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Identification of innovative potential quality markers in rocket and melon fresh-cut produce

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ABSTRACT

Ready-to-eat fresh cut produce are exposed to pre- and postharvest abiotic stresses during the production chain. Our work aimed to identify stress responsive genes as new molecular markers of quality that can be widely applied to leaves and fruits and easily determined at any stage of the production chain. Stress responsive genes associated with quality losses were isolated in rocket and melon fresh-cut produce and their expression levels analyzed by quantitative real time PCR (qRT-PCR) at different time points after harvest at 20 °C and 4 °C. qRT-PCR results were supported by correlation analysis with physiological and biochemical determinations evaluated at the same conditions such as chlorophyll *a* fluorescence indices, total, reducing sugars, sucrose, ethylene, ascorbic acid, lipid peroxidation and reactive oxygen species. In both species the putative molecular markers increased their expression soon after harvest suggesting a possible use as novel and objective quality markers of fresh-cut produces.

1. Introduction

Ready-to-eat (RTE) fresh cut fruits and vegetables are minimally processed produce that are used for a direct consumption and do not undergo further washing or cooking. Because of their characteristics RTEs are very convenient and respond to the changing lifestyles and eating habits of the consumers world-widely. RTEs are fresh and nutritious food that contribute to a healthy balanced diet and represent a good and rather cheap source of vitamins, minerals and antioxidants (Alarcón-Flores, Romero-González, Vidal, & French, 2014). As consequence the production of RTEs showed a positive trend in the market over the last two decades: currently, the fresh-cut salads cover about the 50% of the market volume, fresh-cut fruits account for more than 10% of the share and other fresh-cut vegetables (e.g. crudité, soup mix, stir-fry vegetables) cover the remaining 40%.

The quality of RTEs is defined by sensory characteristics (appearance, aroma, firmness and taste) and nutritional value (Watada & Qi, 1999), parameters that must be preserved during postharvest. However, RTEs are constituted by metabolizing living cells and their shelf-life is limited to few days or a week. Hence, a better comprehension of the physiological, biochemical and molecular mechanisms that control postharvest life in RTEs is pivotal to maintain effectively the quality throughout the supply chain and to reduce losses. It was shown that temperature, storage time, wounding, relative humidity and atmosphere composition affect the rate of quality degradation and the shelf-life of the final produce (Pirovani, Piagentini, Guemes, & Pentima, 1998) and, particularly, abiotic stresses contribute to quality losses (Crisosto & Mitchell, 2002).

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In plants, drought and dark alter the rate of photosynthesis by damaging the photosynthetic apparatus so that the amount of light energy normally absorbed by chlorophylls is dissipated as heat or reemitted as fluorescence. The chlorophyll *a* fluorescence can be used to monitor the effect of several abiotic stresses (Pellegrini, Francini, Lorenzini, & Nali, 2011). Therefore, chlorophyll *a* fluorescence was successfully used as non-destructive method to quantify quality loss (Baldassarre, Cabassi, & Ferrante, 2011; Ferrante & Maggiore, 2007).

Wounding stress generated at harvest, food preparation or transportation promote the acceleration of the metabolic rate of stored sugars (respiration), diminishing the energy resources for the vegetable and the nutritional value for the consumer (Rosen & Kader, 1989).

Water losses, chilling injury and oxidative stresses can lead to the oxidation of phospholipids and other unsaturated lipids. Lipid peroxidation results in the breakdown of structural lipids and in the dysfunction of cell membranes inducing loss of fluidity, lipid crosslinking and inactivation of membrane enzymes (Girotti, 1990). The most important process contributing to membrane disruption is the over production of reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2), that are generated in the cells as response to a stressing factor. Hence, monitoring ROS levels can be an evaluation parameter of stress and quality of the produce.

Physiological disorders in leaves or fruits are also associated to stress related hormones such as ethylene (C_2H_4) and abscissic acid (ABA) and to variations in the levels of antioxidants and nutraceutical compounds such as ascorbic acid (AsA) (Cocetta, Baldassarre, Spinardi, & Ferrante, 2014). Particularly to climacteric fruits, ripening is accompanied by the release of characteristic aroma compounds and by an increment of ethylene biosynthesis (Benedetti, Buratti, Spinardi, Mannino, & Mignani, 2008). Ethylene has beneficial effects on climacteric fruits by stimulating the ripening but it also has harmful effects on marketability by accelerating the softening of fruits and the senescence in leafy vegetables. Furthermore, ethylene tends to accumulate during storage and in tissues under stress conditions and consequently can compromise the produce quality (Hodges & Toivonen, 2008).

In plants responses to abiotic stresses include changes in the regulation of genes involved in biochemical, cellular and physiological processes (Vinocur & Altman, 2005). In the leaf, senescence-associated genes (SAGs) are activated by hormones, detachment, dark incubation and aging, while other genes encoding for metallothionein (*AtSAG17*), CLP proteases (*AtERD1*), cysteine protease (SAG2, SAG12), blue copper binding protein (SAG14), wound-induced protein 12 (SAG20), alcohol and sulfur dehydrogenase (*AtSAG13*, *AtSEN1*) are activated in postharvest by dark, water stress and wounding (Weaver, Gan, Quirino, & Amasino, 1998). In fruits senescence is associated with a higher expression of cell-wall degrading enzymes (e.g. endo-polygalacturonase, endo-1,4-β-mannanase and β-galactosidase) and by the depolymerization of trans-diaminocyclohexane-á-tetra-acetic acid (CDTA)-soluble pectins causing a general decline in firmness and cellular collapse (Brummell, Dal Cin, Crisosto, & Labavitch, 2004).

As RTEs represent an important expanding food category, consumers demand safe, fresh, nutritious and high quality products with extended shelf-life. These demands require that fresh-cut processors use quality management systems based on the use of markers. A good quality marker must be fast, reliable and specific as well as easily measured and sensitive enough to quantify the negative events that influence the quality components of the vegetable produce. Since postharvest quality can only be preserved but not improved, the best markers are those that are able to identify the minimum variation already after the first day of storage.

Several parameters were previously reported as good markers in RTEs and include non-destructive (NIR spectroscopy, photosynthetic indexes, mathematical models, volatile organic compounds profiling (Vikram, Lui, Hossain, & Kushalappa, 2006)) and destructive methods (monitoring the activity of a specific enzyme, a protein or a substrate). Although most of these parameters are optimal biochemical markers, the extraction and quantification procedures are laborious, time-consuming, non-practical for large numbers of samples and require special equipment and technical skills for the analysis (spectrophotometer, HPLC and GC). Moreover, the specificity of these methods largely depends on the substrate whose characteristics are influenced by temperature, matrix and plant species.

The aim of this work was to evaluate the use of stress associated genes activated during postharvest as quality markers for leafy vegetables and fruits by means of qRT-PCR. The use of qRT-PCR respects to other destructive methods has the advantage to be more sensitive and specific since it detects variations at molecular level and requires lower amounts of starting material. The expression of these genes was further linked to the evaluation of biochemical and physiological markers in order to predict the quality or reduced nutritional value in stored produce. We propose the use of these genes as markers of quality in both fresh cut leafy vegetables and fruits. These innovative markers can be adopted in support and integration of those already in use in order to implement the overall assessing and monitoring systems of quality at any point of the distribution chain.

2. Material and methods

2.1. Plant material

Melon (*Cucumis melo* L. cv Macigno) fruits were harvested from a commercial farm in Mantova (Italy) in July. Melons were collected at the optimal commercial stage when an abscission circle was observed on the fruit, screened for uniformity in size and absence of mechanical damage. Fruits were cut into half and seeds removed, each half sliced three times longitudinally and further processed into pieces of 2 cm × 3 cm.

Rocket (*Diplotaxis tenuifolia* L. var. Frastagliata) seeds were sown in polystyrene trays on perlite within containers filled with nutrient solution (mM: 10 N, 2 P, 10 K, 5 Ca, 2.4 Mg, 2.7 S, 0.04 Fe and micronutrients (Hoagland's solution)). Plants were grown in hydroponic systems in growth chambers (26 °C with 400 W/m^2 and 16 h photoperiod). Harvesting of the leaves was performed when the baby leaf commercial stage was reached (30 days after seeding).

2.2. Postharvest storage conditions and sampling

About 40 g of fresh-cut melon pieces (approximately 5–6 pieces) and 40 g of rocket leaves were enclosed in plastic boxes air tight with a plastic foil. Boxes were stored in the dark either at 20 °C or at 4 °C. Sampling was performed in quadruplicate at harvest (day 0) and during postharvest storage every day at 20 °C (day 1, 2, 3, 4). At 4 °C sampling was performed at day 7, 9, 11, 14. All samples were immediately frozen in liquid nitrogen and stored at −80 °C. Low temperatures are normally used to minimize the effects of wounding stress after harvest and to preserve the quality of fresh cut fruits and vegetables during shipping and handling. Nevertheless, cold storage induces quality changes on the final produce, but less rapidly than in produce stored at higher temperatures. Therefore, sampling at 4 °C was performed after 1 week in order to capture the initial stages in which quality starts to decrease also at low temperature.

2.3. Chlorophyll a fluorescence determination

Chlorophyll *a* fluorescence transients were determined on dark adapted leaves kept for 30 min at room temperature using a portable Handy PEA (Hansatech, Norfolk, UK). Measurements were taken on the leaf surface (4 mm diameter) exposed to an excitation light intensity [ultra-bright red light emitting devices (LEDs) with a peak at 650 nm] of 3000 μmol m^{-2} s^{-1} (600 W m^{-2}) emitted by three diodes. Fluorescence detection was measured by fast response PIN photodiode with RG9 long pass filter (Technical manual, Hansatech UK).

2.4. Sugars determination

About 1 g of leaves and 5 g of fruits were homogenized in 5 mL of distilled water (dH_2O) and centrifuged at 4000 rpm for 20 min at room temperature. Sucrose (SUC), total sugars (TS) and reducing sugars (RS) assays were performed according to the resorcinol method, anthrone assay and dinitrosalicylic acid method respectively, as previously described (Cocetta et al., 2015). Melon extracts were diluted 1:20 for SUC and RS and 1:400 for TS. Rocket extracts were diluted 1:100 for TS and used pure for the other determinations. Absorbance was read at 530 nm for RS and at 620 nm for TS using a glucose calibration curve at 0, 1, 2, 3 and 4 mM. Absorbance was read at 500 nm for SUC using a sucrose calibration curve at 0, 0.5, 1, 1.5 and 2 mM.

2.5. Ethylene determination

Ethylene accumulated during storage was measured by taking with a syringe 2 mL of gas from the headspace of each plastic container. Ethylene production was measured by enclosing 8 g of leaves or 130 g of cut melon in airtight glass containers for 1 h at 20 °C, then 1 mL of headspace gas from each jar was withdrawn with a syringe. Gas samples were injected in a Dani 3800 gas chromatograph (DANI Instruments S.p.A, Cologno Monzese-Milano Italy), equipped with a flame ionization detector (FID) and a stainless steel column (100 cm long; 0.32 cm diameter) filled with Porapack Q. The carrier gas was nitrogen at 0.8 bar, the column temperature was set at 100 °C and both the injector and FID temperatures at 210 °C (Benedetti et al., 2008).

2.6. AsA determination

AsA was extracted homogenizing 7 g of tissue in 6% metaphosphoric acid (Rizzolo, Brambilla, Valsecchi, & Eccher-Zerbini, 2002). AsA content was determined by HPLC using a column Inertsil ODS-3 (5 μm, 4.6 × 250 mm) GL Science. Chromatographic data were analyzed with a PerkinElmer TotalChrom 6.3 data processor (PerkinElmer, Norwalk, CT, USA).

2.7. Lipid peroxidation

Lipid peroxidation was determined by using the thiobarbituric acid reactive substances (TBARS) method (Heath & Packer, 1968). About 1 g of tissue was homogenized in 5 mL of trichloroacetic acid (TCA) 0.1% w/v and centrifuged at 4500×*g* for 10 min. The supernatant (1 mL) was mixed with 4 mL of 20% (w/v) TCA, 25 μL of 0.5% thiobarbituric acid (TBA) and dH_2O. After vortexing the mixture was heated at 95 °C (30 min) in a water bath and cooled on ice. Absorbance at 600 nm was subtracted from the absorbance at 532 nm (as an index of non-specific turbidity) and the concentration of TBARS were expressed as malondialdehyde (MDA) equivalents (nmol g^{-1} F.W.), calculated using the Lambert–Beer law with an extinction coefficient εM = 155 mM^{-1} cm^{-1}.

2.8. Superoxide anion radicals and hydrogen peroxide determinations

About 1 g of tissues were homogenized in 3 mL of 50 mM Tris–HCl (pH 7.5) and centrifuged at 4000×*g* for 40 min. The O_2^- assay was performed by mixing 0.1 mL of the extract with 2.890 mL of dH_2O and 10 μL of 50 mM benzene-sulfonic acid hydrate (XTT) and incubated in the dark at 20 °C for 18 h (Able, Guest, & Sutherland, 1998). Absorbance was read at 470 nm. The H_2O_2 assay was performed using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) according to manufacture instructions. Absorbance was read at 560 nm and a H_2O_2 calibration curve was used with 0, 2.5, 10, 20 μM. All data were calculated using the Lambert–Beer law.

2.9. Isolation of quality markers

RNA-Seq on 30-days rocket plants subjected to postharvest stresses (chilling, dark-20 °C, wounding-20 °C and water loss-20 °C) was previously carried out providing sequence information and expression levels of the transcriptome (unpublished data). Expression was calculated as RPKM (Reads per kilobase per million mapped reads) (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008) and differential gene expression (Fold Change = FC) was measured as $\log_2$ FC between treatment and control. Up-regulated genes with a 4-FC increase among postharvest stresses were chosen as quality markers. Thanks to the recent sequencing of the melon genome (Garcia-Mas et al., 2012), the markers in melon were identified by blastN searches of rocket genes against the melon databases (Melonomics: https://melonomics.net/tools/blast/run).

2.10. RNA extraction and qRT-PCR

About 100 mg of grounded tissues were used for the extraction of total RNA using the Spectrum Plant Total RNA Kit with on-column DNase-treatment (Sigma) according to manufacture instructions. RNA concentration and integrity were assessed by NanoDrop N-1000 spectrophotometer (NanoDrop technologies). 5 μg of RNA was reversely transcribed to cDNA using the SuperScript®III cDNA Synthesis Kit according to the manufacturer's instruction (Invitrogen). qRT-PCR analysis was performed using the SYBR® Green PCR Master Mix (Applied Biosystem) in 20 μL on the ABI7300 (Applied Biosystem) consisting of 2 μL of cDNA (1:20 dilution), 10 μL of 1 × Master Mix, 0.4 μM of forward and reverse primers and sterile water up to 20 μL. Primers were designed using Primer3 (Suppl. data S1). Actin was used as internal control to normalize the expression of the target gene. Temperature profiles consisted of an initial step at 50 °C for 2 min followed by denaturation at 95 °C for 2 min and by 40 cycles of denaturation (95 °C for 15 s) and annealing/extension (60 °C for 1 min). The reported values are averages of two independent runs performed on each sample. The expression levels were calculated using the delta–delta Cq (ΔΔCq) method.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6. Data were expressed as the mean of four biological replicates ± standard deviation (SD). Data from each sampling point were compared with the initial values and statistical differences were determined using the *t*-test. Correlation test was performed by a two tailed Pearson correlation analysis and considered as statistically significant at a value of $p \leqslant 0.05$.

3. Results and discussion

The main goal of this research work was to identify quality-associated genes to be used as molecular markers in the produce evaluation during postharvest. This was achieved by identifying those genes whose expression was highly affected by stressful conditions and at the same time showed a good correlation with physiological and biochemical parameters, which are affected by the postharvest conditions.

3.1. Chlorophyll a fluorescence

Since chlorophyll *a* fluorescence was successfully used as non-destructive marker of senescence in basil, broccoli and lettuce (Ferrante & Maggiore, 2007; Meir, Ronen, Lurie, & Philosoph-Hadas, 1997; Schofiled, DeEll, Murr, & Jenni, 2005), it was used to monitor the senescence and the health status of rocket (Fig. 1). Results showed that the maximum photochemical efficiency of photosystem II (Fv/Fm ratio) progressively decreased at both storage temperatures. At harvest the initial value of Fv/Fm was 0.86 ± 0.001 and after 4 days at 20 °C was 0.79 ± 0.016 and after 14 days at 4 °C was 0.73 ± 0.02 (Fig. 1A). Moreover, JIP indexes highlighted differences between the two storage temperatures during storage. The JIP test represents an elaboration of the data recorded in the fluorescence induction curve that provides useful indexes related to leaf health status and degree of stresses (Strasser, Srivastava, & Tsimilli-Michael, 2000). Among the JIP indexes the performance index (PI) that represent an overall evaluation of leaf functionality was 2.6 ± 0.176 immediately after harvest and declined faster at higher temperature with values reaching 1.2 ± 0.191 and 0.93 ± 0.160 at 20 °C and 4 °C, respectively (Fig. 1B). The dissipation energy (DIo/CS) per cross section and per reaction center (DIo/RC) linearly increased with storage time and higher values were observed at 20 °C (Fig 1C and D). Active reaction centers at Fm (RC/CSm) and active reaction centers at Fo per cross section (RC/CSo) declined at both temperature (Fig. 1E and F). Analogous results and trends were observed in lamb's lettuce stored at 4 or 10 °C for two weeks (Ferrante & Maggiore, 2007) or lettuce and spinach at 4 or 8 °C (Baldassarre et al., 2011). These indexes are tightly associated with leaf loss of function hence with senescence and quality losses.

3.2. Ethylene biosynthesis and accumulation

Ethylene is widely used as indicator of senescence and quality in horticultural crops. The ethylene biosynthesis from stored produce and accumulation in the plastic boxes were measured. In rocket, at harvest, the ethylene biosynthesis showed the highest value, 34 pmol kg^{-1} s^{-1} ± 4.20 (Fig. 2A) and progressively declined during storage at 20 °C. By contrast, the accumulation in the plastic boxes was almost undetectable after 1 h at 20 °C and progressively increased after 2 days (Fig. 2B). These results were also observed in lettuce baby leaf stored at 4 or 10 °C (Spinardi & Ferrante, 2012). Ethylene biosynthesis declined because tissues start to senesce and lose the biosynthetic activity. The accumulation in the boxes increases during the first days because there is a higher ethylene evolution then decreases since the gas losses from the plastic box are higher than that is produced by tissues biosynthesis. At 4 °C ethylene biosynthesis was low but remained constant with an increase after 14 days of storage while ethylene accumulation increased after the 9th day of storage and remained constant.

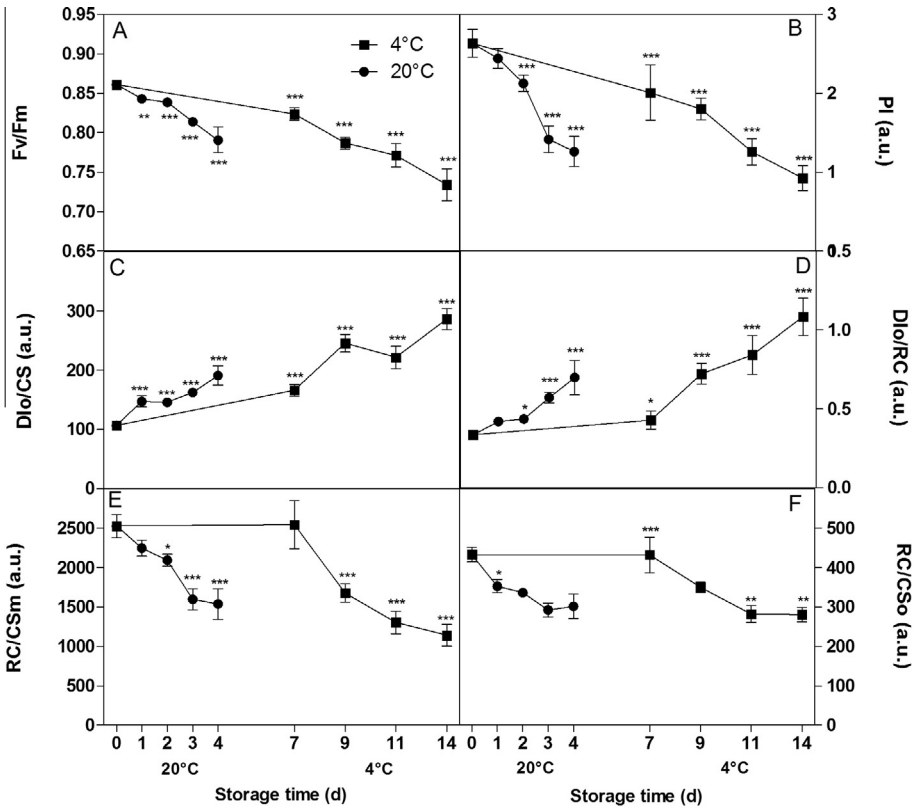

Fig. 1. Chlorophyll *a* fluorescence in rocket stored at 20 °C and 4 °C. The JIP-test parameters per excited cross section (CS) include: Fv/Fm (A), PI (B), DIo/CS (C), DIo/RC (D), RC/CSm (E) and RC/CSo (F). T0 refers to 1 h after harvest. Data are means of 10 measurements per each time point ± SD. Data were subjected to the *t*-test analysis and differences among day 0 and other sampling points were highlighted using asterisks ($P \leqslant 0.05^{*}$; $P \leqslant 0.01^{**}$; $P \leqslant 0.001^{***}$).

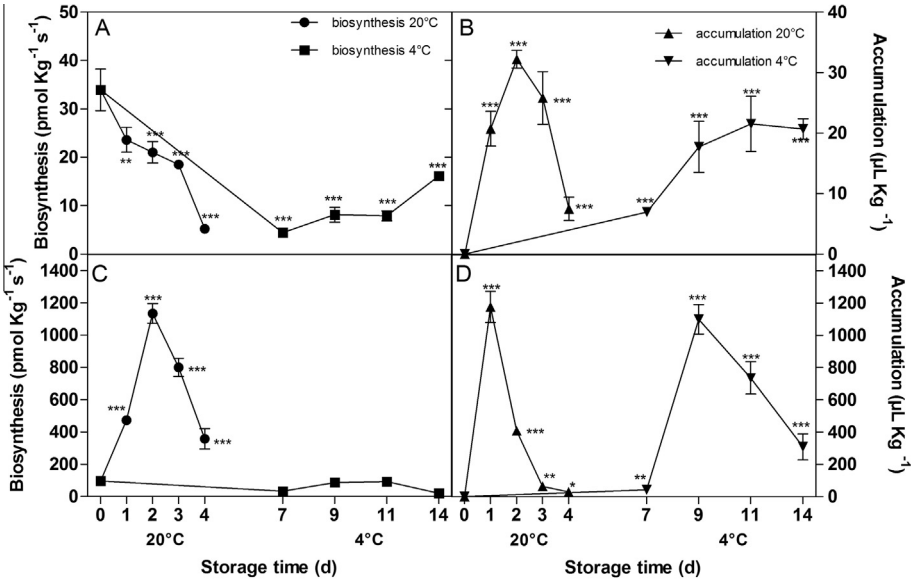

Fig. 2. Ethylene biosynthesis (pmol kg^{-1} s^{-1}) and accumulation (µL kg^{-1}) in rocket (A and B) and melon (C and D) stored at 20 °C and 4 °C. Day 0 refers to 1 h after harvest. Data are means of 4 replicates per each time point ± SD. Data were subjected to the *t*-test analysis and differences among day 0 and other sampling points were highlighted using asterisks ($P \leqslant 0.05^{*}$; $P \leqslant 0.01^{**}$; $P \leqslant 0.001^{***}$).

Melon fruits showed higher ethylene biosynthesis with a peak after two days of storage at 20 °C (Fig. 2C). Ethylene accumulation showed the same pattern, although the peak was at day one (Fig. 2D). At 4 °C ethylene biosynthesis was lower compared to the samples kept at 20 °C. The accumulation, instead, showed the same trend and similar values of the samples stored at 20 °C.

In melon samples stored at 4 °C the production of ethylene was constant and maintained low due to the possible effect of the low temperature on the rate of mRNA transcription of genes involved in C_2H_4 biosynthesis such as the aminocyclopropane-1-carboxylic acid synthase (ACC synthase, ACS) gene. The peak of ethylene production at 20 °C is typically of climacteric fruits, while at 4 °C the peak was delayed and with very low values.

3.3. Evaluation of oxidative stress

Oxidative stress and antioxidant systems were evaluated through the determination of O_2^-, H_2O_2, TBARS content and production of AsA. In compliance with other works (Hodges & Toivonen, 2008), the relation between the increase in oxidative stress and in antioxidant capacity during storage of rocket and melon was not always consistent between the two species and the stressful condition led, in some cases, to an imbalance between the levels of pro- and antioxidant compounds. In line with previous works carried out on orange, broccoli, cucumber, spinach and lamb's lettuce (Ferrante, Martinetti, & Maggiore, 2009; Zhuang, Hildebrand, & Barth, 1997; Karakas & Yildiz, 2007; Huang, Liu, Lu, & Xia, 2008), a relation between TBARS and temperature was observed: in rocket lipid peroxidation increased at 20 °C with a progressive increment from the 1st to the 4th day of storage (Fig. 3A), while at 4 °C a slight increment was observed between 9th and 11th day. In melon, TBARS levels slightly increased from the 1st day at both storage temperatures. A drop was observed at the last day of storage at 20 °C (Fig. 3B). However, in both species the values were below 0.3 nmol g^{-1} F.W. indicating a moderate level of lipid peroxidation.

In rocket the levels of AsA increased soon after the harvest and dramatically decreased after the 2nd day of storage at 20 °C, while increasing linearly at 4 °C starting from day 9 (Fig. 3C). The increase of AsA levels, after cut, was also observed in lamb's lettuce (Ferrante et al., 2009) as a response to the stress. The increment of AsA indicated that tissues were able to respond the stress and were not in the senescence stage yet.

In melon the AsA levels did not change, with the only exception of the last time point at 4 °C, in which levels dramatically decreased (Fig. 3D). The H_2O_2 did not change in rocket and generally declined in melon (Fig. 4A and B). In rocket an increase of O_2^- levels was only observed at day 11 at 4 °C (Fig. 4C). In melon, instead, the increase was found after 2 days at 20 °C (Fig. 4D). The increase of O_2^- confirms the involvement of ROS and AsA in responses to stress during the postharvest of fruits. These results indicate that melon fruits were more sensitive to ROS production than rocket leaves under the storage conditions used.

3.4. Sugars content

Sugars represent the energy source for maintaining the basal metabolism of cells in RTEs. Sugar content depends on the species, organ function and storage conditions. Different vegetables show diverse rates of respiration and, as a direct consequence, different content of sugars in response to storage environmental conditions (Der Agopian et al., 2011; Cao, Yang, & Zheng, 2013).

Significant differences in the amount of TS and RS were observed at 20 °C and 4 °C treatments in both vegetables respect to harvest (Table 1). In rocket the levels of both TS and RS followed a similar trend at 20 °C and 4 °C with a drastic decrease respect to harvest. Reduction of sugars during storage is essentially due to respiration of tissues and in wounded organs the respiration is even higher. In melon, the levels of TS and RS decreased at 20 °C and 4 °C at similar levels with respect to the harvest. In both species sucrose was maintained at constant levels with almost no significant variations at both temperatures. Sucrose is an intermediate compound in sugars degradation, therefore the content depends on how the metabolism of tissues vary during storage time and conditions.

3.5. Identification of molecular markers

Eleven stress responsive genes were selected as putative markers of quality in both species (Suppl. data S2 and S3). The markers

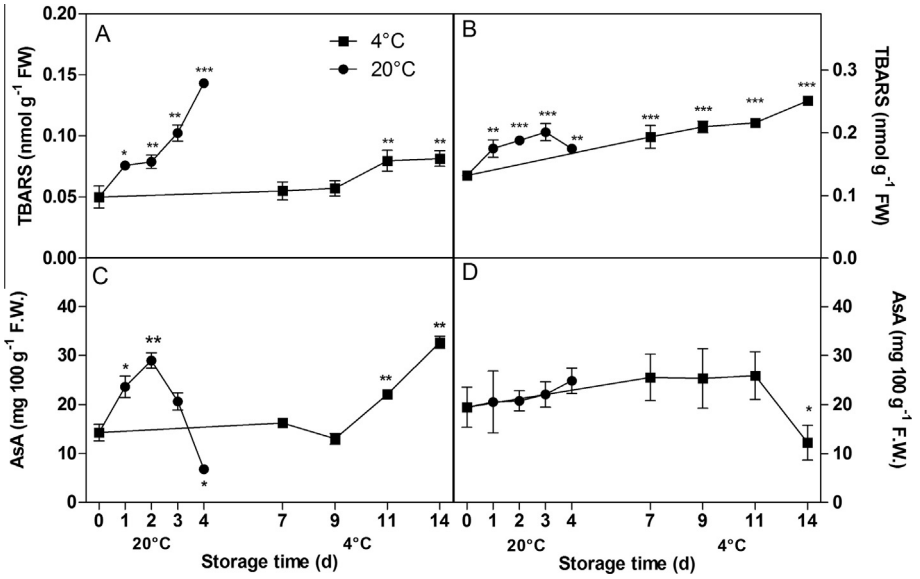

Fig. 3. TBARS equivalents (nmol g^{-1} F.W.) in rocket (A) and melon (B) and AsA content (mg 100 g^{-1}) in rocket (C) and melon (D) stored at 20 °C and 4 °C. Day 0 refers to 1 h after harvest. Data are means of 4 replicates per each time point ± SD. Data were subjected to the *t*-test analysis and differences among day 0 and other sampling points were highlighted using asterisks ($P \leqslant 0.05^{*}$; $P \leqslant 0.01^{**}$; $P \leqslant 0.001^{***}$).

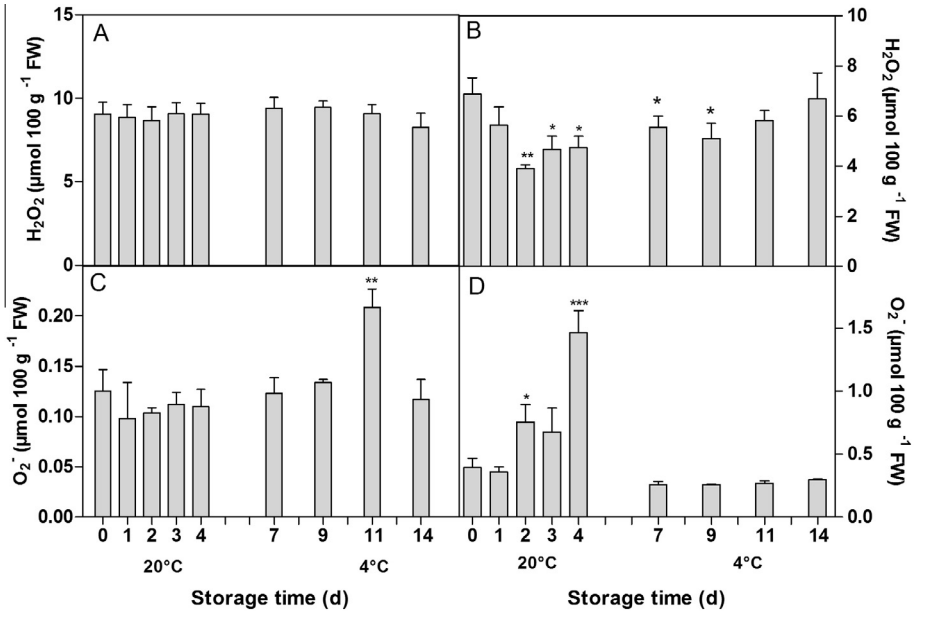

Fig. 4. H_2O_2 (A and B) and O_2^- (C and D) were determined in rocket (A and C) and melon (B and D) stored at 20 °C and 4 °C and measured as µmol 100 g^{-1} F.W. Data are means of 4 replicates per each time point ± SD. Data were subjected to the *t*-test analysis and differences among day 0 and other sampling points were highlighted using asterisks ($P \leqslant 0.05^{*}$; $P \leqslant 0.01^{**}$; $P \leqslant 0.001^{***}$).

encode proteins associated with senescence, stress and degenerative processes, mechanisms that are not mutually exclusive and that drastically induce quality losses (Suppl. data S4): among them the induced genes were related to sugars synthesis (trehalose phosphate synthase, TPS), stress responsive transcription factors (NAC domain-containing protein, NAC29, ANAC059/NAC3), amino acid biosynthesis (asparagine synthetase, ASN), detoxification (lactoylglutathione lyase, GLX) and antioxidants production (4-hydroxyphenylpyruvate dioxygenase, HPD). Unannotated genes were also found such as one uncharacterized protein family gene (UPF) and one unnamed protein product gene (UPP).

The expression levels of the markers were measured by qRT-PCR at each time point and referred as relative to the expression values at the harvest (Fig. 5). All genes were detected at the harvest and over the whole postharvest chain: in both species all genes increased their mRNA abundance soon after the harvest until the 3rd day of storage (at 20 °C) and until the 11th day of storage (at 4 °C).

In rocket (Fig. 5A) the markers related to quality losses were NAC3, StaR-like domain-containing protein (StAR) and Pseudo-response regulator (PRR), the most expressed under 4 °C and 20 °C. NAC3/ANAC059 belongs to the NAC family of transcription factors, one of the largest family in land plants with more than 100 genes identified in *Arabidopsis*. The expression of these genes is induced by dehydration, high salinity and by ABA and methyl jasmonic acid (MeJA) as well as during senescence and dark-induced etiolation (Nakashima et al., 2007; Olsen, Ernst, Lo Leggio, & Skriver, 2005).

Table 1
Amounts of sugars (mg g^{-1}) reported for each day during storage of rocket and melon at 20 °C and 4 °C. Data were subjected to the *t*-test analysis and differences among day 0 and other sampling points were highlighted using asterisks ($P \leqslant 0.05^{*}$; $P \leqslant 0.01^{**}$; $P \leqslant 0.001^{***}$).

Storage (d)	Temperature	Total sugar mg g^{-1}		Reducing sugar mg g^{-1}		Sucrose mg g^{-1}	
Rocket							
0	20 °C	7.39 ± 0.337	–	3.94 ± 0.008	–	0.33 ± 0.080	–
1		5.54 ± 0.084	**	3.69 ± 0.690	*	0.53 ± 0.186	*
2		4.28 ± 0.161	**	2.75 ± 1.808	**	0.40 ± 0.118	*
3		3.44 ± 0.364	**	2.72 ± 1.060	**	0.26 ± 0.037	–
4		2.03 ± 0.050	**	1.09 ± 0.326	***	0.29 ± 0.054	–
7	4 °C	3.04 ± 0.113	***	1.73 ± 0.729	***	0.28 ± 0.108	–
9		2.12 ± 0.041	***	1.20 ± 0.271	***	0.38 ± 0.144	–
11		2.21 ± 0.135	***	1.77 ± 0.030	***	0.35 ± 0.081	–
14		2.69 ± 0.045	***	1.64 ± 0.121	***	0.29 ± 0.127	–
Melon							
0	20 °C	72.19 ± 0.142	–	46.47 ± 0.406	–	22.20 ± 0.438	–
1		51.98 ± 0.307	**	26.80 ± 0.096	**	19.60 ± 0.534	–
2		53.81 ± 0.684	**	33.24 ± 0.201	**	12.41 ± 0.091	–
3		60.84 ± 0.546	**	27.70 ± 0.063	**	21.15 ± 0.129	–
4		46.21 ± 0.702	**	23.18 ± 0.143	***	15.27 ± 0.174	*
7	4 °C	58.10 ± 0.189	**	25.67 ± 0.254	***	16.15 ± 0.536	–
9		51.79 ± 0.307	**	26.19 ± 0.223	***	19.92 ± 0.179	–
11		56.08 ± 0.309	**	34.46 ± 0.260	**	14.52 ± 0.201	*
14		60.34 ± 0.614	**	25.38 ± 0.051	***	22.09 ± 0.148	–

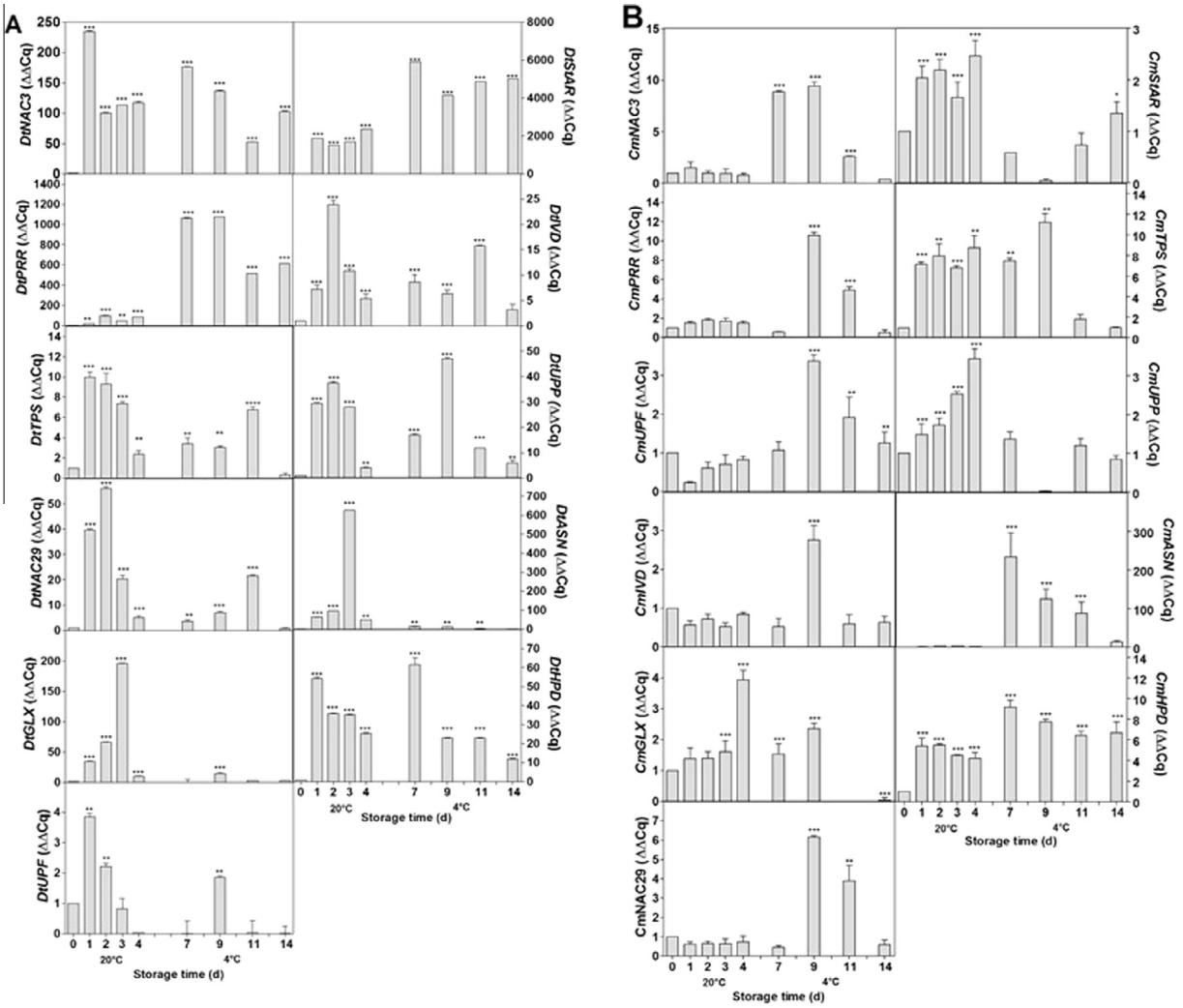

Fig. 5. qRT-PCR of 11 markers in rocket (A) and melon (B) stored at 20 °C and 4 °C. Expression was determined using ΔΔCq. Data are means of 4 replicates per each time point ± SD. Data were subjected to the *t*-test analysis and differences among day 0 and other sampling points were highlighted using asterisks ($P \leqslant 0.05^{*}$; $P \leqslant 0.01^{**}$; $P \leqslant 0.001^{***}$).

StAR proteins are lipid/sterols binding proteins implicated in intracellular lipid transport, metabolism and signaling. In Arabidopsis StAR-related lipid transfer (START) domains are mostly present in transcription factors belonging to the HD family. The interaction between a lipid/sterol and the START domain of HD proteins may positively or negatively affect the transport of the transcription factor to the nucleus, thereby controlling the rate of gene expression (Schrick, Nguyen, Karlowski, & Mayer, 2004).

The PRRs family is a small family of circadian clock-regulated genes with functions in the plant circadian clock. In *Arabidopsis* this family consists of five members (PRR9, PRR7, PRR5, PRR3 and Timing of CAB expression 1 (TOC1)) having different expression peaks at different times during the day (Nakamichi et al., 2010). Circadian rhythms are endogenously generated since the plant needs to synchronize the development, growth and flowering processes with the light cycle of the surrounding environment. The expression of PRR is dependent on light exposure and its pattern could be linked with decrease and loss of photosynthetic activity as well as increase of certain parameters related to chlorophyll *a* fluorescence. The expression of PRR was higher in the dark at 20 °C in rocket and in the dark at 4 °C in melon suggesting that temperature has also an effect on the circadian clock.

In melon (Fig. 5B) all the markers were highly and similarly expressed, but ASN and TPS showed the highest expression levels at 4 °C and 20 °C respectively. The ASN is a key enzyme for the production of the nitrogen-rich amino acid asparagine and consequently is involved in the primary nitrogen metabolism. Asparagine was shown to be involved in plant defense against microbial pathogens in pepper (Hwang, An, & Hwang, 2011) and tomato (Olea et al., 2004) as well as in response to drought stress serving as essential nitrogen and carbon reservoir (Martinelli et al., 2007). Moreover, the production and accumulation of free amino acids represents a source of osmolytes that regulate cell volume under certain types of stress such as water loss and salinity. The *HPD* enzyme (E.C. 1.13.11.27) catalyzes the conversion of 4-hydroxyphenylpyruvate (HPP) to 2,5-dihydroxyphenylacetate (HGA) in the biosynthetic pathway of vitamin E, an antioxidant that maintains the membrane integrity. Loss of function of *HPD* in Arabidopsis caused a decrease in the level of tocopherols. The expression levels of *HPD* increased leading to higher synthesis of tocopherol during leaf senescence (Chrost, Falk, Kernebeck, Molleken, & Krupinska, 1999), while in lettuce the expression of HPD was sensitive to high light and drought stress treatments.

The correlation analysis between gene expression and physiological parameters (Suppl. data S5 and S6) revealed a good association between some molecular markers and physiological and nutritional quality. In rocket the majority of the genes showed correlations with chlorophyll *a* fluorescence and sugars production, while in melon some of the genes were highly and significantly correlated to AsA content, sugars, O_2- and ethylene biosynthesis.

Finally, the best quality markers were represented by genes showing the highest up-regulation (*DtNAC3*, *DtStAR*, *DtPRR*, *CmANS* and *CmUPF*) with respect to harvest indicating a higher sensitivity compare to physiological-based technologies. Moreover, good quality markers were also represented by genes having a good correlation with physiological determinations such as *DtStAR*, *DtPRR*, *DtUPF* for rocket and *CmHPD* and *CmUPP* for melon.

4. Conclusion

This work provides a quantitative description of the physiological variations and the molecular mechanisms that are connected to quality changes in two horticultural produces during postharvest by using both classical parameters and new molecular markers. Our results showed that storage time and temperature had a direct effect on the quality and shelf-life of rocket and melon by inducing the over-expression of stress responsive genes. The differential expression pattern of the markers was supported by the determination of physiological parameters whose behavior was in line with the produce deterioration. Among the classical quality markers the best ones were chlorophyll *a* fluorescence and sugars for rocket and AsA, TBARS and superoxide anion for melon. Despite physiological determinations were showing different behaviors between the two species, most of the genes were effectively and constantly expressed soon after the harvest suggesting that common markers can be possibly applied to evaluate quality in both vegetables although leafy greens and climacteric fruit are very different biological organs. On the basis of our results, the best quality markers were represented by *DtNAC3* and *DtStAR* in rocket and *CmASN* and *CmHPD* in melon. These markers can be taken in consideration for the further development of new quality predictive technologies as well as quality monitoring systems on fresh produce markets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.04.143.

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