Ripening transition and storage environmental impact on sucrose accumulation and associated responsive genes in strawberries

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Abstract

To obtain the best taste of woodland strawberries (Fragaria vesca) at the moment of consumption, it is essential to analyse the dynamics of sugar accumulation during ripening transition stages and explore how to control sucrose reserves after harvest. For this purpose, we analysed the accumulation of major sugars, sucrose-related olygomers and the expression of homologues genes involved in sucrose metabolism in attached strawberries at different ripening stages and after harvest. Measurements were taken during early and late phases of low temperature storage (LT) with and without CO2 pretreatment and further shelf-life at 20 ºC (SL). Our results show an increase in major sugars and sucrose in dark red (DR) strawberries associated with up-regulation of FvSPS1 and down-regulation of FvVINV2. The CO2-treated fruit showed high levels of sucrose, an FvSS1 up-regulation together with a modulation of homologues of FvVINV. Moreover, fruit treated with CO2 during SL (LTC) revealed a reduction in FvVINV homologue expression maintaining the sucrose reserves. High amounts in fructose and myo-inositol at LTC were observed, following a similar trend to that found in DR strawberries. We suggest that CO2 pre-treatment promotes a carbohydrate conserving state which has important implications for reducing weight loss and preserving sweetness.

1. Introduction

Woodland strawberries (*Fragaria vesca*) are characterised by a delicious taste and an abundance of non-structural carbohydrates. During development and ripening, strawberries undergo substantial changes to multiple sensorial and biochemical attributes, acquiring good quality and high commercial value at harvest (Roch et al., 2019). Being a non-climacteric fruit, strawberries have to be harvested in an optimal state for consumption since ripening does not continue once they have been harvested. Thus, the harvest date has a decisive influence on determining the duration of storage life and fruit quality. Soluble sugar content is one of the most important quality traits since sugar accumulation affects both fruit growth and quality (Hancock, 1999; Schwieterman et al, 2014). At the same time the organoleptic quality of the fruit that is largely established by its sweetness (Colquhoun et al., 2012), depends on the content and composition of sugars since different types of soluble sugars contribute differently to the relative degree of sweetness (Kroger et al, 2006; Schwieterman et al 2014). Thus, specific sugars or groups of sugars must be characterized and quantified during ripening transition in the period around the anticipated optimum harvest date, storage and shelf-life, in order to consider their potential role as indicators of optimum harvest date.

Strawberries accumulate various types of soluble sugars which vary throughout fruit development. The main sugars are sucrose, glucose and fructose, followed by myo-inositol and trehalose (Zhang et al., 2011). In immature fruit, changes during early fruit development mainly occur in glucose and some polyalcohol sugars such as *myo* -inositol (Ogiwara et al., 1998a, 1998b). During later fruit development, sucrose, glucose and

fructose become the predominant soluble sugars, with galactose and some cell wall sugars also being observed in ripe fruit (Menager et al., 2004; Fait et al., 2008; Basson et al., 2010; Zhang et al., 2011). Despite this, little is known about the changes that take place during different stages of ripening in the oligosaccharides levels derived from sucrose and galactose, such as fructo-oligosaccharides (FOS) and galactose-oligosaccharides (RFOS).

Sucrose content has been shown to be more responsible than any other individual compound for a greater variation in sweetness intensity and overall liking (Schwieterman et al, 2014). It is, therefore, essential to investigate sucrose levels during ripening transition as this may reflect high acceptance of strawberries at harvest. However, delaying harvest day has a negative impact as it shortens postharvest life. Consequently, adequate environmental conditions surrounding the fruit are needed to control sucrose content and avoid, as much as possible, rapid deterioration of the fruit following harvest. High CO_2 treatment has been used as coadjuvant technology to alleviate physiological disorders caused by storage at severe low temperature. Its application over only a short period of time at the beginning of storage is a commercially available technology which is used to reduce fungal decay and water loss. In addition to being essential for sweetness and a substrate for FOS synthesis, sucrose has an important role as a major osmotically active solute. It plays an important role in regulating water within cells which join with other major soluble carbohydrates such as glucose, fructose and*myo* -inositol (Blanch et al., 2015b, Vimolmangkang et al. 2016). FOS are olygomers that result from extended sucrose metabolism, where fructosyl units are bound by β linkage to sucrose. This linkage favours the formation of helical structures. FOS have different protective effects against environmental stress in plants (Valluru and Van den Ende, 2008; Hincha et al., 2000). We have previously reported the implication of FOS on fruit water status in strawberries more than a reserve carbohydrate, spite this fruit is a non-fructan accumulating plant (Blanch et al., 2012). Their specific structure and biophysical properties support the ability of these compounds to reorganize water-hydrogen bonding networks which might be a factor contributing to cellular water stabilization (Furiki, 2002). On the other hand, several reports indicate an important role of RFOS in response to osmotic stress (Ishitani et al., 1996; Loewus and Murthy, 2000; Sengupta et al., 2015). Some of these oligosaccharides might act as osmoprotectants, protecting against damage caused by the osmotic imbalance induced by several kinds of stress (Hare et al., 1998; Verslues et al., 2006; Sperdouli and Moustakas, 2012). This can function to stabilize cellular membranes by replacing water molecules and thereby keeping membrane surfaces hydrated (Verslues et al., 2006; Valluru and Van der Ende, 2008). In addition, beneficial effects of FOS in the diet as a health-promoting food ingredient have been recognized in humans (Sabater-Molina et al., 2009; Closa-Monasterolo et al., 2013; Tousen et al., 2013; Yao et al.,2014; Singh et al., 2017). Other sugars such as trehalose also act to protect membranes and proteins from damage caused by different stress conditions, including low temperatures (Fernandez et al. 2010, Delorge et al., 2014, Lunn et al., 2014).

Low temperature and high CO_2 are known to impact fruit metabolism by reprogramming gene expression to involve numerous transcription factors and activating abiotic stress genes (Rosales et al., 2016; Romero et al 2016; Wang et al., 2017, Zhu et al 2018, Jin et al. 2018, Li et al 2019a, Zhu et al 2019). However, the effect of high CO_2 on the expression of genes involved in sucrose metabolism and underlying sucrose levels, during low temperature storage and further shelf-life, remains unknown. In addition, although information about sucrose metabolism gene expression during development is available, little is known about the different stages of ripening transition. During strawberry fruit development sucrose is imported from photosynthetic tissues, through apoplast, to the berry, entering as sucrose or being hydrolysed into glucose and fructose by cell wall invertase (CWINV) (Koch, 2004; Fait et al., 2008; Basson et al. 2010). Thus, cytoplasmatic sucrose can be reversibly cleaved by sucrose synthase (SS) or irreversibly hydrolysed by invertases (Winter and Huber, 2000; Koch, 2004). Different groups of intra or extracellular invertases can be discerned. These include CWINV and soluble vacuolar invertase (VINV), which have an acidic optimal pH, soluble cytoplasmic invertase (NINV) that has a neutral to alkaline optimal pH, and soluble apoplastic invertase (Roitsch et al., 2003). CWINV and VINV are closely phylogenetically related through their activities, which are regulated at both a transcriptional and post-translational level (Wan et al., 2018). Invertases are highly homologous to fructan exo-hydrolases involved in fructan degradation. However, in contrast to invertases, fructan exo-hydrolases

cannot use sucrose as a substrate (Van den Ende et al 2004; Van den Ende 2013). Thus, FOS can be degraded by fructan 1-exohydrolases (1-FEHs), whilst plant FEHs lack invertase activity. They are enzymes with a single function and probably evolved from CWINV, serving only to degrade fructans. In contrast, FOS biosynthetic genes from dicots, sucrose: sucrose 1-fructosyltransferase (1-SST) and fructan: fructan 1-fructosyltransferase (1-FFT), evolved from VINV, use sucrose and fructans, respectively, as preferential donor substrates (Lasseur et al., 2009). On the other hand, RFOS is derived from UDP-glucose which is simultaneously involved in sucrose synthesis through the major enzyme, sucrose-phosphate synthase (SPS).

SPS reversibly catalyses sucrose-6-phosphate formation from fructose-6-phosphate and UDP-glucose (Huber and Huber, 1996; Nguyen-Quoc and Foyer, 2001). UDP-glucose can also join with glucose-6-phosphate (G-6-P) to form trehalose-6-phosphate (trealose-6-P) and, subsequently, trehalose (Ponnu et al., 2011). The importance of SPS in carbohydrate metabolism and development has been confirmed in *Arabidopsis* and rice mutant lines and muskmelon interference lines, and through heterologous expression in tomato, tobacco and cotton plants (Worrell et al., 1991; Galtier et al, 1993; Baxter et al., 2003; Haigler et al., 2007; Tian et al., 2010; Volkert et al., 2014; Seger et al., 2014; Hashida et al., 2016). Different potential benefits of SS over invertases have also been previously reported (Zeng et al. 1999; Bologa et al., 2003; Koch et al. 2000). The efficiency of SS in ATP net yield has been extensively reported (Stitt and Steup, 1985; Sachs, 1994; Stitt, 1998; Baroja-Fernández et al., 2009).

Whilst the SS pathway produces phosphorylated glucose, the unidirectional invertase pathway releases glucose, which must then be phosphorylated at the expense of ATP in order to enter the glycolytic pathway. It has been reported that hypoxia caused by cellular oxygen deficiency (Gibbs and Greenway, 2003; Greenway and Gibbs, 2003; Narsai, et al., 2011), generally upregulates the expression of genes which code enzymes involved in sugars, with the only exception being seen in invertases.

The objective of the present work was firstly to analyse whether sucrose, major water-soluble sugars and related oligosaccharides, together with sucrose metabolism gene expression dynamically change during ripening transition in attached strawberries. A second objective was to explore the impact of environmental conditions on controlling sucrose reserves and sucrose metabolism gene expression, after storage and shelf-life. For this purpose, we analysed the accumulation of different sugars and expression of the homologue genes involved in sucrose metabolism in strawberries at three different ripening stages. The effect of low temperature (0 ^{o}C) and CO_2pre-treatment (17% CO_2 for 2 days) on sucrose retention in strawberries, as well as the underlying molecular mechanisms, were analysed during early and late phases of LT, and further SL. Effectiveness of pre-treatment with high CO_2 levels for reducing weight loss and maintaining other major soluble carbohydrates was determined. The effect of ripening stage, storage and shelf-life on the accumulation of short-chain RFOS (raffinose, degree of polymerization (DP) 3, and estaquiose, DP4) and short-chain FOS (1-kestose and 6G-kestose, DP3, DP4, DP5) was also analysed.

2. Material and methods

2.1. Plant material

For ripening transition assay, *Fragaria vesca* 'Mara des Bois' strawberries were harvested in 2017 from a commercial planting in San Sebastian de los Reyes (Madrid, Spain) were selected by colour and transported to the laboratory and classified into three ripening stages as 3/4 red (AR), full red (FR) and dark red (DR). Immediately after harvest, half of the collected fruits were frozen in liquid nitrogen and the other half of fruit was used for quality parameter assessments.

The postharvest treatment assay was performed using fruit harvested in 2018 of an intermediate stage between FR and DR from the same commercial planting. Two hours after harvest, selected strawberries free from physical defects were transferred into covered polyethylene boxes of 200 g of fruit equipped with inlet-outlet ports. Strawberries immediately after harvest were the control sample of storage period analysis (0d). One lot of 20 samples of freshly harvested fruit placed directly at 20 ^{o}C during two days and they were the shelf-life control (SLC). A total of 80 boxes of fruit were equality divided into 2 lots. Two lots of 40 subsamples each were stored at 0 $\pm$1 ^{o}C (Low temperature, LT) in two containers with a capacity of 1

m^3 and 90 % relative humidity (RH). One container was fluxed with a continuous flow of air during 2 days or 7 days (2d Air and 7d Air), and the other one under continuous flow of 17% CO_2 in air for 2 days (2d CO_2). At the end of CO_2 treatment, 10 samples were evaluated and 30 were air-ventilated for 5 additional days (2d CO_2+5d Air). Following 7 days of storage at 0 ºC, fruit from each treatment were transferred to a chamber at 20 ºC and the atmosphere was saturated with water vapour to reach 80 % RH. After 1 and 2 days of shelf-life at 20 ºC (SL), samples from those stored in air at low temperature (LTA) or under high CO_2 (LTC) were evaluated. Fruits were rapidly frozen in liquid nitrogen and stored at -80 ºC. Observation of external appearance of fruit of each treatment was made every day and total soluble solids, titratable acidity and weight loss were also determined. All strawberries were kept in the dark throughout the trials.

2.2. Extraction and Chromatographic determination of glucose, fructose, sucrose, myo-inositol, threhalose, FOS and RFOS.

For carbohydrates analyses, 2 g of frozen strawberry powder (wet) were extracted with 5 ml of distilled water, homogenized and centrifuged at 10000 rpm for 30 min at 4 ºC, and determined by UPAEC-PAD with a Carb2-250 (250 x 4mm) column, as previously described (Blanch et al., 2015b). Samples were analysed on Bioscan module (817 IC Metrohm, Herisau, Switzerland) equipped with a pulsed amperometric detector, (IC Pump 812) and coupled degasser (IC-837). Isocratic elution was carried out with 100 mM NaOH- 10 mM NaOAc, and the flow rate through the column was 0.5 mL/min, leading to sampling times of 60 min. For FOS and RFOS analyses, 3 g of frozen strawberry powder (wet) were homogenized in 4 ml of ultra-pure water and the mixture was boiled under reflux in a water bath for 15 min. After cooling, the samples were sonicated for 10 min at 40 ºC and the pH of each sample was adjusted to 7.5 with 10% NH_4OH. The samples were then centrifuged at 14700g for 15 min at 4 ºC and the supernatants were filtered thought a 0.45 μm pore size membrane. The analyses of extracts and calibration standards were performed using an Agilent 1200 liquid chromatography coupled to an Agilent Triple Quadrupole MS detector G6410B (HPLC-QqQ-SIM, Agilent Technologies, Palo Alto, CA, USA). The chromatographic separation was achieved on an HYPERCARB (100 mm X 2.1μm X 5μm) column, with a mobile phase consisting of water containing 5 mM ammonium formate (A) and performing an elution using acetonitrile (B) in gradient as follows: %B: 0 min, 5%; 5 min, 10%; 10 min, 10%; 20 min, 50%; 30 min, 5%; 40, min, 5% at 25 ºC with an injection volume of 5 μL.

2.3. Fruit weight losses, total soluble solids and titratable acidity.

Fruit weight losses were measured on 60 polyethylene boxes or punnets of 200 g of fruit equipped with inlet-outlet ports for each storage condition, using analytical balance. The weight was monitored on the same boxes all along the distribution chain: early and late phases of LT and during SL for 1 and 2 days following 7 days of cold (LTA and LTC). Three replicates per treatment (10 berries per sample) were ground in an electric juice extractor. The obtained clear juice without achenes was used for the determination of total soluble solids (TSS), pH-value and titratable acidity (TA). TSS was measured using a digital and temperature-compensated refractometer (Atago, Co, Tokyo, Japan) that detects reducing sugars and other soluble compounds. In the juice of strawberry fruit, the pH-value was determined with a pH meter. Titratable acidity was determined by tritration with 0.1 N NaOH to pH 8.1 (Mettler DL-70, Mettler –Toledo, Spain) and the results were expressed as citric acid equivalents, which is the main organic acid in strawberry fruit, and expressed per fresh weight.

2.4. Expression analyses.

The total RNA of three biological replicates samples was extracted from 0.5 g of fruit powder according to Yu et al. (2012). Each biological replicate sample contained a fruit mixture of at least 50 fruits. The cDNA was prepared by reverse transcription of 1 μg of total RNA using the Maxima cDNA Kit with dsDNase kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer instructions. Quantification was performed by real-time quantitative RT-PCR (qPCR) using iCycler iQ Real-Time PCR Detection System (BIORAD) and quantified using Real Time Detection System Software (version 2.0). The amplification reactions were carried out in a final volume of 12 μL containing 6 μl of NZY qPCR Green Master Mix (2x) (NZYTech, Ltd), 1 μL of each primer (10 μM) and 1 μL of the cDNA. The PCR profile

used was 2 min at 50 °C, 95 °C for 10 min, followed by 40 cycles of 20 s at 95 °C and 30 s at 55 or 60 ºC. Three technical replicates were made from each of the genes studied. Gene expression was determined by 2^{-CT} method using a *F. vesca* Actin-97-like (XM_004307470) as housekeeping gene. The specific primers used are described in Supplementary Table S1 and PCR amplicons were sequenced to confirm specificity.

2.5. Identification of genes involved in sucrose metabolism in Fragaria vesca.

In order to identify each *Fragaria vesca* paralogues, the Hidden Markov model (HMM) profiles from Pfam (https://pfam.xfam.org/) or InterPro (http://www.ebi.ac.uk/interpro/) databases were used as a query against predicted protein sequences of *Fragaria vesca* from genome database for Rosaceae (GDR, https://www.rosaceae.org/). The profiles used as query was as follows: SPS (family IPR012819, Superfamily IPR023214, domains IPR000368/IPR001296/IPR006380, PF00862/PF00534/PF05116), SS (family IPR012820, domains IPR000368/ IPR001296, PF00862/PF00534) and invertases(family IPR001362; domains PF00251/PF08244) (CWINV, Superfamily IPR013148/IPR013189, Domains IPR013320/IPR023296, UniProtKB Q9ZP42; VINV, Family IPR001362, Superfamily IPR23296, Domains IPR013148, UniProtKB Q08IC1). Thus, for SPS 4 homologues were found clustering together (FvSPS1, FvH4_2g28820. FvH4_4g13520, FvSPS2). For SS, 4 homologues which clustering together (FvSS1, FvH4_1g07260, FvSS2, FvH4_4g18710) were analyzed and the other 2 homologues (FvH4_1g27400, FvH4_5g18160) belonging to another cluster were not analyzed. For CWINV, 3 homologues which clustering together (FvCWINV1, FvH4_6g33780, FvCWINV2) were analyzed. In the case of FvH4_6g19270 was not analyzed because it was not in the same cluster but also the expression level in fruit was very low. For VINV the only 2 homologues found in a cluster were analyzed (FvVINV1, FvVINV2).

In order to perform functional phylogenetic trees, the paralogues obtained were used to find near orthologues in monocots and dicots in which the specific enzymatic activities have been described.

2.6. Phylogenetic analysis.

Phylogenetic analysis was performed using MEGA X (Kumar et al., 2018) that relies on the Maximum Likelihood method and JTT matrix-based model. The trees with the highest log likelihood (A: -11895.94, B: -7003.22, C: -18522.86) are shown. The tree was obtained applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value (Jones et al., 1992). These analysis involved (A: 8, B: 8, C: 20) amino acid sequences respectively. There were a total (A: 1124, B: 947, C: 752) positions respectively in the final dataset. Phylogenetic tree analysis for SPS and SS orthologues was performed using*Arabidopsis* protein sequences, selecting only the closest ones between both species without analyzing the furthest clusters. Regarding invertases, the phylogenetic tree performed for CWINV orthologues was using protein sequences with 6-FEH activity from *Arabidopsis* : AtCWINV1 (At3G13790), AtCWINV3 (At1G55120), AtCWINV5 (At3G13784) and*Beta vulgaris* : Bv6-FEH (AJ508534); 1-FEH activity from*Cichorium intybus* Ci1-FEH (AJ242538), Ci1-FEH2a (AJ295033); and 6-1-FEH activity from *Arabidopsis* AtCWINV6 (At5G11920) and*Triticum aestivum* Ta6-1-FEH (AB089269). For VINV orthologues was using the closest protein sequences from *Arabidopsis* : AtVI2 (AT1G12240), AtVI1 (AT1G62660); protein sequences with 1-SST activity from *Allium cepa* : Ac1-SST (AJ006066), *Cichorium intybus* : Ci1-SST (U81520), *Helianthus tuberosus* Ht1-SST (AJ009757); and protein sequences with 6-GFT activity from *Allium cepa* : Ac6-GFT (ACY07838) and *Asparagus officinalis* Ao6-GFT (AB084283). Accession numbers used for all analyses are according to the following databases nomenclature respectively (gene_V4, gene_V2, NCBI_LOC, NCBI_PROT) (Supplemental Information S1).

2.7. Statistical analyses.

A completely randomized design was used in this experiment and repeated in triplicate. One-way ANOVA was performed using GraphPad Prism software Multiple comparisons of every mean to a control mean was determined using Dunnett's test at $P < 0.05$ (*),$P < 0.01$ (**), $P < 0.005$(***) and$P < 0.001$ (****) (ANOVA test). All data plotted in figures are presented as mean ± standard deviation.

3. Results

3.1. Changes in sugars during ripening transition stages.

The date of harvest has a pronounced influence on the storage potential of fruit. To determine optimum harvest dates, it is essential to know the changes that occur in sugar levels during ripening transition stages. This is important as they influence and determine storage life duration and fruit quality, especially sweetness. We performed chromatographic determinations of sucrose, glucose, fructose and other sugars associated with hexose metabolism, such as myo-inositol and trehalose. These are compatible solutes involved in osmotic adjustment and protect fruit properties when subjected to changing environmental conditions. In addition, FOS and RFOS were also characterized and quantified by MS during ripening transitions stages. In 'Mara des Bois,' glucose and fructose accounted for the vast majority of change in sugars, followed by sucrose. Myo-inositol and trehalose were found to contribute to a lesser extent (Figure 1). In AR strawberries (66 % redness), sucrose content was similar to that found in FR strawberries but significantly lower in comparison with DR fruit (Figure 1A). Sucrose content was approximately half that of glucose and fructose, and demonstrated marked increases during the DR stage, reaching values of 11.18±1.10 mg/g fresh weight. A significantly high level of glucose (Figure 1B) and fructose (Figure 1C) was also quantified at the DR stage, relative to the AR and FR stages. The increase in sucrose, glucose and fructose was similar in all cases, approximately doubling in quantity. Similarly, higher levels of myo-inositol and trehalose were quantified at the DR stage as compared to the AR and FR stages (Figure 1D and E). With respect to FOS, the main polymer is 1-kestose (DP3) (Figure 1F). This showed levels that were six times more abundant than levels of the isomer 6G-kestose (Figure 1G), and more than sixty times more abundant than nystose (DP4) (Figure 1H). In the case of 1-kestose, 6G-kestose and nystose, levels peak at the FR stage and decline at the DR stage. In comparison, Kestopentaose (Figure 1I) is found at much lower concentrations generally, with levels declining progressively during the ripening transition and being lowest at the DR stage. With respect to RFOS content, no clear trends can be observed in raffinose at the different ripening transition stages (Figure 1J). On the other hand, estaquiose, which is 25 times less abundant, showed a minor decrease in concentration following the AR stage (Figure 1K).

3.2. Identification of sucrose metabolism genes in Fragaria vesca. Effect of ripening transition on sucrose metabolism gene expression.

In order to identify the putative homologues involved in sucrose metabolism in *Fragaria vesca* , data-mining approaches were performed using BLAST search queries targeting HMM profiles of individual protein domains. SPS (EC 2.4.1.14), SS (EC 2.4.1.13) and invertases (EC 3.2.1.26) (CWINV, UniProtKB Q9ZP42 and VINV, UniProtKB Q08IC1) are key enzymes involved in sucrose metabolism within plants. The number of individual enzyme families differs among the plant species, being found in plants encoded by different multigene families. The paralogues found were used alongside the closer *Arabidopsis*orthologues in order to form functional phylogenetic trees (Figure 2). Four homologues were found to cluster SPS into four distinct groups, with FvSPS1 being more similar than FvH4_2g28820 to AtSPS1F and AtSPS2F orthologues. On the other hand, FvH4_4g13520 clustered with AtSPS3F, whilst FvSPS2 was seen to cluster with AtSPS4F (Figure 2 A). Four homologues were also found to cluster SS into three groups. In this instance, FvSS1 and FvH4_1g07260 formed a group close to the cluster formed by AtSUS4 and AtSUS1, whilst FvSS2 clustered with AtSUS3 and FvH4_4g18710 clustered with AtSUS2 (Figure 2 B). Likewise, three homologues were found to cluster together for CWINV. Here it was found that FvCWINV1 and FvH4_6g33780 were closer than FvCWINV2. These were separated from 6-FEH of *Beta vulgaris* and *Arabidopsis*cluster formed by AtCWINV3, 1 and 5 (Figure 2 C). These in turn are seen to be significantly separated from the cluster formed by 1-FEH activities. However, two different soluble invertase VINV groups were found to cluster into two groups, with FvVINV2 being close to a cluster formed by AtVI2 and AtVI1 (figure 2 C), and qualitatively separated from clusters formed by 1-SST and 6-GFT activities.

Tissue expression pattern during berry ripening and development has been described by the fruitENCODE project (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA381300) and *Fragaria vesca* eFP Browser (Darwish et al. 2013; Kang et al. 2013; Hollender et al., 2014). This provides a basis from which we can select which genes to analyse. From this, genes with the greatest expression in fruit tissues or with significant

changes in relative gene expression between different developmental and ripening stages were selected (see asterisk marked genes in Figure 2). These were then analysed using qPCR in 'Mara des Bois' strawberries at the three different ripening transition stages (Figure 3 E). *FvH4_2g28820, FvH4_4g13520, FvH4_1g07260, FvH4_4g18710* and *FvH4_6g33780* were not analysed as they demonstrated low expression in fruit.

Expression of *FvSPS1* , which is involved in sucrose synthesis, significantly increased in both FR and DR strawberries (Figure 3 A), whilst no changes in *FvSPS2* expression were observed. With regards to the expression of *FvSS* homologues involved mainly in sucrose breakdown, our results indicate that *FvSS1* and*FvSS2* are expressed at all of the analysed ripening stages. Specifically, *FvSS1* was more abundantly expressed than*FvSS2* , with expression of both increasing up until the stage of ripeness (Figure 3 B). On the other hand, transcriptional analysis of the invertase gene families involved in sucrose cleavage shows a significant increase in *FvCWINV1* and *FvVINV1* expression at the end of the ripeness stage (DR, Figure 3 C y D). In contrast,*FvCWINV2* and *FvVINV2* expression significantly decreases in DR strawberries, in comparison to AR and FR fruit. In fact, a greater decrease in *FvVINV2* expression (2.5-fold) was observed in DR relative to AR (Figure 3 D). These results seem to indicate that sucrose metabolism is highly active during the DR stage, with an increase in*FvSPS1* and a decrease in *FvVINV2* expression leading to the accumulation of sucrose.

3.3. *Changes during early and late storage at 0 oC with and without CO_2.*

3.3.1 *Changes in sugar levels*

We were interested to understand the way in which sugar levels are altered by postharvest environmental factors during storage. For this reason, we analysed sugar levels in strawberry fruits immediately after harvest (0d), stored in air at 0o C for 2 days (2d Air) or 7 days (7d Air), or stored in these same conditions but with an additional CO_2 pre-treatment (2d CO_2 and 2d CO_2+5d Air). As shown in Figure 4 A, a significant decrease in the content of sucrose was quantified in fruit stored in air at the early stage of LT storage at 0 oC (2d). The sucrose content decreased by 23 % respect to the initial value after 2 days at 0 oC, whereas it was maintained at the end of CO_2 treatment. Nonetheless, following transfer to regular air conditions the effects of high CO_2 on retaining sucrose was progressively lost. Higher levels of sucrose were still recorded in comparison to air-stored fruit after 7 days at 0 oC. Similarly, our results indicate that there were significant decreases in glucose and fructose concentration by day 2 at 0 oC in fruit stored in air (2d Air, Figures 4 B-C) relative to freshly harvested fruit (0d). In contrast, a significant decrease in glucose and fructose was not observed in CO_2-treated fruit. With regards to myo-inositol abundance, a significant increase (28%) in myo-inositol was quantified in CO_2-treated fruit at LT storage (2d CO_2 and 2d CO_2+5d Air, Figure 4 D). In addition to a loss of hexoses and sucrose, there was a 42% decrease in trehalose concentration in strawberries stored at 0 oC in air by day 2, compared with fruit at harvest (Figure 4 E). There was also a similar significant decrease in trehalose levels at the end of CO_2- treated fruit. However, after 7 days at 0 oC, CO_2-treated fruit reached values similar to those quantified in fruit at harvest and 32 % greater than those found in air-stored fruit. With respect to FOS, only 1-kestose (DP3) showed significant changes during low temperature storage (Figure 4 F). Content of 1-kestose significantly increased at the end of CO_2 treatment, while slightly decreased at the early stage of low temperature storage at 0 oC (2d) compare to freshly harvested fruit. Opposite trends were observed following prolonged storage at 0 oC (7d), with this variable increasing in air-stored fruit. With respect to 6G-kestose and FOS concentrations with higher DP, no clear trends were observed in changes during low temperature storage (Figures 4 G-I). In contrast, in the case of RFOS, significant increases were seen in the concentration of both raffinose (DP3) and estaquiose (DP4) in air-stored fruit, both during early storage and after prolonged storage at low temperature (Figures 4 J-K). Such increases were less pronounced in CO_2-treated fruit. In the case of RFOS of DP4, only significant increases were quantified in air-stored fruit.

3.3.2 *Changes in expression of genes involved in sucrose metabolism during LT*

In order to verify changes to sucrose metabolic status during storage we analysed the expression of genes previously involved in the synthesis or breakdown of sucrose. With regards to synthesis, our results indicate

a slight yet significant increase in *FwSPS1* expression at the end of CO_2 treatment. A similar increase was also observed after 7 days in air-stored fruit, with homologues being expressed to a greater extent that *FwSPS2,* which showed a minor decrease (Figure 5 A).

From the beginning of cold storage and following sucrose breakdown, air-stored fruit already showed an upregulation of *FvSS1*expression at low temperature and significantly higher transcription levels (Figure 5 B). However, application of high CO_2prevented this increase in *FvSS1* expression from occurring. In contrast, whilst *FvSS2* expression was greatly up-regulated at low temperatures, CO_2 treatment failed to maintain initial rates of *FvSS2* expression. With regards to expression of putative cell-wall type invertase genes, transcription of the *FvCWINV1*gene occurred in time-dependent manner at low temperature, with greater homologue expression in comparison to *FvCWINV2* (Figure 5 C). The increase in *FvCWINV1* expression observed at the end of LT period (7d) is particularly striking. This was seen to be 8 times greater than its expression in freshly harvested fruits. Such a sharp increase in*FvCWINV1* expression is largely dictated by high CO_2 treatment, even after 5 days of transference to air. In a similar way, a significant increase in *FvCWINV2* expression was only observed at the end of the LT period. Likewise, a similar pattern of *FvVINV1* and *FvVINV2* expression was observed. Expression of both was increased in air-stored fruit, particularly during late LT at 0 oC, whilst high CO_2 treatment prevented this rise and resulted in notable lower expression (Figure 5 D).

3.4. Changes after cold removal and transfer to 20 degC (SL).

3.4.1 Changes in sugar levels during SL

We were interested to know whether the high sucrose levels in CO_2 -treated strawberries during LT storage were preserved during SL. Higher levels of sucrose were quantified in LTC relative to SLC. Sucrose content was also 50% higher than in LTA (Figure 6 A), reaching values of 6.2+-0.5 mg g^{-1} FW. However, no significant differences in glucose or fructose levels were found in LTC when compared to SLC and LTA (Figures 6 B-C). On the other hand, a slight yet significant increase in myo-inositol was found in LTC in comparison with SLC, with lower levels of trehalose being seen in both LTA and LTC (Figure 6 D). Asides from a slight decrease in 1-kestose during LTC, no significant differences in FOS or raffinose were quantified when values were compared during SL (Figures 6 F-J). The higher levels of estaquiose quantified in air-stored fruit during storage at 0 oC seems to be maintained after transfer to 20 oC (Figure 6 K). The highest level of estaquiose, therefore, was quantified in LTA.

3.4.2 Changes in the expression of genes involved in sucrose metabolism during SL

Expression of the main genes involved in sucrose metabolism was analysed in order to identify whether transcriptional regulation of these genes changes in response to SL. A decrease in *FvSPS1* , the main homologue expressed, was observed in LTA strawberries when compared with SLC and LTC (Figure 7 A). Moreover, a significant decrease in*FvSS1* expression was found in the main homologue expressed in LTA and LTC, compared with SLC (Figure 7 B). Thus, these results seem to indicate that CO_2 pre-treatment maintains sucrose synthesis after transfer to 20 oC, whereas LT reduces sucrose breakdown. Changes in the relative gene expression of invertases during SL are indicated in Figures 7 C and D. Interestingly, whilst no changes to*FvCWINV* homologues were observed, a significant decrease in the expression of both *FvVINV* homologues was quantified in LTC compared to SLC. Furthermore, *FvVINV2* expression was found to significantly increase during LTA. These results indicate that CO_2 pre-treatment significantly reduces sucrose degradation during SL. Overall, the present analysis of gene expression could explain the high level of sucrose detected in LTC during SL.

3.5. Sugar and acid balance in strawberries at the moment of consumption.

As would be expected given the aforementioned higher sugar levels when fruit were transferred to 20 oC for 1 day, TSS content was higher in LTC than in LTA and exceeded values found in SLC following two days of storage at 20 oC (Table 1). In LTA strawberries, maximum TSS levels are found during day 1. Following 2 days at 20 oC, there is a pronounced decrease in TSS that does not occur in LTC (data not shown). As can

be seen in Table 1, titratable acidity values which are expressed as % of citric acid, decreased in strawberries previously stored at LT, with this decrease being higher in LTC than in LTA. The present results indicate that elevated CO_2 reduced organic acid content and increased the total sugar to acid ratio, reflecting lowered sourness in the strawberry. As can be seen in Table 4, 'Mara des Bois' accumulates high levels of TSS during the DR stage. Although high TSS may also correlate with a high risk of fungal disease, fruit is considered to have a richer flavour when it has a good balance of sugars and organic acids, with the sugar/acid balance largely depending on the maturity degree (Schwieterman et al., 2013; Basson et al., 2010; Fait et al., 2008; Jouquand et al., 2008). The highest sugar/acid ratio was found in DR with values similar to those found in CO_2-treated fruit, being these previously scored as the most highly in sweetness and sensory evaluations (data not shown).

3.6 Impact of high CO_2 on sugar accumulation and weight loss reduction

The marked accumulation of major soluble sugars in CO_2-treated fruit suggests that is has better osmotic adjustment. This has a significant influence on weight loss reduction in fruit. Detailed data relating to weight loss are presented in Figure 6. During LT storage, average weight loss throughout the early stage of storage at 0 oC was significantly lower in CO_2-treated strawberries in comparison to fruit stored in air. After 7 days of storage at 0 oC, the percent of weight loss was also more accentuated in fruit stored in air than in the fruit pre-treated with CO_2 for 2 days. In this case, a reduction in weight loss of 47-50% was recorded due to high CO_2 treatment. Weight loss did not exceed 1% at any stage during LT storage (Figure 8). When fruit were transferred to 20 oC for 1 day, significantly less weight was lost in fruit previously stored at low temperature than those directly kept at 20 oC. However, weight loss was not significantly different between fruit with or without CO_2. When SL was prolonged for two days, water loss was significantly higher in fruit stored in air than in CO_2-treated fruit. Average weight loss in SL+LTA was 1.0%, compared to 0.8% in SL+LTC and 1.5% in fruit stored directly at 20 oC. CO_2-treated strawberries showed the lowest weight losses after 2 days at 20 oC. Present results show that pre-treatment with 17% CO_2 for 2 days limited water loss during SL.

We speculate that increased soluble carbohydrate concentration is engendered by a lower consumption of hexoses. This may be due in part to its low availability as present results evidenced that reduced sucrose breakdown by FvSS1, FvVINV1 and FvVINV2 avoiding water loss.

4. Discussion

Changes in sugars during ripening transition and after harvest.

Sweetness is strongly associated with the general concept of fruit quality. Thus, it is important to understand how this trait is affected by both fruit ripening stages and their specific interaction with the environmental after harvest. The improvement of flavour and fruit quality at harvest may be possible, through greater understanding of the ripening stages that are particularly sensitive to sucrose accumulation and have a positive impact on sensorial quality. The present results show increased sucrose content in DR, which is in accordance with previous works that reported higher sucrose levels during the ripe stage (stage 6, Zhang et al., 2011). In a similar way, in other varieties, glucose and fructose were also found to be the major sugars followed by sucrose and myo-inositol (Macias-Rodriquez, et al., 2002). Previous works reported an increase in myo-inositol during the ripe stage, followed by a decrease during the over-ripe stage (stage 7, Zhang et al., 2011). Similar sucrose levels, however, were quantified at the AR and FR stages, suggesting that another role may be important other than the contribution to sucrose. With respect to FOS during ripening transition stages, our results show that DP3, 1-kestose, 6G-kestose and DP4 show similar trends, with significant increases being seen in fruit during FR relative to strawberries at the AR stage. We previously reported a correlation between FOS and changes in fruit water status (Blanch et al., 2012). The present results about dynamic changes in FOS during ripening point to this contribution. In consideration of the high levels of sucrose observed, alongside those of other major sugars during the DR stage, it would be interesting to analyse the molecular mechanisms involved in the synthesis and degradation of sucrose. Furthermore, it is essential to analyse the dynamics of sucrose accumulation and to explore the way in which sucrose reserves might be controlled following harvest through modifications to the environment surrounding fruits.

One of the most effective technologies for delaying biochemical and physiological processes after harvest is to apply low temperature storage at 0 oC. Indeed, the negative correlation between the storage duration at LT and taste quality requires application of coadjutant technologies such as pre-treatment with high CO_2. Present results indicate maintenance of sucrose levels in CO_2 pre-treated fruit during low temperature storage, whilst significant decreases occurred in fruit at the early stage of storage at 0 oC. Moreover, the decrease in hexose sugars detected is also coupled with the decrease in sucrose previously mentioned, indicating that a net consumption of sugars mainly occurs during the early stage of LT storage. However, a decrease in glucose or fructose was not observed during the late stage of LT in either regular or CO_2-treated fruit. An increase in sucrose due to CO_2 treatment has already been reported and the opposite trend was observed during postharvest storage at low temperature (6 oC) in several cultivars of strawberries (Drake et al., 1997; Cordenunsi et al., 2003). Further, a decrease in myo-inositol has been observed during low temperature in air-stored fruit in a similar way as was previously reported under unfavourable storage conditions (Blanch et al 2015b). We suggest that myo-inositol could be a precocious marker of a relevant metabolic condition for measuring optimal storage conditions. Likewise, the increase in RFOS observed, particularly in air-stored fruit, could be linked to the response of strawberries to storage at 0 oC. Several authors have indicated that RFOS seems to play an important role in plant responses to osmotic stress (Ishitani et al., 1996; Loewus and Murthy, 2000; Sengupta et al., 2015). Given the present findings of a marked increase in RFOS in air-stored fruit at during early and late storage at 0 oC, we suggest that RFOS was linked to cold stress. In the case of trehalose levels, CO_2-treated fruit showed higher levels that fruit stored in air, only at the end of low temperature storage. These results agree with those reported in the skin tissue of CO_2-treated table grapes. In this case, higher levels of trehalose were quantified in comparison with untreated fruit after prolonged low temperature storage (Vazquez-Hernandez et al., 2018). In fact, a protective role has been attributed to trehalose through the stabilization of membranes and proteins (Garg et al 2002, Fernandez. et al. 2010). According to present outcomes regarding sucrose, trehalose and RFOS, we speculate that treatment with high levels of CO_2 seems to favour the accumulation of sucrose during the early stage of LT, as opposed to accumulation of trehalose and RFOS.

Changes in sucrose metabolism gene expression.

Sucrose metabolism is controlled mainly by SPS, SS and invertases (Winter and Huber 2000; Wan et al., 2018; Stein and Granot, 2019). The reaction catalysed by SPS plays an important role in the regulatory steps controlling sucrose synthesis depending on the plant species considered (Huber and Huber, 1996; Do Nacismento et al., 1997; Winter and Huber 2000; Choudhury et al., 2010; Volkert et al., 2014). Further, SPS plays a crucial role in many physiological processes including the alteration of the cell wall ultrastructure (Geigenberger et al., 1999; Coleman et al., 2009). Present results showed that *FvSPS1* expression significantly increased in both FR and DR strawberries. Although a marked increase in sucrose was only seen in DR strawberries, we cannot rule out the possibility of post-translational modulation of its activity. Concerning both of the *FvSS* homologues analysed, we detected a slight increase in their expression. Although sucrose synthase is capable of both degrading and synthesizing sucrose, its primary function might be sucrose degradation (Nguyen-Quoc and Foyer, 2001). Whilst the role of each homologous remains to be elucidated, present results suggest that *FvSS* could play a role in sucrose cleavage in strawberries during ripening transition. In this sense, SS has been identified as an indicator of sink strength in growing tomato fruits (Sun et al., 1992) and as playing a crucial role in sucrose utilization in fruit development (Wang et al., 1993). Present data show increased *FvCWINV1* and *FvVINV1* expression in DR fruit compared to AR and FR fruit (2 and 3-fold respectively; Figures 3 C-D), whilst *FvVINV2* expression was considerably decreased (6 fold). It is possible that whilst these two genes are from the same family, they have developed different functions. The possible involvement of these activities hydrolysing oligosaccharides containing fructose (Wan et al., 2018), seem not to be an option considering the levels of FOS and sucrose observed during ripening transition stage. Thus, the large sucrose increases detected in DR may be largely explained by the compensatory balance between the increase in *FvSPS1* and the sharp decrease in *FvVINV2,* together with all of the activities involved in sucrose metabolism.

Regarding the gene expression analysed at LT and SL, no major changes in the *FwSPS1* expressions were

detected in CO_2treatments. On the other hand, the slight increase of its expression observed at the end of CO_2 treatment together with the maintenance of *FwSPS1* expression in LTC match with the highest levels of sucrose detected. Considering all of these results together, the increase in sucrose, specifically in DR and in SLC, seems to not only be explained by changes in *SPS* expression. Thus, our results showed increased *SS* gene expression in strawberries stored at 0 oC during early and prolonged storage at 0 oC, with the lowest sucrose values being quantified in this work. In contrast, *FvSS1*expression in CO_2-treated fruit was maintained, resembling levels observed immediately after harvest, and modulating the sharp increase of *FvSS2* expression observed in un-treated samples. In fact, the low *FvSS1* expression observed in LTA and LTC during SL could explain the fact that higher sucrose levels were observed in LTC which were similar to those observed in CO_2 pre-treated fruit. As the SS pathway produces already phosphorylated glucose which does not, therefore, have to be phosphorylated at the expense of ATP in order to enter into the glycolytic pathway, the breakdown of sucrose into hexoses in the cytosol via SS is more energy-efficient. It has been reported that SS sustains glycolysis under hypoxic conditions (Germain et al., 1997; Ruan et al, 2012). In addition to its involvement in rapid sucrose degradation, SS has been linked to biosynthetic processes of cell wall polysaccharides by directly supplying UDP-glucose as a substrate for secondary cell wall formation (Salnikov et al., 2001; Li et al., 2019b; Coleman et al., 2009). Present results indicate that *SS* may play a role in modulating sucrose cleavage in strawberries stored in air at low temperature. It is possible that higher *SS* expression in strawberries stored at 0 oC in air may contribute to sustaining glycolysis, whilst also providing UDP-glucose for the synthesis of RFOS. Present results indicate that low temperature cause increase in the levels of raffinose and estaquiose. These results are in line with the enhancement of RFOS in plants in response to different kind of stress. The sharp increase in *FvCWINV1* expression observed in fruit stored in air at 0 oC is also interesting. Present results show an apparent association between LT damage and up-regulation of cell wall invertases. In fact, increased cell wall invertase activities are typically associated with stress responses (Roitsch et al., 2003). Interestingly, the high *FvCWINV1* expression observed here in addition to *FvCWINV2* expression were controlled by high CO_2 pre-treatment (Figure 5 C). The decrease observed in sucrose, glucose and fructose alongside the significant increase in expression of both *FvCWINV* and *FvVINV* in air-LT strawberries, suggests that storage at 0 oC leads to a reprogramming of carbon metabolism with a preference for sucrose-consumption reactions. Sucrose breakdown at 0 oC possibly occurs in fruit in order to meet demands for an increased substrate supply due to an increased respiration rate. In the same way, increased expressions of*FvVINV* homologues in fruit stored at 0 oC in air also suggests an association between LT damage and up-regulation of vacuolar invertase. Such up-regulation could possibly be integrated within the context of general damage caused by low temperature in the active transport systems of ions, soluble sugars and other metabolites. We suggest that the low utilization of hexoses in CO_2 pre-treated fruits should contribute towards generating sufficient cell turgor, preventing sucrose degradation or rendering it unnecessary (Blanch et al., 2015b). It has been proposed that VINV plays a key role in regulating plant cell expansion through osmotic regulation (Sergeeva et al., 2006; Tang et al., 1999; Neumann et al., 2002; Wang and Ruan 2016; Wan et al., 2018). Distinct types of vacuoles with different functions have also been reported (Frigerio et al., 2008; Zouhar and Rojo, 2009; Marcos Lousa et al., 2012), some of which are synthesized at the end of the plant's life cycle and are denominated senescence-associated vacuoles (Otegi et al., 2005). On the other hand, it is interesting to note that during SL,*FvVINV* expression observed in LTC remains very low compared to SLC, *FvVINV2* expression remains particularly repressed following 2 days at 20 oC in LTC samples, when considered relative to fruit stored in air (LTA) at low temperature. These results could explain the higher level of sucrose observed in LTC, with these samples being optimum for consumption. Thus, decreased expression of invertases in CO_2-treated fruit could be integrated within the context of a general protective mechanism that controls total soluble sugar, with repression of this mechanism affecting postharvest transpirational water loss.

Conclusions

In order to obtain better taste quality in strawberries at the moment of consumption, we analysed the dynamics of sucrose accumulation during ripening transition in strawberries and considered the way in which sucrose reserves after harvest are controlled by environmental factors. Analysis of carbohydrates

and the expression of sucrose-related genes revealed that short-term treatment with high levels of CO_2 is particularly efficient in the accumulation of sucrose and soluble carbohydrates, suggesting activation of a carbohydrate-conserving mode in CO_2-treated strawberries. To our knowledge, this is the first time that a synchronized down-regulation of genes encoding sucrose breakdown has been highlighted, which mainly involves *FvVINV* homologues, with an increase in sucrose and soluble carbohydrate content. The lower expression of *FvVINV2* observed in CO_2-treated strawberries might be related with a less consumption of sucrose what would drive to a lower weight loss. This fact is supported not only by the negative correlation between solutes and *FwVINV2* expression in air-stored strawberries, but also by the findings observed in DR strawberries. In this ripening stage, a more active sucrose metabolism is recorded relative to FR strawberries, suggesting an essential role of the date of harvest in sucrose metabolism. The decrease of sucrose at 0 oC is linked to a high consumption of released hexoses due to upregulation of *FvSS* , *FvVINV* homologues and, especially,*FvCWINV2* encoding genes involved in sucrose breakdown.

Supporting information

Supporting data are available at *Plant, Cell & Environment online* .

Supplementary Table S1.

List of primer sequences used in this study.

Supplementary Figure S1.

Accession numbers of SPS, SS and CWINV homologues according to different databases.

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Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Quality parameters from 'Mara des Bois' strawberries. Changes in the content of Malic Acid determined as Total Soluble Solids (TSS), pH, and Titratable Acidity (TA). Experiments were performed with strawberry fruit at 0d: immediately after harvest; SLC: freshly harvested fruit placed directly at 20 oC during two days; LTA: samples transferred 1 day at 20 oC from air at low temperature; LTC: samples transferred 1 day at 20 oC from CO_2 pretreatment, and with different stages of ripening strawberry fruit immediately after harvest at AR: 2/3 red, FR: full red and DR: dark red. Data are presented as the means +- SE of three technical determinations for each three biological replicates (each one contains 10 fruits); different letters (a–c) within rows indicate significant differences at $P< 0.05$ using Duncan's Multiple Range test.

	TSS (%)	**TA (%)**	**pH**	**TSS/TA**
0d	8.02 ± 0.19 a	1.45 ± 0.02 b	3.15 ± 0.01 a	5.53
SLC	8.32 ± 0.28 ab	1.39 ± 0.07 b	3.17 ± 0.02 a	6.00
LTA	8.33 ± 0.23 ab	1.23 ± 0.05 a	3.40 ± 0.02 b	6.74
LTC	8.58 ± 0.29 b	1.17 ± 0.07 a	3.39 ± 0.02 b	7.31
AR	7.95 ± 0.14 a	1.98 ± 0.00 c	3.19 ± 0.01 a	4.02
FR	8.65 ± 0.05 b	1.76 ± 0.00 b	3.26 ± 0.01 b	4.91
DR	10.40 ± 0.00 c	1.47 ± 0.00 a	3.50 ± 0.01 c	7.09

Figure captions

Figure 1. Amounts of sugars quantified for each ripening stage. Experiments were performed with strawberry fruits immediately after harvest at ripening stages AR: 3/4 red, FR: full red and DR: dark red. A) Sucrose (mg g-1 FW); B) Glucose (mg g-1 FW); C) Fructose (mg g-1 FW); D) Myo-inositol (µg g^{-1} FW); E) Trehalose (µg g^{-1} FW); F) 1-Kestose (µg g^{-1} FW); G) 6G-Kestose (µg g^{-1} FW); H) Nystose (µg g^{-1} FW); I) Kestopentaose (µg g^{-1}FW); J) Raffinose (µg g^{-1} FW); K) Estaquiose (µg g^{-1} FW)). Graphs represent the average of at least three independent replicates (each one contains 10 fruits) quantified. Error bars indicate

± SEM; ***$P < 0.001$, ****$P < 0.0001$; Student's t-test of AR versus FR or DR value.

Figure 2 . Phylogenetic analysis of main sucrose metabolism proteins. Protein sequence phylogeny using the Maximum Likelihood method and JTT matrix-based model for *Fragaria vesca* and*Arabidopsis thaliana* in: A) SPS, B) SS, C) CWINV and VINV proteins families. HMM profiles to each protein family from Pfam or InterPro databases were used as a query against the *F. vesca*v4.0.a2 genome to find the closest *F. vesca* homologues. These paralogues were used for find the most closed *Arabidopsis*orthologues.

Figure 3. Expression pattern of the main sucrose metabolism genes during different ripening stages. Experiments were performed with strawberry fruits immediately after harvest at ripening stages AR: 3/4 red, FR: full red and DR: dark red. Expression levels of: A)*FvSPS1* and *FvSPS2* , B) *FvSS1* and *FvSS2* , C)*FvCWINV1* and *FvCWINV2* and D) *FvVINV1* and*FvVINV2* . E) Pictures of strawberry fruits at different ripening stages. Graphs represent the average of three independent biological experiments quantified by qPCR. Error bars indicate +- SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's t-test of AR versus FR or DR value.

Figure 4. Amounts of sugars quantified for each day during storage at 0 oC. Experiments were performed with strawberry fruit at 0d: immediately after harvest; 2d Air, 7d Air: air stored fruits during 2 or 7 days; 2d CO_2: fruits treated during 2 days with CO_2; 2d CO_2+5d Air: fruits treated during 2 days with CO_2 plus 5 days in air. A) Sucrose (mg g^{-1} FW); B) Glucose (mg g^{-1} FW); C) Fructose (mg g^{-1} FW); D) Myo-inositol (µg g^{-1} FW); E) Trehalose (µg g^{-1} FW); F) 1-Kestose (µg g^{-1} FW); G) 6G-Kestose (µg g^{-1} FW); H) Nystose (µg g^{-1} FW); I) Kestopentaose (µg g^{-1} FW); J) Raffinose (µg g^{-1} FW); K) Estaquiose (µg g^{-1} FW)). Graphs represent the average of at least three independent replicates (each one contains 10 fruits) quantified. Error bars indicate ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; Student's t-test of 0d versus each treatment value.

Figure 5. Expression levels of the main sucrose metabolism genes during storage at 0oC. Experiments were performed with strawberry fruit at 0d: immediately after harvest; 2d Air, 7d Air: air stored fruits during 2 or 7 days; 2d CO_2: fruits treated during 2 days with CO_2; 2d CO_2+5d Air: fruits treated during 2 days with CO_2 plus 5 days in air. Expression levels of: A) *FvSPS1* and *FvSPS2* , B)*FvSS1* and *FvSS2* , C) *FvCWINV1* and *FvCWINV2*and D) *FvVINV1* and *FvVINV2* . Graphs represent the average of three independent biological experiments quantified by qPCR. Error bars indicate +- SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; Student's t-test of 0d versus each treatment value.

Figure 6. Amounts of sugars quantified during shelf-life at 20 oC. Experiments were performed with SLC: freshly harvested fruit placed directly at 20 oC during two days; LTA: samples transferred 1 day at 20 oC from air at low temperature; LTC: samples transferred 1 day at 20 oC from CO_2 pretreatment. A) Sucrose (mg g^{-1} FW); B) Glucose (mg g^{-1} FW); C) Fructose (mg g^{-1} FW); D) Myo-inositol (µg g^{-1} FW); E) Trehalose (µg g^{-1} FW); F) 1-Kestose (µg g^{-1} FW); G) 6G-Kestose (µg g^{-1} FW); H) Nystose (µg g^{-1} FW); I) Kestopentaose (µg g^{-1} FW); J) Raffinose (µg g^{-1} FW); K) Estaquiose (µg g^{-1} FW). Graphs represent the average of at least three independent replicates (each one contains 10 fruits) quantified. Error bars indicate ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's t-test of SLC versus LTA or LTC value.

Figure 7. Expression levels of the main sucrose metabolism genes during shelf-life at 20 oC. Experiments were performed with SLC: freshly harvested fruit placed directly at 20 oC during two days; LTA: samples transferred 1 day at 20 oC from air at low temperature; LTC: samples transferred 1 day at 20 oC from CO_2pretreatment. A) Sucrose (mg g^{-1} FW); B) Glucose (mg g^{-1} FW); C) Fructose (mg g^{-1} FW); D) Myo-inositol (µg g^{-1} FW); E) Trehalose (µg g^{-1} FW). Graphs represent the average of three independent biological experiments quantified by qPCR. Error bars indicate ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Student's t-test of SLC versus LTA or LTC value.

Figure 8. Changes in weight loss (%) during storage and during shelf-life compared to strawberry fruit immediately after harvest. Fruit weight losses were measured in strawberry fruit at 2d Air, 7d Air: air stored fruits during 2 or 7 days; 2d CO_2: fruits treated during 2 days with $CO_,$, 2d CO_2+5d Air: fruits treated during 2 days with CO_2 plus 5 days in air; SLC: freshly harvested fruit placed directly at 20 oC during two

days; LTA: samples transferred 1 day at 20 oC from air at low temperature; LTC: samples transferred 1 day at 20 oC from CO_2 pretreatment. Graphs represent the average of at least 16 (n=16-52) independent experiments. Error bars indicate +- SEM; $^{**}P < 0.01$, $^{****}P < 0.0001$. Student's t-test of each comparison is indicated.

Additional Files

NAME	SEQUENCE	GENE
FvSPS1_F	GATACGCTCGTTTCTGGTCTCT	*FvH4_1g02490*
FvSPS1_R	GCGTACCAATGTCTTCCTCAAC	
FvSPS2_F	GCAAAAGTGGGAGTGAAATG	*FvH4_2g00340*
FvSPS2_R	CCCTCCATACTCCGTGATG	
FvSS1_F	CATCTACAGCCAAGAGGA	*FvH4_1g09360*
FvSS1_R	GCTTTCTCCTCATTGTCCT	
FvSS2_F	CTCTTATGGCCGATTTCTTTC	*FvH4_2g26000*
FvSS2_R	GCCTTTCAAGCTTTGAGACATA	
FvCWINV1_F	GCTTGGACAACACTAGGAA	*FvH4_6g33590*
FvCWINV1_R	AGTCCAGACCATCCTTTCTT	
FvCWINV2_F	TTGGTGGAGAAGGCAAAGT	*FvH4_6g33620*
FvCWINV2_R	ATACTCCACGCACTTCCTGA	
FvVINV1_F	TGAAGCAGGAGCAGTTGTTG	*FvH4_7g27630*
FvVINV1_R	AGGGGTCAGCTCTGAAAGAA	
FvVINV2_F	CGATCATTGACAGCTTTGCT	*FvH4_5g00290*
FvVINV2_R	TTGCTGCATCAGGTGAAAAT	
FvACT_F	GGGTTTGCTGGAGATGATG	*FvH4_7g22410*
FvACT_R	CACGATTGGCCTTGGGATTC	

Table S1. List of primer sequences used in this study.

Supplementary Figure S1

Accession numbers (gene_V4, gene_V2, NCBI_LOC, NCBI_PROT)

SPS homologues : *FvSPS1* (*FvH4_1g02490* ,*gene31122-v1.0-hybrid.t1* , LOC101307526, XP_004287272.1),*FvSPS2* (*FvH4_2g00340* , *gene31164-v1.0-hybrid.t1* , LOC101310276, XP_004290259.1), (*FvH4_2g28820* ,*gene11606-v2.0.a2-hybrid.t1* , LOC101292871, XP_004292975.1), (*FvH4_4g13520* , *gene06523-v1.0-hybrid.t1* , LOC101298507, XP_004296966.1).

SS homologues : *FvSS1* (*FvH4_1g09360* ,*gene12940-v1.0-hybrid.t1* , LOC101306372, XP_004287669.1),*FvSS2* (*FvH4_2g26000* , *gene11077-v2.0.a2-hybrid.t1* , LOC101313949, XP_004290813.1), (*FvH4_1g07260* ,*gene35311-v2.0.a2-hybrid.t1* , LOC101292831, XP_004287544.1) (*FvH4_4g18710* , *gene07050-v2.0.a2-hybrid.t1* , LOC101298794, XP_011462741.1).

CWINV homologues : *FvCWINV1* (*FvH4_6g33590* ,*gene23034-v2.0.a2-hybrid.t3* , LOC101304277, XP_004297549.1), FvCWINV2 (FvH4_6g33620, *gene37783-v2.0.a2-hybrid.t2* , LOC101307779, XP_004297561.1), (FvH4_6g33780,*gene06912-v2.0.a2-hybrid.t1* , LOC101311110, XP_004303654.1).

VINV homologues : *FvVINV1* (*FvH4_7g27630* ,*gene21228-v1.0-hybrid.t1* , LOC101302788, XP_004307805.1), FvVINV2 (FvH4_5g00290, *gene32247-v2.0.a2-hybrid.t1* , LOC101315234, XP_004298661.1)

Figure 1

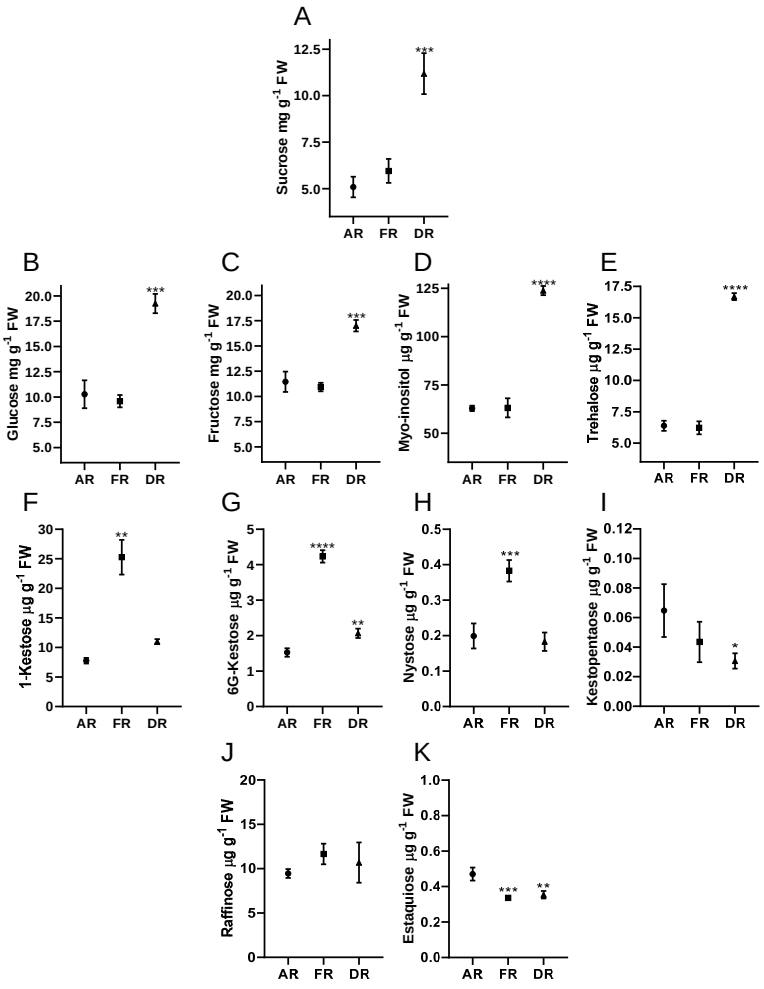

Figure 2

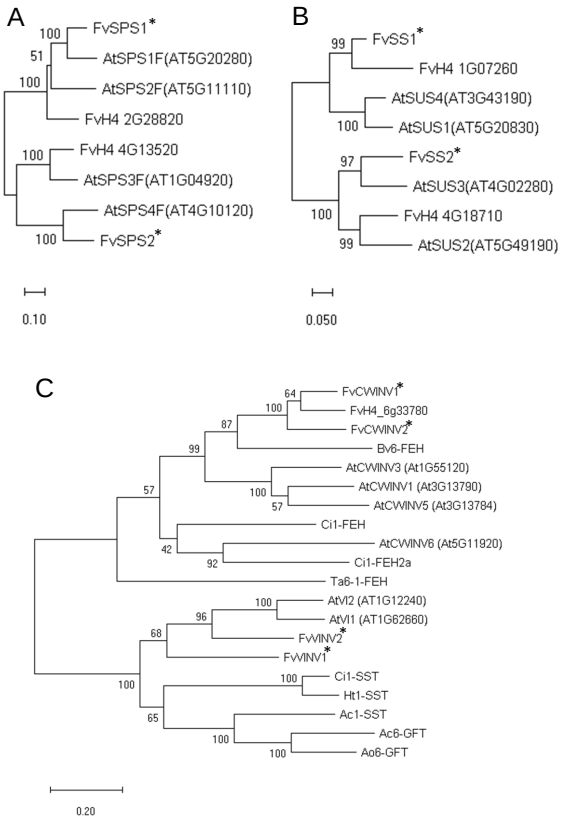

Figure 3

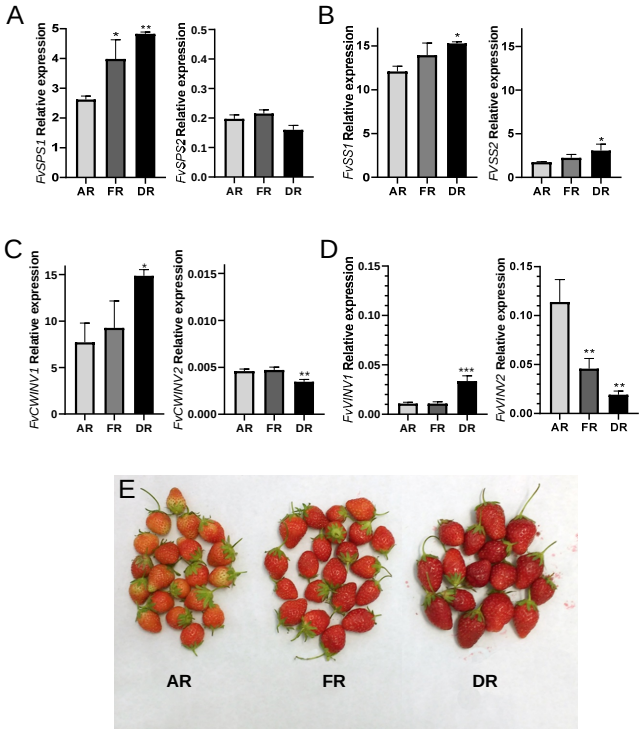

Figure 4

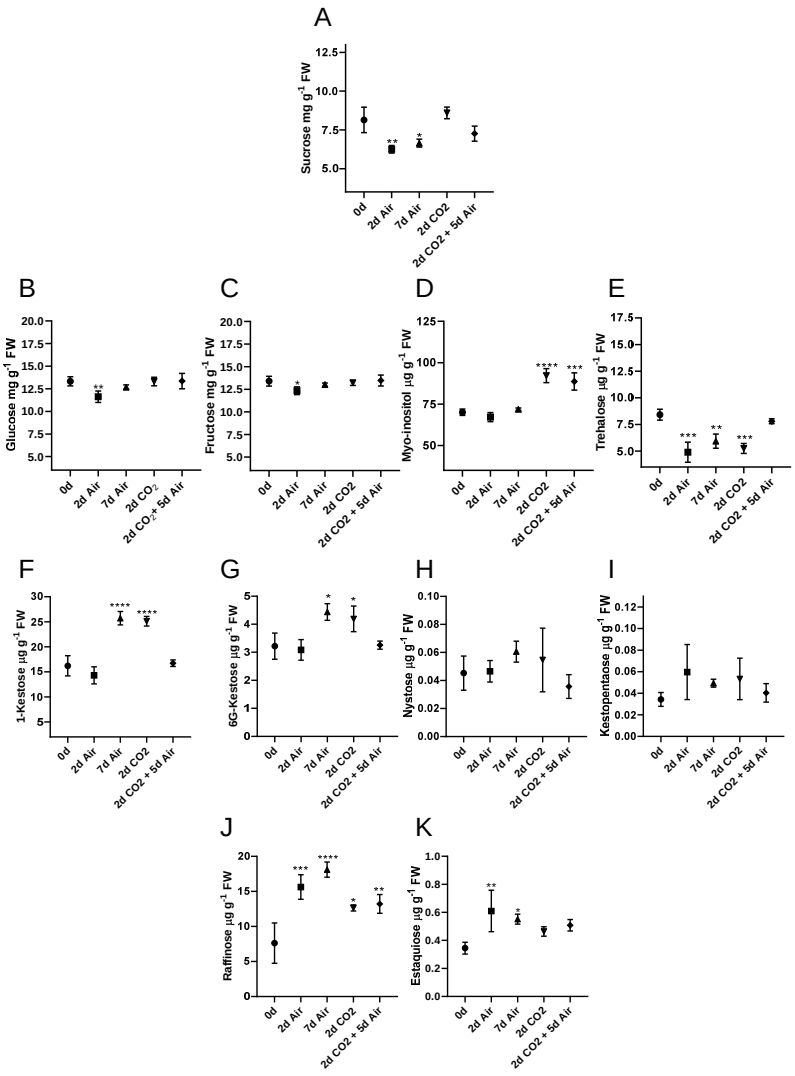

Figure 5

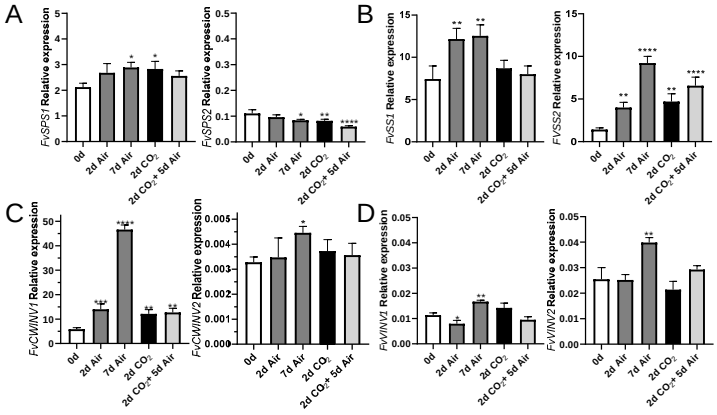

Figure 6

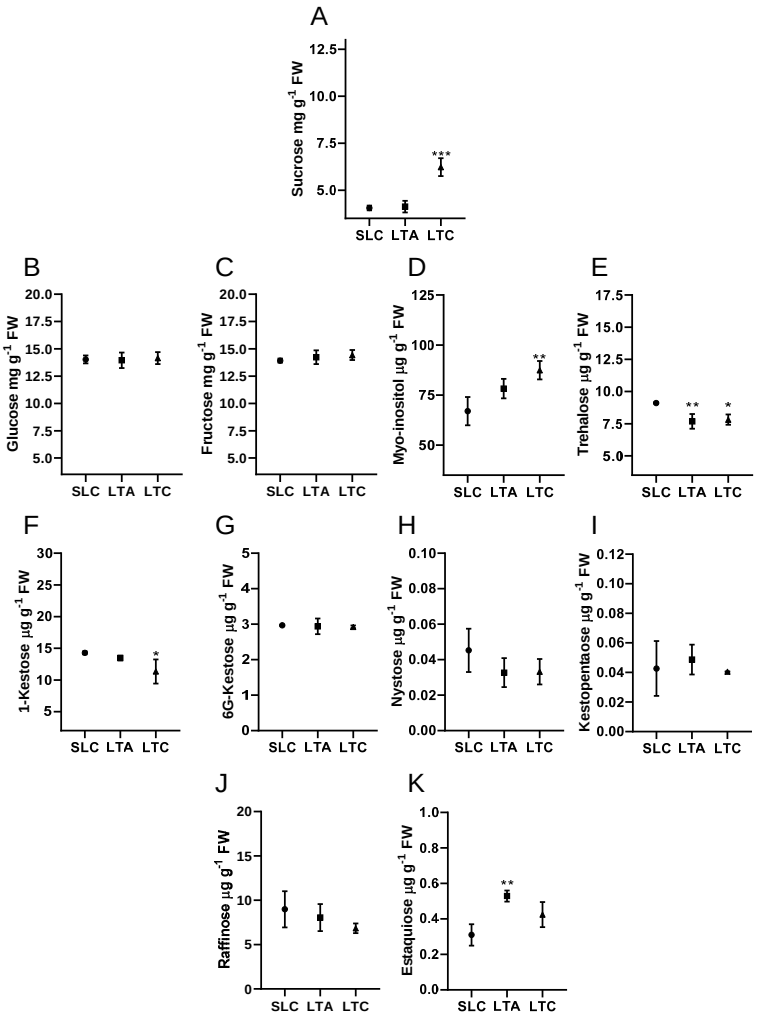

Figure 7