars (glucose and sucrose) and malonaldehyde (MDA) content of peach fruit at harvest from the different genetic classes in the parents and progeny from the 'Belbinette x Nectalady' F1 population. Fruit harvested at different maturity stages according to flesh firmness and DA-values (M1, M2 and M3) expressed in days after full bloom (DAFB). Data shown are means ± Standard deviation.

Genotype	**Maturity**	**DAFB**	**I_{AD}**	**Malic acid** mg g^{-1}	**Citric acid** mg g^{-1}	**Glucose** mg g^{-1}	**Sucrose** mg g^{-1}	**MDA** nmol g^{-1}	**AC** AU g^{-1}
Sr1Sr2	**M1**	140.4±6.80	1.9±0.43ab	2.4±0.65 bcd	2.0±0.81 ab	8.1±0.03 ab	33.6±4.19 cdef	8.6±2.23	1.9±0.09
	M2	155.4±10.13	1.2±0.44cd	4.0±0.87 ab	0.5±0.12 c	7.1±1.32 bc	48.2±6.67 bc	11.5±1.40	1.7±0.09
	M3	169.8±10.52	0.7±0.32d	3.9±0.51 abc	0.6±0.16 c	5.9±0.59 bc	52.3±11.08 b	15.0±1.88	1.3±0.10
Sr1sr	**M1**	145.1±7.82	1.8±0.23ab	2.2±0.57 bcd	2.1±0.59 a	8.7±0.55 ab	35.5±5.62 cde	10.6±2.12	1.5±0.08
	M2	162.7±10.42	1.0±0.46cd	5.2±1.12 a	0.7±0.17 c	7.6±0.13 abc	72.5±5.86 a	12.6±3.63	1.4±0.40
	M3	181.3±8.54	0.3±0.19d	5.1±1.57 a	0.6±0.22 c	6.1±0.38 bc	53.22±4.29 b	15.7±2.24	1.2±0.20
Sr2sr	**M1**	143.1±5.81	2.0±0.18ab	1.5±0.52 cd	1.4±0.18 abc	8.7±0.45 ab	22.1±1.27 efg	5.2±2.11	1.6±0.06
	M2	162.6±8.04	1.3±0.47cd	3.3±0.73 abcd	1.4±0.25 abc	7.2±0.43 bc	35.5±1.39 cde	8.9±1.56	1.2±0.09
	M3	188.0±6.26	0.6±0.41d	2.6±0.12 bcd	0.9±0.28 bc	5.2±0.74 c	43.7±2.55 bcd	10.2±0.07	0.7±0.05
srsr	**M1**	146.0±0.00	2.0±0.03a	1.0±0.37 d	1.1±0.44 abc	8.9±0.19 ab	14.5±4.96 g	6.2±0.68	1.6±0.15
	M2	169.0±0.00	1.9±0.04b	1.7±0.55 bcd	1.3±0.42 abc	10.2±0.62 a	18.3±1.82 fg	6.2±1.25	1.4±0.14
	M3	188.0±0.00	1.7±0.06c	1.8±0.55 bcd	0.7±0.41 c	10.4±0.36 a	30.4±6.46 defg	7.1±0.70	1.2±0.03
p (genotype x maturity)		-	<0.0001	<0.0001	0.0005	<0.001	0.0002	ns	ns

Figures

Figure 1. Visual appearance of peach fruit from the different genetic classes in the progeny from the ‘Belbinette x Nectalady’ F1 population at the maturity stage 2 (M2).

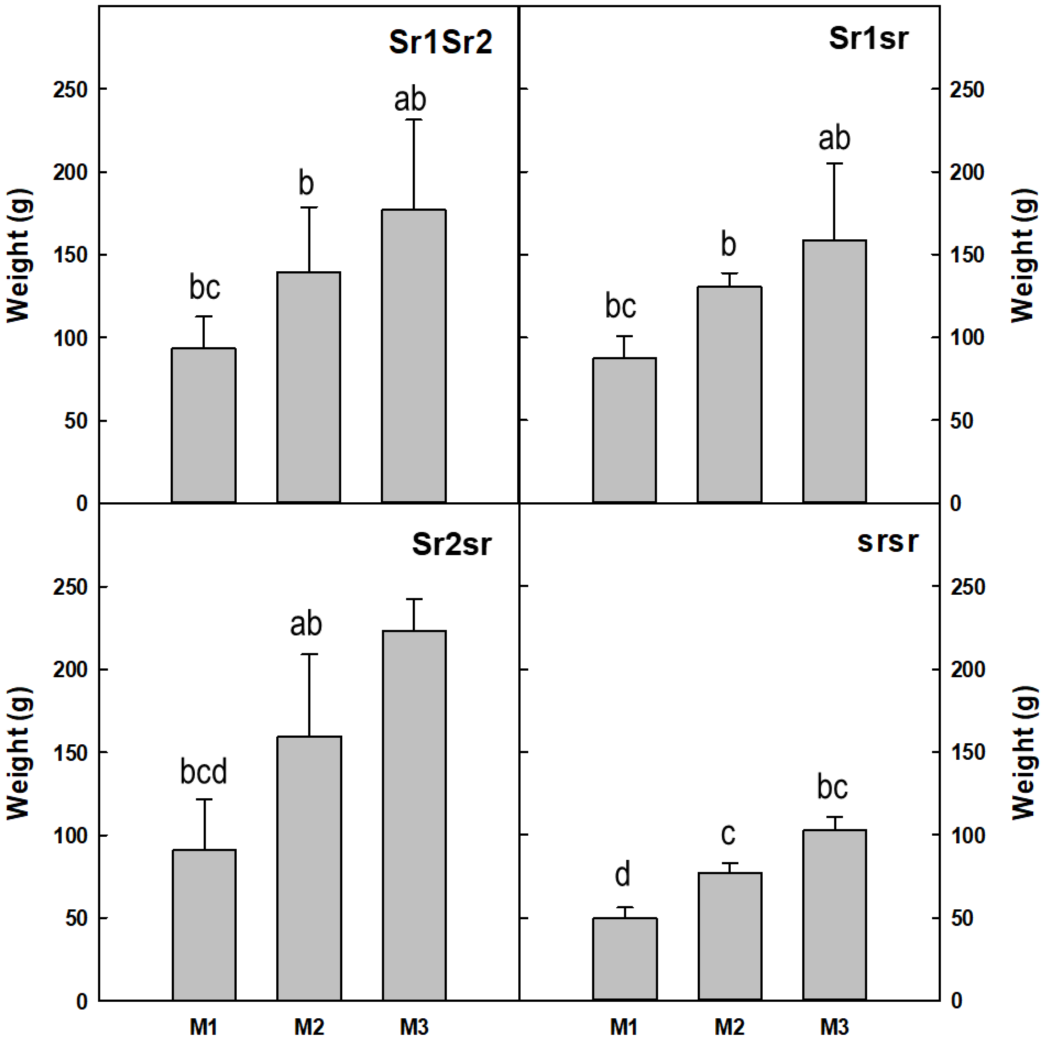

Figure 2. Fruit weight (g), at different maturity stages (M1, M2 and M3), of peach fruit from the different genetic classes in the progeny from the 'Belbinette x Nectalady' F1 population. Data shown are means ± Std. dev. Means with the same letter are not significantly different according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

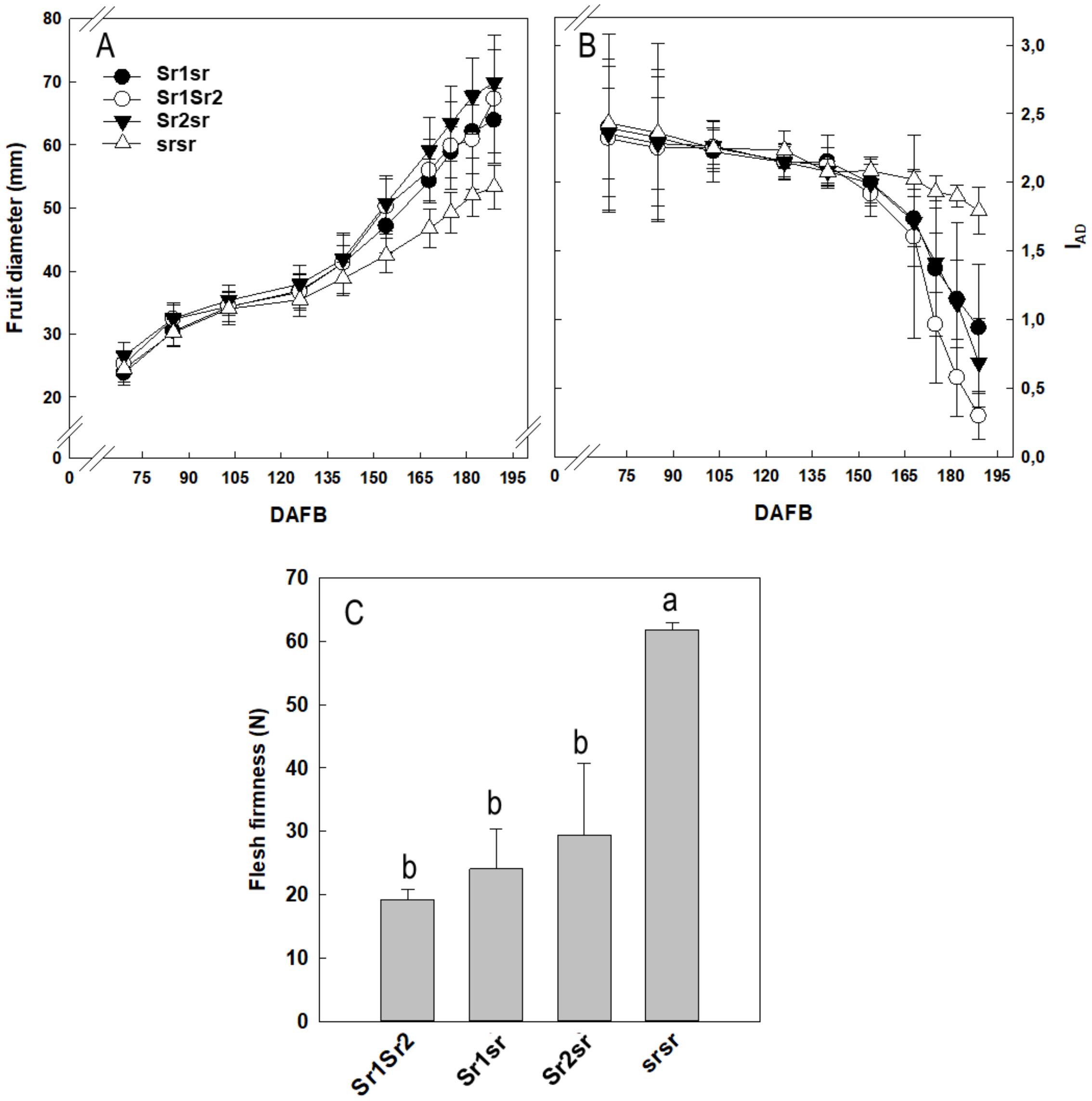

Figure 3. Changes in fruit diameter (mm; A) and I_{AD} values (B) from fruit set to harvest in the four genetic classes of the progeny from the 'Belbinette x Nectalady' F1 population. Data shown are means ± Std. dev. LSD values for the interaction genetic class x DAFB are 10.1 and 0.69 for (A) and (B), respectively. (C) Flesh firmness at 190 days after full bloom (DAFB) for the four genetic classes of the progeny from the 'Belbinette x Nectalady' F1 population. Values represent the mean mean ± Std. dev and means with the same letter are not significantly different according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

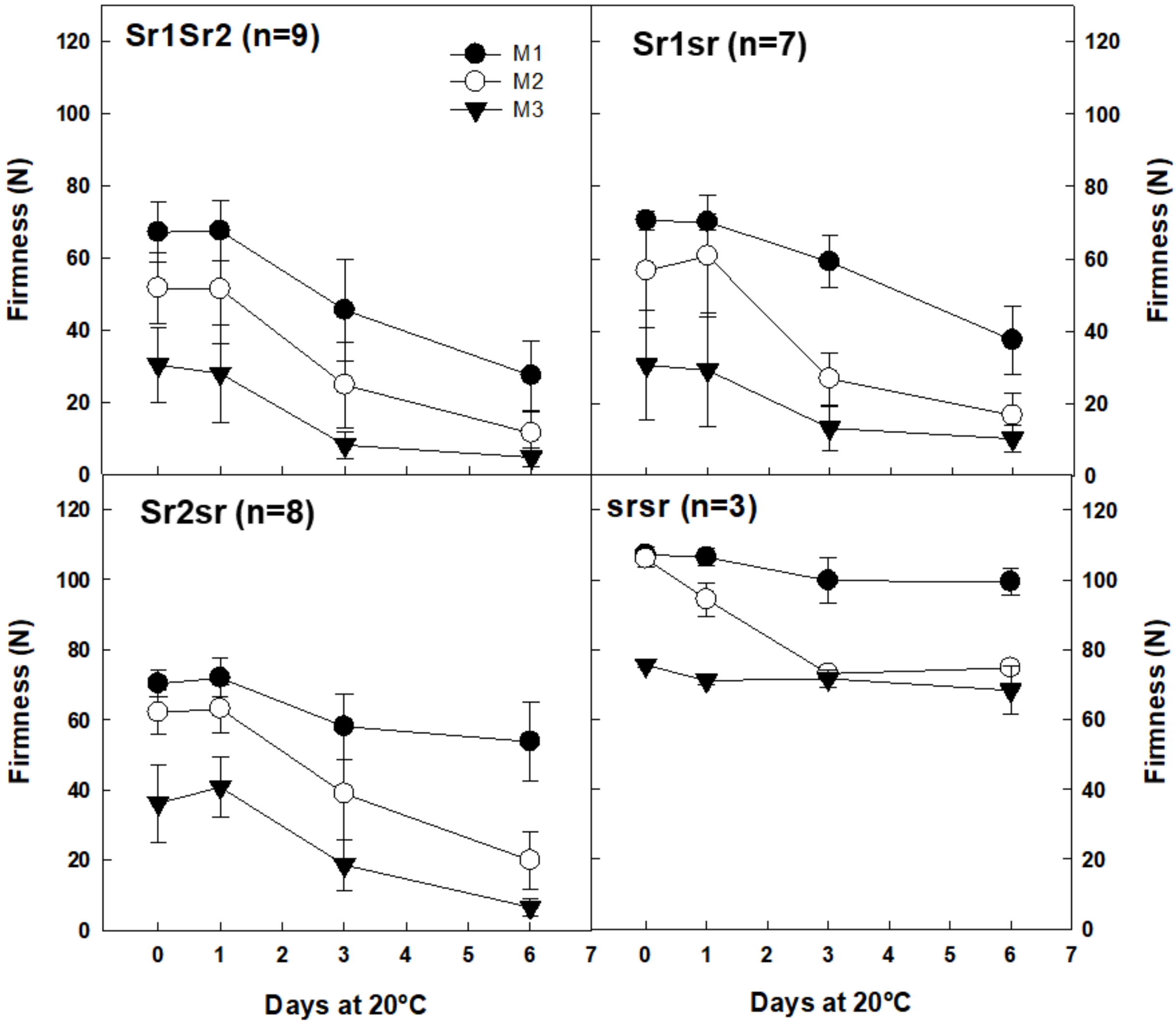

Figure 4. Changes in flesh firmness of peach fruit from the different genetic classes in the progeny from the 'Belbinette x Nectalady' F1 population, and harvested at different maturity stages (M1, M2 and M3), during shelf life at 20ºC (70% RH). Data represents the mean ± Std. dev.

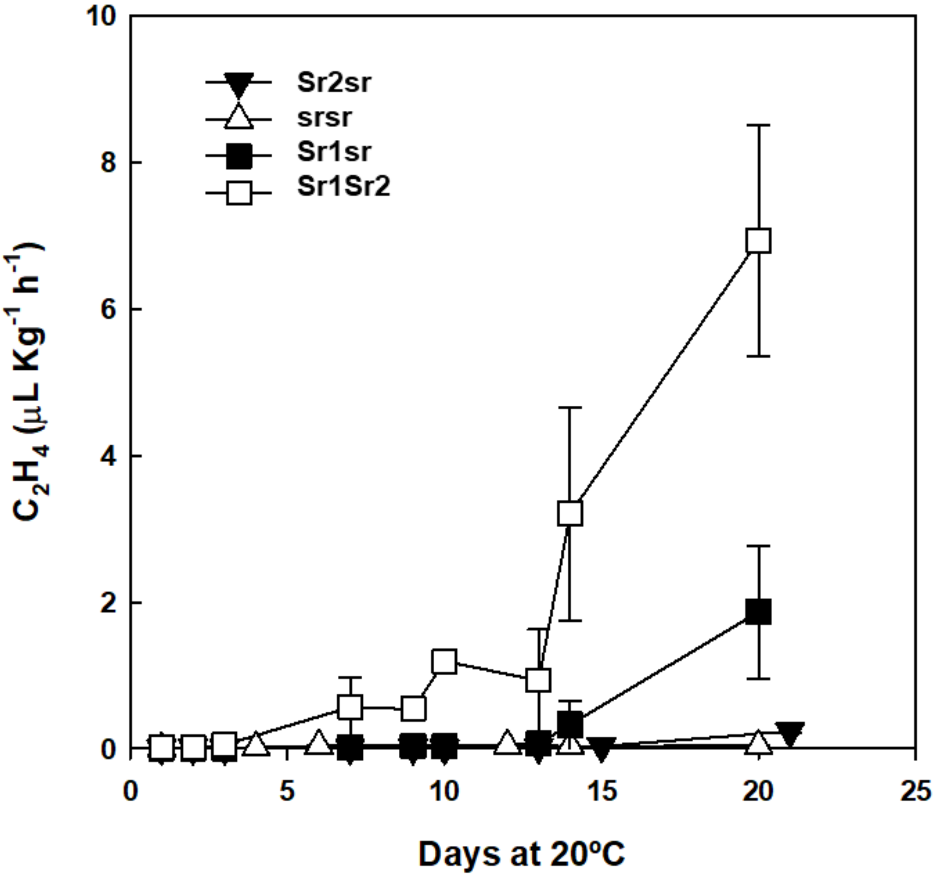

Figure 5. Ethylene production of early harvested peach fruit (M1) from the different genetic classes in the progeny from the ‘Belbinette x Nectalady’ F1 population during shelf life. Data represents the mean ± Std. dev. LSD value for the interaction genotype x days at 20ºC is 0.87.

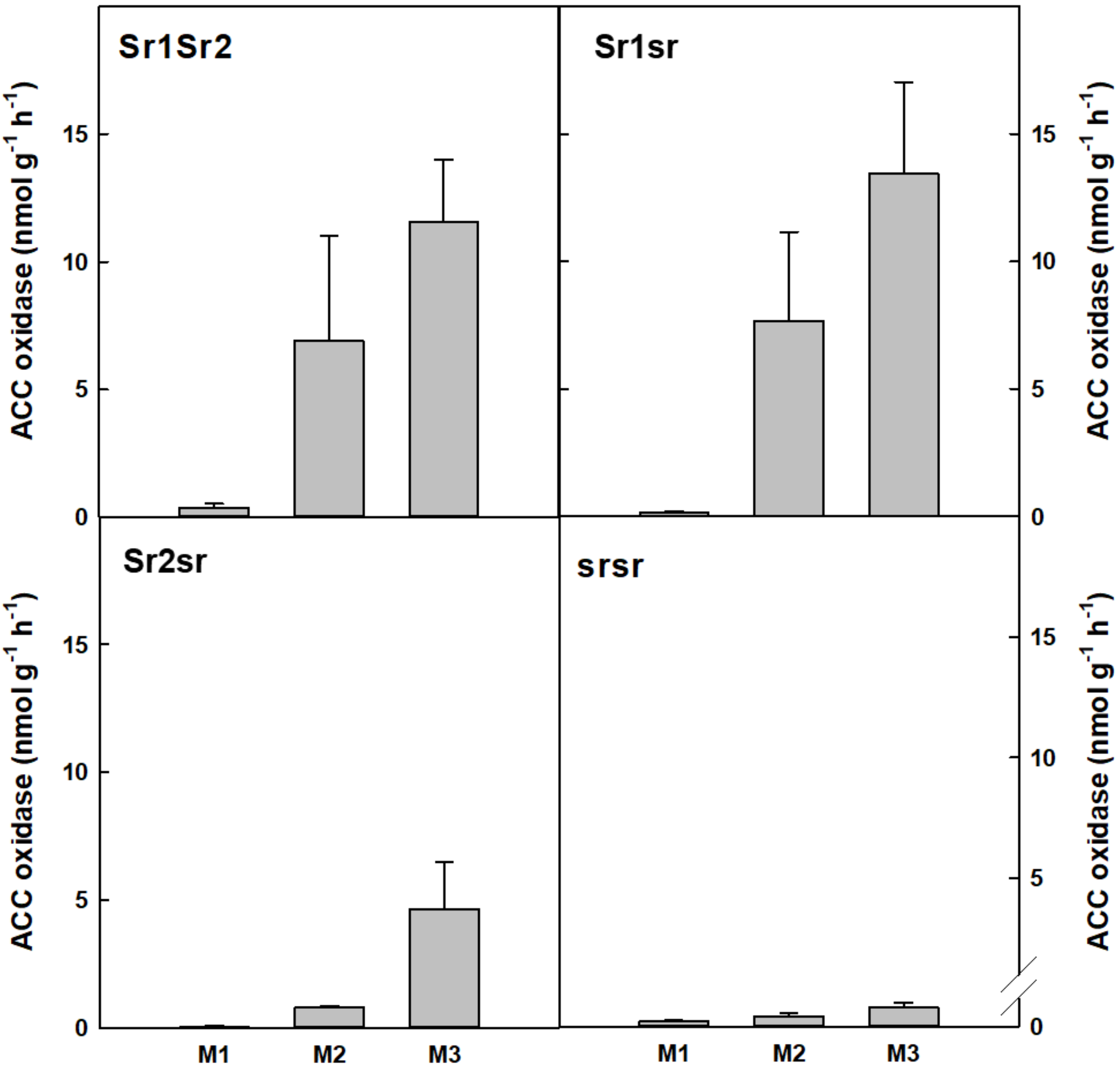

Figure 6. ACC oxidase activity, at different maturity stages (M1, M2 and M3), of peach fruit from the different genetic classes in the progeny from the 'Belbinette x Nectalady' F1 population. Data shown are means ± Std. dev. Means with the same letter are not significantly different according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

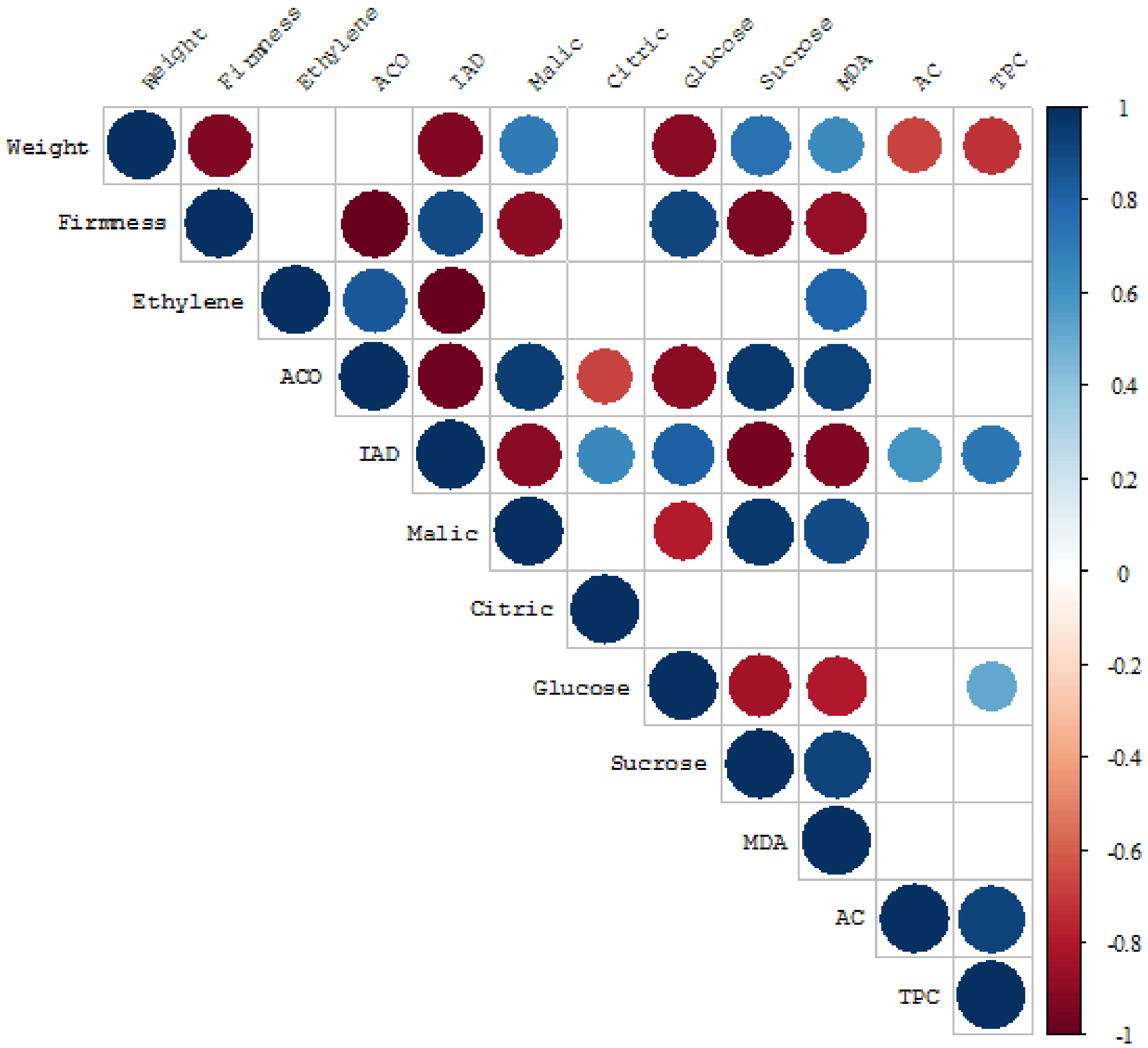

Figure 7. Visualization of Spearman's rank correlation matrix (significance level $p<0.05$) between the different biochemical and quality traits analysed in the progeny from the 'Belbinette x Nectalady' F1 population.