RESEARCH PROJECT REPORT– TALENTIA POSTDOC

Contracting Entity: University of Granada
Beneficiary: Raquel Rosales López

Project Title: Elucidating the impact of low temperature storage on tomato fruit flavor: possibilities for genetic improvement?
Mobility Scheme:

___ INCOMING
X OUTGOING (Host Institution abroad: Wageningen University and Research)

Duration of the contract and the project: From **1st October** of 2015 to **30th September** of **2017**
Key dates:

INCOMING	**OUTGOING**		
Start date Contracting Entity (only INCOMING projects)	**Start date Contracting Entity** (only OUTGOING projects with mobility periods modified by Resolution)	**Start date Host Institution abroad**	**Start date Contracting Entity** (only OUTGOING projects, after period abroad)
		1st, October, 2015	1st, January 2017

Object Reporting Period: From **October 1st** of **2016** to **September 30th** of **2017**

Content:

[Signature]

Signed: Raquel Rosales Lopez
_____
Beneficiary person

[Signature and stamp]

[Signature and stamp]

Approved: Dolores Garrido Garrido
Scientific Supervisor at Contracting Entity

Approved: Arnaud G. Bovy
Scientific Supervisor at Host Institution (only OUTGOING projects)

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

1.- EXPECTED GOALS

We hypothesized that there is genetic variation in the response of tomato fruits to low temperature storage and that the concomitant loss of tomato flavour quality induced by cold storage and the assessment of this variability could be used to improve the organoleptic quality of tomato stored at low temperature. Thus, the expected goals of this project were:

1) To evaluate the impact of cold storage on tomato flavour in a diverse set of genotypes, identify those showing a better performance in terms of maintenance of fruit flavour and general quality
2) To study the transcriptional changes related with the loss of flavour in cold-stored fruit by means of RNA-Seq in selected contrasting genotypes, according to the impact of cold storage on their original organoleptic quality.
3) Determine allelic variation in flavour-related candidate genes and associate candidate gene haplotypes with low temperature tolerance.

2.- ANALISYS OF ETHICAL ISSUES

This project does not involve collection of human specimens, recruitment of patients, storage of personal data or genetic information, the development of genetically modified organisms, the use of animals or experimental work relating to a developing region.

For further details, we refer you to the ethical reports submitted together with this research report to the ‘Agencia Andaluza del Conocimiento’ and signed by the head of Breeding department in Wageningen UR (Dr. Richard G.F. Visser) and the head of the Plant Physiology department in the University of Granada (Dr. Juan Manuel Caba Barrientos).

3.- METHODOLOGY

Abbreviations

BFp = Firmness as the breaking point of the pericarp.
CS = Cold stored fruit (5º C)
FH = Fresh harvested fruit
GRAS = Generally recognized as safe
HCA = Hierarchical cluster analysis
PCA = Principal component analysis
RH = relative humidity
SPME-GC-MS = solid phase microextraction-gas chromatography-mass spectrometry
SSC = soluble solids content
TA = titratable acidity
USFDA = United States food and drug administration
VOC = volatile organic compound

Plant Material

Tomato plants from eight different genotypes were grown under commercial standard conditions and were provided by ENZA ZADEN (Enkhuizen, The Netherlands). Fruit at red-ripe stage were harvested in three biological replicates (fruit from each replicate came from different plants). Upon

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union’s Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

arrival to the lab, fruits free of visual defects, and uniform in size were selected and stored at chilling (5 ºC) or non-chilling (15 ºC) temperature and 80 % RH for 7 days then transferred at 20 ºC for 1-day recovery. Tomato fruit were sampled at 0h and 7 days of storage at 5 ºC or 15 ºC plus 1-day recovery at 20 ºC. These samples were named T0, cold-stored (CS), and control. For each genotype, we performed at least two cold storage experiments.
Moreover, the day before the sensory panels were going to be carried out, a new batch of fruit, at the same stage of ripeness than T0, was harvested and placed at 20 ºC for 1 day to be compared with CS and control fruit. We called this sample FH (fresh harvest) to differentiate it from the non-stored, T0 sample.
For VOC and primary metabolites analysis a quarter of 10-12 fruits per replicate and sample were frozen in liquid N and stored at -80 ºC until analysis.
All instrumental measurements were determined in all four fruit groups: T0, CS, Control, and FH.

Quality parameters
Soluble solid content (SSC) was measured using a refractometer and expressed as º Brix.
Titratable acidity (TA) was determined by titration with NaOH and expressed as mmol acid per 100g FW. For each sample and biological replicate, the juice of 12-15 fruits was used to measure SSC and TA.
Firmness and juiciness were measured in 10mm diameter discs of tomato pericarp according to Verkerke et al., 1998 (Acta Hort. 456. ISHS 1998). Firmness represents the force needed to break the fruit pericarp (BFp, N). Juiciness indicates the amount of juice pressed from the fruit pericarp and was expressed as % juice (g juice/100 g fresh weight). Juiciness and BFp were measured in 3 technical replicates of 5 fruit for each storage condition and biological replicate.

The flavor level of each sample was calculated using these four parameters by means of the Instrumental Flavor Prediction Model (Wageningen UR Greenhouse Horticulture, 2011).
Sensory analysis
Within a variety, the stored fruits were compared with fresh harvest fruits by flavor panels. Liking was scored by a consumer panel (n≈50) on a 0-100 scale. Flavor attributes were scored by a trained sensory panel (n≈25) on a scale ranging from 0 to 100. All fruits were placed at 20 ºC the night before testing to equalize eating temperature. Per sample, pieces of 6 different fruits were tasted and samples were served according to a randomized design.

Odor Threshold experiment
Volatile compounds (95-99 % purity) were obtained from Sigma-Aldrich. Z-3-Hexenal was in a 50% solution of triacetin (a triglyceride used as food additive, approved as a GRAS food additive by the USFDA).
Tomato matrix: less than 1h before the test was carried out, Merlice fruits previously refrigerated for at least 24h at 4 ºC (fridge), were juiced with a slow-juicer (Omega Products, Inc. PA, USA) and used as the partially deodorized tomato juice in which the different VOCs were added.
Sensory method: detection thresholds of the tomato aroma compounds were determined according to the ‘ascending method of limits’ of the American Society for Testing and Materials (ASTM, 1991, Reapproved 2011. DOI: 10.1520/E0679-04R11). For each VOC, panelists were given four sets of three samples, one per VOC concentration in ascending order. From the set of three samples, one containing the compound and two blank tomato purees with no added compound, panelists were asked to indicate which of the three samples was different. The method provides a ‘best estimate threshold’ (BET) for each panelist, based on the geometric mean of the highest concentration missed

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

and the next highest concentration. A group threshold is then calculated as the geometric mean of the individual BETs.

Volatiles organic compounds (VOCs) were profiled and identified according to Tikunov et al., 2005 (Plant Physiol. 139, 1125-39). First the VOC profiles were obtained by SPME-GC-MS (solid phase microextraction-gas chromatography-mass spectrometry), then the chromatograms were aligned using MetAlign software (http://www.wur.nl/nl/Expertises-Dienstverlening/Onderzoeksinstituten/rikilt/show/MetAlign.htm), followed by multivariate comparative analysis of metabolic phenotypes at the level of individual molecular fragments, and multivariate mass spectral reconstruction using MSClust software.
For the 10 VOCs used in the odor threshold experiment, absolute content was quantified using standard curves.

Primary metabolites analysis was performed by GC-TOF-MS (Lisec et al., 2006. Nature Protocols 1: 387-96) and in some cases confirmed by ion chromatography pulsed amperometric detection.

RNA-Seq experiment
Three biological replicates of tomato fruit cv. Campari were harvested at red-ripe stage. Upon arrival to the lab, fruits were stored at 5 ºC (CS) or 15 ºC (control) and 80 % RH. After 7 days of storage, half of the stored fruit for each condition were sampled and the other half transferred at 20 ºC for 1-day recovery. Tomato fruit were sampled at 0h, 7 days of storage at 5 ºC or 15 ºC, and 7 days of storage at 5 ºC or 15 ºC plus 1-day recovery at 20 ºC. These samples were named T0, T7-CS, T7-Control, T7+1-CS, and T7+1-Control. Moreover, the day before the sensory panels were going to be carried out, a new batch of fruit, at the same stage of ripeness than T0, was harvested and placed at 20 ºC for 1 day (fresh harvest, FH) to be compared with CS and control fruit. For each replicate and condition, the pericarp of 10 fruits were pooled and ground in liquid N and stored at -80 ºC until analysis.

RNA Extraction
Total RNA was extracted according to Verwoerd et al., (1989, Nucleic Acids Research, 17, 2362), treated with RNAse-Free DNAse and purified using RNasy® MiniEluteTM Cleanup columns (Qiagen, Hilden, Germany) following manufacture's instractions. The quality and quantity of RNA was determined by agarose gel electrophoresis, NanoDrop Lite spectrophotometer (Thermo Fisher) and RNA integrity number (RIN) in an Agilent Bioanalyzer (Santa Clara, CA, USA). High quality RNA samples were desiccated using RNAstable (Biomatrica, Inc., San Diego, CA) and shipped to INRA-get-PlaGe (Toulouse, France) for sequencing.

Sequencing data processing and gene expression analysis
A total of 18 libraries corresponding to three biological replicates from each condition (T0, T7-CS, T7-Control, T7+1-CS, T7+1-Control, and FH) were constructed following Illumina TruSeq Strand mRNA protocol [21] and sequenced using Illumina HiSeq3000 in paired-end (2 x 150 bp). The quality of the single reads generated by Illumina was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the high-quality reads (Phred quality scores, $Q > 20$) were mapped against the *Solanum lycopersicum* genome version SL2.50/iTAG2.4 (Fernandez-Pozo et al., 2015. Nucleic Acids Res. 43: D1036-41). The data were normalized by the FPKM method (Mortazavi et al., 2008), and the FPKM data were used to quantify the relative gene expression. Differential expression transcripts were identified using DESeq2 (Love et al., 2014) R package with the RLE normalization method (Maza et al., 2013 Commun. Integr. Biol.

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

6: e25849). Transcripts with an adjusted padj (p-value adjusted for multiple comparisons using Bejamini-Hochberg method) < 0.05 and a log2 fold change (FC) ± 1.5 based in three biological replicates were considered as DEGs. Principal component and clustering analysis were performed with Mev software.

Statistics

All statistical analyses were performed in R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.) Depending on the experiment data were subjected to uni-, or multifactorial analysis of variance (ANOVA). When appropriate, means were separated by LSD test. For metabolic data, hierarchical cluster analysis (HCA) and Principal component analysis (PCA) were performed in GeneMaths software package (http://www.applied-maths.com). Data were log_2 (primary metabolites and VOCs data) or mean transformed (sensory data) before statistical analysis.

4.- ACTIVITIES

ACTIVITY	MONTH																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
a.1	X	X	X	X	X	X	X	X	X	X	X	X												
a.2																X	X	X	X	X	X			
a.3													X	X	X									
a.4														X				X			X	X	X	X
a.5																								
a.6																								
a.7																								
a.8																								
a.9																								
a.10																								
a.11																								
a.12																								
add																								

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

a.1.- Activity Tittle: **1st year cold storage experiments**
Duration:12 months
Description:

The focus of this first year of grant was to establish the impact of low temperature on tomato organoleptic quality. To do that, we performed different experiments with a range of tomato genotypes in which we evaluated the differences in primary metabolites, aroma compounds and sensory quality between fruit stored at chilling and non-chilling temperature. From these experiments we concluded that when fruit are harvested ripe the impact of cold storage on flavor is not as severe as previously reported, and that the effect of cold storage on aroma profile and sensory quality is dependent on cultivar. Low quality cultivars showed a significant decrease in lipid, phenolic, and amino acid derived volatiles compared to fruit stored at non-chilling temperature, while in high quality cultivars the changes were restricted to phenolic and amino acids derived volatiles. Metabolic changes in CS fruit from cultivars with a high organoleptic quality (high content of soluble sugars and organic acids and aroma compounds) were not perceive as negative by the expert panel when compared to fruit stored at non-chilling temperature.

For further details, please see first year's report

a.2.- Activity Tittle: **2nd year cold storage experiments**
Duration: 6 months

Description:

Based on the first-year observations, we hypothesize that i) storage, even at the recommended temperature for tomatoes (15 ºC), had an effect on the organoleptic quality of these fruits and ii) that the changes induced by low temperature in high quality modern cultivars might not be as important as it was in older cultivars used in previous literature. Thus, to stablish the severity of the cold-induced loss of flavor in modern tomato we designed new cold storage experiments including fundamental changes with the ones from first year:

1- To include a fresh harvest sample to be compared with fruit stored at chilling and non-chilling temperatures by the flavor panels. This would help us to compare analytical to sensory data, as well as to better establish the effect of storage regardless of temperature.

2- Evaluate tomato liking by consumer panels when possible, depending on fruit availability.

Eight modern tomato cultivars contrasting in terms of organoleptic quality were used:

- Low brix and low aroma = Merlice and Roterno
- Medium brix and aroma = Campari and Brioso
- High brix and aroma = EC15-40720 and Ministar.
- Very high brix and aroma = Piccolo and Axiany

In addition to the modern cultivars, the heirloom variety Ailsa Craig was used to evaluate the effect of storage time and low temperature in an old cultivar. However, the quality of the full ripe fruit obtained under our growing conditions was below the standards of our flavor panels, masking any effect that cold storage may have had on fruit flavor. In this sense, before storage, Ailsa Craig had a very low liking scored in the consumer panel, more than 5 points lower than Merlice, the modern cultivar with the poorest organoleptic quality (Table 1). Moreover, the low firmness of the fruits (20 points below the average) made it hard for the panelists to judge other sensory attributes. Thus, to be able to judge the effect of storage and low temperature on Ailsa Craig flavor quality, new experiments

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

are being designed using fruits harvested at earlier developmental stages (breaker) and with shorter storage periods (3 to 5 days).

For each cultivar, fruit were stored for 7 days at chilling and non-chilling temperature. Moreover, for some long shelf life cultivars (Campari, Merlice, Ministar, and E15C-40720), fruit were stored for an additional week. This report will focus on the data concerning all 8 genotypes after 7 days of storage.

Results:
Fruit quality and volatile organic compound content (VOCs) were evaluated in fruit before storage (T0) and after 7 days storage plus 1-day recovery at 20 □C (control and CS), and compared to fruit acclimated for 1 day at 20 □C (FH). It is important to highlight that in some genotypes such as Ministar and E15C40720, there was some differences in quality parameters between T0 and FH fruit (Table S1). These differences were also evident on the aroma profile in most of the genotypes (Fig. S1), which could be attributed to: i) differences between harvests, and ii) the fact that FH fruit were maintained for 24h at 20 □C, i.e. in the same conditions as control and CS fruit, to even eating temperature among the three treatments for the flavor panels.

Thus, the effect of storage is evaluated by comparing fruit before storage (T0) to stored-fruit (control and CS). On the other hand, the comparison FH with control and CS fruit is analyzed to be able to directly compare the sensory analyses data with the instrumental ones.

Our data showed that the effect of storage at chilling and non-chilling temperature on quality parameters of tomato fruit was dependent on cultivar (Table S1). Particularly, 7 days of storage had no effect on quality parameters in Merlice. In Roterno, Piccolo, and Axiany, 7-day stored fruit (at both 15 □C and 5 □C) had lower TA, firmness (only in Roterno), and juiciness (Roterno and Axiany) than non-stored fruit (Table 2). In the cocktail kind of cultivars Campari and Brioso, fruit stored at 15 □C had lower TA and firmness than T0 and cold-stored fruit (Table 2). On the other hand, cold-stored fruit from the cocktails cultivars Ministar and E15C-40720, showed lower juiciness and firmness than T0 and control fruit (Table 2).

Regarding the fruit aroma profile, in general, this year's experiment results confirmed our previous findings.Lipid derived volatiles did not show a significant reduction in fruits stored for 7 days at 5 □C except for Roterno (Table 3). In this cultivar, the levels of Z-3-hexenal and E-2-Hexenal were significantly lower in CS fruit compared to FH and control fruit. Carotenoid derived volatiles showed an increase in fruits stored at 15 □C, especially in fruits of truss tomato (Merlice and Roterno), possibly suggesting a more intense carotenoid breakdown at this condition. The VOCs most affected by low temperature storage in all cultivars were amino acid and phenolic derived volatiles. Most of these volatiles showed lower levels in cold-stored fruit than in FH or control fruit. the phenolic derived volatiles phenylacetaldehyde, 2-phenylethanol, and 2-phenylnitroethane increased in fruit stored at 15 □C compared to FH fruit in most of the cultivars. In addition, the short chain phenolic volatiles methyl salicylate and guaiacol were only detected in significant amounts in the cherry type tomatoes (Axiany and Piccolo). In both cultivars these two volatiles showed a significant reduction in 7-day stored fruit at both 15 and 5 C. Thus, it could be concluded that 7 days of storage and low temperature altered the aroma profile of the eight tomato genotypes tested with some differences among genotypes.

The sensory analysis showed a clear effect of 7-day storage in aroma attributes between non-stored and stored fruit in the cocktail type tomato, Campari and Brioso. In the cultivars Piccolo and Ministar, panelists found a higher aroma presence in control fruit than in either FH or CS ones, while they rated Axiany control fruit as having a higher fruity and rosehip aroma than CS fruit (Table 3). In Merlice, Roterno, or E15C-40720, after 7 days of storage panelists found little differences in the aroma

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

attributes among FH and fruit stored at chilling or non-chilling temperature.

To evaluate whether the metabolic changes induced by storage and low temperature made an impact on consumers preferences, overall liking was evaluated by consumer panel (Table 3). Our results revealed that Merlice and Roterno (low SSC and aroma) fruit stored at low temperature for 7 days were less preferred than FH or fruit stored at 15 □C. In the case of Campari and Brioso, non-stored fruit were rated higher than stored fruit at both 15 □C or 5 □C; although it was not significant in the case of Campari (P = 0.0531). On the other hand, Piccolo and Axiany fruit stored for 7 days at non-chilling or chilling temperature were as preferred as non-stored fruit. The data seem to indicate that the high sugar levels of these two genotypes may overpower tomato flavor and masking the changes in their aroma profile induced by storage.

a.3.- Activity Tittle: **Odor threshold experiments**
Duration: 3 months
Description:
In an attempt to shed some light on which metabolic changes were more relevant for the final sensory quality of the fruit, we performed an experiment to establish the odor threshold of some of the most important volatiles for tomato flavor as well as to calculate their absolute amount in our samples.

Results:

Ten tomato aroma compounds were selected based on their contribution to tomato flavor and their changes after storage: Hexanal, Z-3-Hexenal, 1-Penten-3-one, Benzaldehyde, Phenylacetaldehyde, 2-Phenylethanol, Methyl salicylate, 2-Isobutylthiazole, 3-Methylbutanol, and 2-Methylbutanol. The odor thresholds of all 10 VOCs were identified in our panel of tomato experts following the 'best estimate threshold' method. The results showed that the odor threshold of important VOCs such as the phenolic derived benzaldehyde, and the amino acid derived 3- and 2-methylbutanol, were very high for our panelists, and the levels of these aroma compounds were below the odor threshold in the 8 genotypes tested (Table 4). Thus, although the production of these VOCs was reduced after cold storage in most of the genotypes, this reduction in volatile levels may very well not be perceived by panelists. Changes in volatiles that are above or close to the odor threshold should have a bigger contribution to tomato flavor. That is the case for hexanal, phenylacetaldehyde and methyl salicylate (Table 3 and 4). The odor threshold of Z-3-hexenal is similar to the one previously reported in tomato homogenate and the levels in our tomato samples are above this threshold; however, we could not accurately quantify this important volatile in our samples due to partial conversion into E-2-hexenal which most likely occurred during or as a result from the preparation of the odour thresshold samples. Regarding 2-phenylethanol, although some of our cultivars presented high amounts of this volatile it was still below its odor threshold in tomato homogenate (Table 3 and 4). Interestingly, we have data that indicate that our expert panel was able to detect significant differences in rosehip aroma among tomatoes from near introgression lines (NILs) differing in their levels of 2-phenylethanol, so we believe that we may have overestimated the odor threshold for this compound. The discrepancy between the odor threshold calculated for 2-phenylethanol alone in partially deodorized tomato homogenate and the one in fresh tomato samples may be explained by the olfactory and perceptual interactions that occur in complex volatile mixtures and that are not taken into account with the odor threshold method. In this sense, it has been observed that the perception of aroma is not due to the additive effect of each individual volatile compound, but to the interaction of different volatile compounds causing a summation of the individual contributions (additivity) or antagonism (suppression) and affecting perception. For instance, the threshold concentrations for mixtures of methyl salicylate and guaiacol were consistently lower than thresholds reported separately for each

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

volatile (Yoder et al. Journal of Sensory Studies 27 (2012) 161-167). Furthermore, it has been observed that although taste and olfactory receptors are different and recognize different chemicals, there also exists an interaction in the perception between volatile and non-volatile compounds. So, it has been described that the presence of sugar or organic acids alters the taste panel perception of aromatic descriptors of samples with the same concentration of volatile compounds. In conclusion, these results are an example of the complexity of flavor perception and the difficulty in predicting the effect of changes in volatile compounds on flavor and consumer preference.

a.4.- Activity Tittle: **RNA-Seq analysis**

Duration: About 6 months. Cold storage experiment for Campari was performed in November 2016; RNA extraction and preparation for shipping to Toulouse was done in March 2017; c-DNA libraries and sequencing was done during June and July 2017; and data analysis was done during August and September 2017.

Description:

To study the transcriptional changes related with the loss of flavour in tomato fruit due to storage and low temperature, a RNA-Seq analysis was performed. Campari fruit was the first genotype selected due to the clear difference detected by the expert panel between fresh harvested and stored fruit at both chilling and non-chilling temperature. The experiment consisted of 3 biological replicates of 6 different conditions: Day 0 fruit (T0), fruit stored for 7 days at 5 ºC (T7-CS) or 15 ºC (T7-control), fruit stored for 7 days at 5 ºC or 15 ºC and allowed to recover for 1 day at 20 ºC (T7+1-CS and T7+1-Control, respectively), and fresh fruit harvested at day 7 of storage and placed at 20 ºC for 1 day to be compared with T7+1-CS and T7+1-Control fruit by the sensory panels (FH). A total of 18 libraries corresponding to three biological replicates from each condition were sequenced using Illumina HiSeq3000 and after pre-processing and trimming, 266.6 million high quality reads were obtained (an average of 14,809,503 per sample) (Table 5) and mapped against *Solanum lycopersicum* genome version SL2.50/iTAG2.4 (Fernandez-Pozo et al., 2015. Nucleic Acids Res. 43: D1036-41). DEGs were defined by reads per kilobase per million (RPKM) with FC ± 1.5 and pdaj < 0.05.

Results:

To analyze the complexity of the transcriptomic data and to cluster samples according to their gene expression profile, we first performed a principal component analysis (PCA) over the expression data of the 18 biological samples (Fig. 1). The analysis showed that in all conditions the gene expression profile of the three independent biological replicates clustered together in the firsts three components that explain 72.15% of the variability; thus the experiment was considered reliable for further analysis. The PCA revealed that 7 days of storage at 5 ºC affected the transcriptomic profile of tomato fruit much more that storage at 15 ºC. Interestingly, storage at 20 ºC had a big effect on Campari tomato fruit and after only 1 day of acclimation at this temperature, the expression pattern of fresh harvested, cold-stored and control fruit changed, and they clustered close to each other in PC2 (15.6% of the variation). Moreover, differences between T7+1-CS and FH fruit were larger than those between T7+1-control and FH ones, similar to the pattern derived from the volatile content (Fig S2).

Gene expression was compared using pairwise analysis. A summary with the number of up- and downregulated DEG in each comparison is summarized in table S3. The comparison of the transcriptome of cold-stored fruit compared to day 0 fruit, revealed that after 1-day acclimation at 20

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

ºC there were 3 times less DEG than in fruit right after cold storage. On the other hand, the comparison with FH fruit showed that the expression profile of T7+1-control fruit was more similar to the FH ones (57 DEGs) than T7+1-CS fruit (320 DEGs).

Over 60 genes known to be involved in tomato volatiles metabolic pathways were mapped in the RNA-Seq analysis, from which 15 were DEG between stored fruit and T0 or FH fruit (Table 6). Lipoxygenase (LOX) and alcohol dehydrogenase (ADH) are involved in the biosynthesis of lipid derived volatiles. In Campari fruit a gene encoding for a Lox-C, the only LOX isoform expressed in tomato fruit, was down-regulated in fruit cold-stored for 7 days compared to fruit at day 0, while the expression recovered after 1-day acclimation at 20 ºC. The same pattern was observed for *ADH-2* and *ADH-1* (Table 6), agreeing with the fact that the levels of lipid derived volatiles such as Z-3-hexenal, hexanal, or 1-hexanol were not reduced in T7+1-CS fruit compared to FH. The levels of carotenoids derived volatiles was not affected by 7-day storage and FH, T7+1-Control and T7+1-CS showed similar values (Table 3). RNA-Seq analysis showed that only one gene involved in the biosynthesis of these VOCs, *Carotenoid cleavage dioxygenase 4A*, was DE in Campari fruit during storage. The expression of this gene was very low in Campari fruit at harvest, increased exclusively in cold-stored fruit (T7-CS) and was completely repressed after 1-day acclimation at 20 ºC in stored and non-stored fruit (FH, T7+1-Control and T7+1-CS). Regarding the biosynthesis of phenolic derived volatiles, 5 genes were DE in at least one of the pairwise comparisons of Campari fruit including a gene encoding for salicylic acid methyl transferase (SAMT), the enzyme that catalyzes the synthesis of methyl salicylate. The levels of this volatile were reduced in Campari fruit after 7 days of storage compared to FH fruit. *SAMT* gene expression was down-regulated in cold-stored fruit even after 1 day of recovery at 20 ºC, however this reduction was only significant when compared to T0 fruit (Table 6). The differential expression analysis also revealed 4 DE genes encoding for branched-chain-amino-acid aminotransferase (BCAT), an enzyme reported to be involved in the biosynthesis of amino acid derived volatiles. Three of these genes (Solyc12g088220, Solyc01g098700, and Solyc07g021630) were down-regulated in cold-stored fruit (T7-CS) compared to non-stored fruit (T0 and FH) and their expression partially recovered after 1-day acclimation at 20 ºC, which was not enough to restore the levels of amino acid derived volatiles in T7+1-CS fruit. It is important to specify that the biosynthesis pathway of this group of volatiles has not yet been elucidated and some authors have suggested that the catabolism of amino acids by means of the BCATs is unrelated to the synthesis of amino acid derived volatiles (Kochevenko et al. 2012. Molecular Plant 5, 366-375).

5.- PERSONS AND INSTITUTIONS INVOLVED

Wageningen University and Research

Wageningen campus
Arnaud Bovy
Yury Tikunov

Bleiswijk campus
Wouter Verkerke
Maike Hanenberg

ENZA ZADEN

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

Martijn Van Stee

Universidad de Granada
Dolores Garrido

6.- PUBLICATIONS, CONGRESS, DISSEMINATION

Congresses:

Hanenberg M., Rosales R., Tikunov Y., Bovy A., Verkerke V. The effect of storage and temperature on flavor of three modern tomato varieties. 12th Pangborn Sensory Science Symposium. Rhode Island, USA, 20-24 August 2017.

Rosales R., Tikunov Y., Hanenberg M., Verkerke V., Bovy A. Cold storage of tomato: the good, the bad and the ugly. XIV Solanaceae and 3rd Cucurbitaceae joint Conference (COLCUC2017). Valencia, Spain, 3-6 September 2017.

We are preparing two manuscripts to publish the main outcomes of this project. The first one will include the metabolic and sensory data from the screening of eight modern, commercial tomato cultivars in response to 7 seven days of storage at chilling and non-chilling temperature. A second publication will focus on the response of Campari fruit (good quality genotype) to cold storage and will include metabolic, sensory and transcriptomic data.

During my stay in Granada I collaborated with Dr. Dolores Garrido and Dr. Fatima Carvajal in the analysis of RNA-Seq data in zucchini fruit after postharvest and together we prepared a manuscript that is under revision in BMC-Genomics titled “Transcriptomic changes in Cucurbita pepo fruit after cold storage: Differential response between two cultivars contrasting in chilling sensitivity”

7.- RESULTS AND APPLICABILITY

We have found that the changes in quality parameters of tomato fruit induced by storage were dependent on cultivar, with genotypes in which storage had no effect such as Merlice, others in which the storage itself reduced TA and firmness such as Roterno, and others in which either storage at 15 □C or 5 □C had an effect on quality parameters such as Brioso and E15C-40720, respectively. Moreover, according to our data, storage at chilling temperatures promotes a decrease in aroma compounds while storage at the recommended temperature for tomato increased the levels of some VOCs, with differences among cultivars. The reduction in lipid derived volatiles in modern tomato cultivars, especially in genotypes with high organoleptic quality, was not as important as previously reported for older genotypes harvested before fully ripe stage. Carotenoid derived volatiles on the other hand, showed an increase in fruits stored at 15 □C, especially in fruits of truss tomato (Merlice and Roterno), while the VOCs more affected by low temperature storage in all cultivars were amino acid and phenolic derived volatiles
Interestingly, the perception of those changes by consumer and even experts was also dependent on cultivar. In this sense, in low organoleptic quality cultivars the metabolic changes that took place in CS fruit were perceived as negative by consumers. In some high organoleptic quality genotypes stored fruits, including both chilling and non-chilling temperatures, were less appreciated than FH fruit; while in genotypes with very high levels of SSC, stored fruit were undistinguishable from FH fruit,

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union’s Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

suggesting that high sugar levels could mask the changes in aroma profile induced by storage and low temperature.
In general, transcriptomic data from Campari fruit agreed with metabolic data and revealed that 7-day cold storage had a clear effect on gene expression and that 1-day recovery at 20 □C could partially compensate for these changes.
Our data indicate that acclimating cold-stored fruit at room temperature for 1-day could partially recover transcriptional and metabolic changes induced by low temperature, which could be enough in high organoleptic quality cultivars, such as Campari, to keep perceiving the flavor of cold-stored fruit as favorable as non-stored fruit. Although these findings need confirmation, they could mean that for high quality cultivars no further breeding efforts would be necessary to improve their organoleptic quality after cold storage, saving important time and money to tomato industry.
Moreover, our work could assist in the selection of high organoleptic quality genotypes that not only do have a good performance under cold storage but also under non-chilling temperature storage.

8.- CONCLUSIONS AND CONTINUATION PROPOSALS

During this two-year grant, we have evaluated the impact of cold storage on tomato flavor in a wide set of modern genotypes. One of our first observations was that in general, the loss of tomato flavour quality induced by cold storage in ripe tomato from modern genotypes is not as severe as it was anticipated based on previous literature. Thus, our focus had to be redirected to establish the severity of the metabolic changes induced by low temperature storage, and specially to how these changes affected the final organoleptic quality and liking of tomato. This involved including sensory analyses that were not initially planned in the project and postponed the objective 3 of the original project, since as we above mentioned, we could not record an important cold-induced loss of flavour in any of the tasted genotypes.
Our studies identified three patterns of response to low temperature and storage in terms of maintenance of fruit flavor and general quality:

- Genotypes in which there was an effect of storage rather than cold: high VOC levels, medium TSS content, such as Campari or Brioso.
- Genotypes in which the metabolic changes induced by low temperature storage were undetected by consumers: high organoleptic quality genotypes, such as Piccolo or Axiany.
- Genotypes in which the metabolic changes induced by low temperature storage were negatively perceived by consumers: low organoleptic quality genotypes, such as Merlice or Roterno.
- These suggests that the high sugar content of high quality genotypes masked the cold-induced metabolic changes.

There is still important work to be done regarding this line of work:

Identify key metabolic factors that could better explain consumers preferences to tomato flavor by using multivariate analysis and gene-metabolite correlation networks in our current data set.

Establish the odor threshold of other important tomato aroma compounds and confirm the ones calculated so far.

Compare the transcriptomic data we have from Campari with the ones from Merlice and Axiany, as representatives of a genotype in which the impact of cold storage on tomato flavor was perceived as negative or undetected by consumers, respectively.

Moreover, the outcomes of our research have generated new questions that need to be explored in the future. In this sense, our data seems to indicate that cold storage is not so negative for the organoleptic

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.

quality of modern tomato genotypes when harvested fully ripe (standards recommendations for these genotypes). Consequently, we propose that further efforts be directed to study the effect of storage in general on tomato flavor quality.

9.- DOCUMENTATION ENCLOSED

DOCUMENT	Description
Figures	Figures and Tables for activities a2 to a4
Supplementary material	Supplementary figures and tables for activities a2 to a4

10.- ANNUAL EXPENSES (

Material Fungible

Concepto	Importe	Justificación

Viajes y dietas

Concepto	Importe	Justificación

Project approved by the Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency, co-funded by the European Union's Seventh Framework Program, Marie Skłodowska-Curie actions (COFUND – Grant Agreement nº 291780) and the Ministry of Economy, Innovation, Science and Employment of the Junta de Andalucía.