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Characterization of blueberry monodehydroascorbate reductase gene and changes in levels of ascorbic acid and the antioxidative capacity of water soluble antioxidants upon storage of fruits under various conditions

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ABSTRACT

Blueberry (*Vaccinium corymbosum*) is among the richest fruits in ascorbic acid (AA), which is the most important antioxidant involved in the ascorbate–glutathione cycle. In this cycle monodehydroascorbate reductase (MDAR) is the enzymatic component involved in the regeneration of reduced ascorbate. Here we report on the isolation of a full-length cDNA from blueberry encoding a protein of 433 amino acids homologous to the MDARs of *Pisum sativum* and *Vitis vinifera*. To assess changes in the expression of blueberry MDAR after harvest, a storage trial was initiated, and the major results were: (1) A dramatic loss in AA occurred under all storage conditions. However, storing fruit under low O_2, combined with high CO_2 level (up to 18%) resulted in better preservation of AA. (2) The antioxidative capacity of water soluble antioxidants (ACW) decreased under all storage conditions, even after 3 weeks storage time, and decreasing O_2 levels did not result in preservation of the ACW. The northern blot hybridization showed a clear differential expression between freshly harvested and stored fruits as well as between fruits stored under various storage condition, in accordance with the above-mentioned changes.

In conclusion, this study clearly indicates that consumption of freshly harvested blueberries is highly recommended over the stored berries. Moreover, further work is needed to elucidate reasons for the quality loss over the storage period, in particular at the molecular level.

1. Introduction

Ascorbic acid (AA) is the most important water soluble antioxidant needed for healthy diet by humans (FAO/WHO, 2002). While humans, plus other primates and some birds, are unable to synthesize AA (Naidu, 2003), plants, mainly fruits and vegetables, are the major source of dietary Vitamin C (Smirnoff et al., 2001). Recent studies suggest that an AA intake of 60–200 mg day^{-1} may carry health benefits (Carr and Frei, 1999; Levine et al., 1999). In plants, AA has diverse physiological roles (Smirnoff, 1996) as one of the major antioxidants which act as scavenger for free radicals (Halliwell and Gutteridge, 2000), and/or as an electron donor to ascorbate peroxidase (APX) to scavenge hydrogen peroxide involved in the ascorbate–glutathione cycle (Asada, 1992; Noctor and Foyer, 1998). Moreover, it is known that stressed plants increase their production of harmful reactive oxygen species (ROS) (Yoon et al., 2004), and plant cells will suffer oxidative stress, if the generation rates of ROS exceed their detoxification rate (Foyer and Noctor, 2000). In response plants evolved various defense mechanisms, where the primary components of the detoxification system are antioxidants like ascorbate, glutathione, carotenoids, and flavonoids, in addition to enzymes involved in the ascorbate–glutathione cycle such as MDAR (EC 1.6.5.4), dehydroascorbate reductase, glutathione reductase, and ascorbate peroxidase (APX) (Asada, 1996). AA is synthesized from L-galactono-1,4-lactone (GL) through the action of GL dehydrogenase (Wheeler et al., 1998) and oxidized to monodehydroascorbate (MDA) through the action of ascorbate peroxidase (APX). However, MDA is converted back to ascorbate by MDAR. In the case that MDA is not reduced by MDAR, MDA may disproportionate non-enzymatically to ascorbate and dehydroascorbate (DHA), which in turn is hydrolyzed to 2,3-diketogulonate (Nishikawa et al., 2003).

As mentioned above, the regeneration of reduced ascorbate is the major role of MDAR which used NAD(P)H as an electron donor (Leterrier et al., 2005). Concerning MDAR, plant cDNAs of MDAR have been identified in various plant species including cucumber (Sano and Asada, 1994), pea (Murthy and Zilinskas, 1994), and tomato (Grantz et al., 1995). Furthermore, it has been reported that

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Arabidopsis thaliana has five genes for MDAR (Obara et al., 2002; Chew et al., 2003), and that the catalytic activity MDAR is a FAD enzyme; it is the only known enzyme that uses an organic radical as a substrate (Sakihama et al., 2000).

Although MDAR is just one enzyme among many which influence AA and antioxidative capacity of water soluble antioxidants (ACW) levels, its role is significant. Accordingly, the purposes of this research were to assess changes in the levels of AA and ACW, and to isolate and characterize MDAR, which might be responsible for these changes.

2. Materials and methods

2.1. Fruit material and storage conditions

Blueberries (cv. 'Bluecrop') were obtained for 2 consecutive years (2003 and 2004) from a cooperative packing house in the Lake Constance area, Southwest Germany. Fruits were picked, selected for uniformity without decay and external injuries, cooled to $1 \pm 0.5\,^{\circ}C$ within 12 h, and stored in small CA chambers under the following conditions (% CO_2 + % O_2): 00 + 18 (cold storage), 06 + 18, 12 + 18, 18 + 18, 24 + 18, 06 + 02, 12 + 02, 18 + 02, and 24 + 02. These storage conditions were selected to combine the increasing concentrations of CO_2 (from 0% up to 24%) with high (18%) and low (2%) oxygen to assess the specific effects of each gas on storability and quality changes, although under commercial conditions the storage condition 12–18% CO_2 and 18% O_2 is widely adopted. However, for the molecular analysis, number of treatments was reduced to include only storage conditions which have promising results, either for commercial usage or for understanding the metabolism of fruit under the most stressful CO_2 levels. Moreover, berries were cooled within 12 h (overnight) to simulate the time needed for cooling under commercial conditions. The gas concentrations were measured by a paramagnetic O_2 (Magnos 3K) and an infrared CO_2 analyzer (Uras 3G, both Hartmann & Braun, Germany) and regulated to set points by a computerized system using nitrogen from a N_2-separator, CO_2 from a gas cylinder, and O_2 from air.

2.2. Ascorbic acid determination

All steps of this determination procedure were carried out in cold and dark conditions to minimize the rapid degradation of AA. At each sampling date, representative fruit sections were obtained from at least 15 fruits per replicate, two replicates for each treatment, and quickly immersed in liquid nitrogen and stored at $-28\,^{\circ}C$ until analysis. For analysis, frozen tissue samples were ground to powder with liquid N_2. Six grams of fruit powder was added to 15 mL of 3% HPO_3 solution and homogenized with Ultra Turrax (T25 basic; IKA Labortechnik, Staufen, Germany) for 30 s. Following centrifugation at $14{,}000 \times g$ for 20 min, the supernatant was filtered with 13 mm HPLC syringe filter, 0.45 μm (Alltech Associates Inc.) into eppendorf cuvettes, and 25 μL of it was injected to Bischoff (LC-CaDI 22-14) HPLC with the following conditions: column: Prontosil 60-5-C18-H; size: 4.0 mm × 125 mm; particle size: 5.0 μm; eluent: tetra-n-butylammoniumhydrogensulfate (2.5 g) + methanol (55 mL) in 1 l H_2O; flow: 800 μL min^{-1}; temperature: 25 °C; pressure: 14–17 MPa; sample volume: 20 μL. AA preparation (20 mg 100 mL^{-1}) was used as a standard to calculate the AA content of the fruit.

2.3. Antioxidative capacity of water soluble compounds (ACW)

Determinations were carried out using the PHOTOCHEM system (Analytik Jena AG, Germany) with the PCL-method (method of photo-chemiluminescence). Here the photochemical excitation of radical forms is combined with luminometric detection. Standard kits from Analytik Jena AG were used to measure water soluble antioxidants.

To measure the water soluble antioxidants the same samples extracted for measuring the AA concentration were used. Homogenized fruit samples (in 3% HPO_3 solution; see above) were obtained and diluted with dd H_2O at a ratio of 1:100. Ten microliters of the diluted sample was added to the reagents provided by the kit. The mixture was then placed in the PHOTOCHEM system and the readings obtained are expressed as AA – equivalents in nmol.

2.4. Statistical analysis

Results of the parameters above were subjected to analysis of variance (ANOVA) using the CoStat-software (CoHort Software, Monterey, CA, 1998), and mean separations were calculated by Duncan's Multiple Range Test at $P \leq 0.05$

2.5. RNA-extraction

RNA was extracted according to the protocol of Chang et al. (1993). The extracted RNA was purified using RNeasy spin columns from QIAGEN GmbH (Hilden, Germany), and its quantity and quality were assessed using the Spectrophotometer and 1% agarose gel, respectively.

2.6. Gene fishing

Gene Fishing Kit from Seegene, Inc. (Seoul, Korea) was used to assess the differentially expressed genes. In brief, Differential display reverse transcription PCR (DDRT-PCR) was performed with total RNA derived from blueberry plants and reverse transcribed using M-MLV reverse transcriptase enzyme. Using the GeneFishing DEG Kit 101 (Seegene, http://www.seegene.com), the blueberry MDAR cDNA fragment was amplified with the provided arbitrary primer dT-ACP1 (5′-CTGTGAATGCTGCGACTACGATXXXXX$(T)_{18}$-3′) to synthesize the first strand cDNAs. The cDNAs were subjected to two-stage PCR using the primers provided in the kit, and PCR products were further electrophorized on 2% agarose gel containing EtBr. Bands reflected differentially expressed genes were incised and DNA was extracted using QIAquick Gel extraction kit.

Cloning: Each extracted DNA fragment was cloned using the GeneJET™ PCR Cloning Kit (Fermentas GmbH, St. Leon-Rot, Germany). Competent *E. coli* cells were cultured after ligation on LB medium, plated after plasmid insert on Petri dishes containing Amp-LB-medium and further incubated overnight at 37 °C. The size of insert in the cultured cells was evaluated through Colony PCR. A broth of the selected colonies was incubated in liquid LB media containing Ampicilin overnight at 37 °C, and in the next day the DNA from the culture was purified using the Wizard® Plus SV Minipreps (DNA Purification System) from Promega (Promega GmbH, Mannheim, Germany). The purified DNA was sent for sequencing to the GATC company (www.gatc-biotech.de), Germany.

2.7. RACE PCR

2.7.1. Cloning of the blueberry monodehydroascorbate reductase (MDAR) cDNA

At the beginning the cDNA clone comprising the 3′ end of MDAR was sequenced, and subsequently the synthesis of 5′ RACE-ready cDNAs was carried out according to Zhu et al. (2001) using the BD Smart RACE cDNA Amplification Kit (Clontech). Subsequent PCR reactions were performed using the UPM Primer-Mix supplied with the Kit in combination with gene specific primer: 5′-GGCGATAGCCTTGTTTTCTTCCGGTGTC-3′ derived from the target of

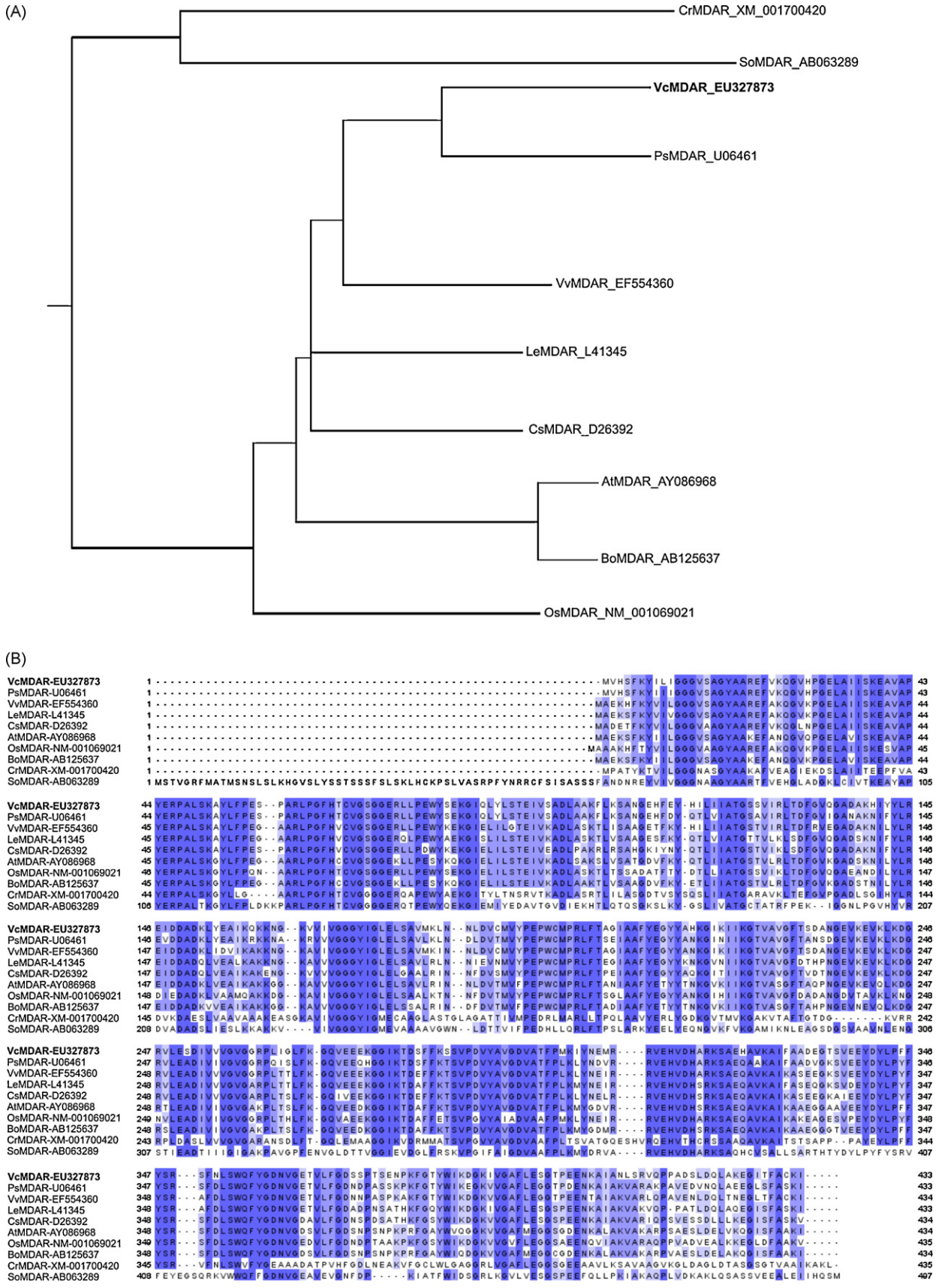

Fig. 1. Analysis of the monodehydroascorbate reductase (*VcMDAR*). (A) Neighbour-joining tree showing the phylogenetic relationships between plants MDAR proteins. MDAR from *Vaccinium corymbosum* cv. Bluecrop is indicated in bold. Species abbreviations are At (*Arabidopsis thaliana*), Bo (*Brassica oleracea*), Cr (*Chlamydomonas reinhardtii*), Cs (*Cucumis sativus*), So (*Spinacia oleracea*), Os (*Oryza sativa*), Ps (*Pisum sativum*), Le (*Lycopersicon esculentum*), Vc (*Vaccinium corymbosum*), Vv (*Vitis vinifera*). (B) Multiple protein sequence alignment of *VcMDAR* homologues from plants.

MDAR mRNA. Full products were excised from the gel, cloned and sequenced.

2.8. Northern blot hybridization

Samples served for this test were obtained from fruits stored for 3 weeks. Twenty micrograms of total RNA was mixed with an equal volume of RNA-denaturing buffer (500 μL formamide, 120 μL deionized formaldehyde (37%), 200 μL 10× MOPS, and 1 μL ethidium bromide). The solution was prepared just prior to use, and incubated at 65 °C for 10 min. Then, 1/10 volume of RNA-loading dye was added and the RNA was separated on agarose formaldehyde gel (1.25% agarose, 1× MOPS, 6.5% formaldehyde (37%)). As a size marker the peqGOLD High Range RNA Ladder (PeqLab, Erlangen) was used. Samples were transferred onto a Hybond-N+ nylon membrane (GE Healthcare, München, Germany) using a Turboblotter (Schleicher & Schuell, Dassel, Germany) with 20× SSC. RNA was fixed via UV cross-linking (Biolink BLX, Biometra, Göttingen, Germany). Hybridization was carried out with a ^{32}P labelled DNA probe. The Rediprime II Random Prime Labeling System (GE Healthcare, München, Germany) was used for generation of the probe following the manufacturer's instructions. Pre-hybridization was carried out at 67 °C for 4 h, and subsequent hybridization was carried out at 67 °C overnight. After hybridization the membrane was washed once with primary washing buffer for 10 min and three times for 10 min with secondary washing buffer at 67 °C. Signals were detected using the Molecular Imager FX (Bio-Rad, USA).

3. Results

3.1. Characterization of the monodehydroascorbate reductase and alignments

The homologues of blueberry protein sequences were used to identify MDAR homologues by TBLASTN search. The Blueberry cDNA with highest similarity to the plant MDARs was identified, cloned, sequenced as full-length cDNA, and termed MDAR of *Vaccinium corymbosum* (accession no. EU327873) encoding a protein of 433 amino acids. The prediction of protein domains in the Pfam database (Bateman et al., 2004) identified all functional domains present in all plant MDAR homologues (Table 1). Most important family is the pyridine nucleotide-disulphide oxidoreductase (pyr_redox_2 and pyr_redox); this family includes both class I and class II oxidoreductases and also NADH oxidases and peroxidases. This domain is actually a small NADH binding domain within a larger FAD binding domain. The amino acid sequence deduced from the nucleotide sequence of the ORF was aligned with those of MDAR from other plant species (Fig. 1). A substantial degree of identity was found to amino acid sequences of MDAR from *Pisum sativum* (84.5%), *Vitis vinifera* (83.6%), *Lycopersicon esculentum* Mill. (78.8%), and *Cucumis sativus* (75%). In contrast, the amino acid sequence of the *V. corymbosum* showed lower than 50% identity to those of *Chlamydomonas reinhardtii* (38.8%), and *Spinacia oleracea* (39.3%).

3.2. Quantitative determination of ascorbic acid and antioxidative capacity of water soluble compounds (ACW)

3.2.1. Ascorbic acid

A dramatic loss in AA occurred under all storage conditions (Table 2). It is obvious that storing fruit under low O_2, combined with high CO_2 level (up to 18%) resulted in better preservation of AA. However, the highest CO_2 level (24%) was injurious and resulted in lower AA content. Changes in AA content over the entire storage period of 6 weeks reflected a significant and accelerated degradation of this form of antioxidant. Storing fruit for a short period (3 weeks) guaranteed lower rates of loss, and increasing CO_2 level (up to 18%), and decreasing O_2 in store resulted in a partial preservation of AA. Generally there is a negative correlation between CO_2 level (including the injurious 24%) and AA content.

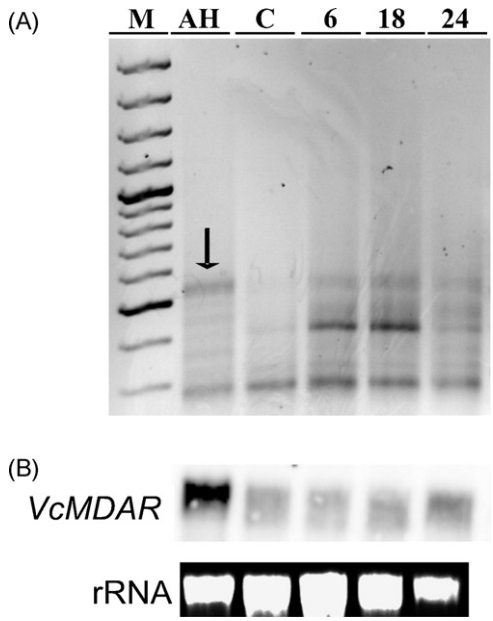

Fig. 2. Molecular analysis of the *Blueberry* monodehydroascorbate reductase (*VcMDAR*). (A) Differential display reverse transcription PCR (DDRT-PCR) was performed with total RNA derived from the *Blueberry* plants treatments: harvest (HT), cold storage (C), 6%: 6% CO_2 + 18% O_2 (6), 18%: 18% CO_2 + 18% O_2 (18), 24%: 24% CO_2 + 18% O_2 (24), using the GeneFishing DEG Kit 101 (Seegene, http://www.seegene.com). The *VcMDAR* cDNA fragment was amplified with the provided arbitrary primer ACP/dT-ACP2. A *VcMDAR* full-length cDNA was identified from the database by BLASTN searches and was sequenced. (B) RNA gel blot (20 μg each) from the *Blueberry* plants treatments: at harvest (AH), cold storage (C), 6%: 6% CO_2 + 18% O_2 (6), 18%: 18% CO_2 + 18% O_2 (18), 24%: 24% CO_2 + 18% O_2 (24) hybridised with *VcMDAR* probe. The ethidium bromide stained gel below indicates equal loading of the samples.

3.2.2. Antioxidative capacity of the water soluble compounds (ACW)

The level of ACW decreased under all storage conditions (Table 2). Extending the storage period for another 3 weeks resulted in a further significant loss in ACW, in particular with fruit stored under cold storage (1 ± 0.5 °C), and under the highest CO_2 level combined with high O_2 (24% CO_2 + 18% O_2). Decreasing O_2 levels did not result in preservation of ACW, although the low O_2 level (2%), combined with 6–12% CO_2 gave significantly better results than cold storage. The best results were obtained with increasing CO_2 levels up to 12%, but with the high O_2 level (18%). Correlations tests over the entire storage period revealed a negative correlation ($r = -0.5$) between O_2 level and ACW, but a slightly positive correlation ($r = +0.35$) between CO_2 and ACW. Correlation tests revealed also here a negative correlation between CO_2 and ACW, either under high O_2 levels ($r = -0.28$) or under low O_2 levels ($r = -0.85$). Northern blot hybridization confirmed, although partially, the quantitative assessment of AA and ACW (Fig. 2).

4. Discussion

The concentration of water soluble antioxidants, in particular AA, in fruits decreased with prolonged storage, and irrespective of the specific impact of each storage condition, it is clear that increasing CO_2 and/or decreasing O_2 partial pressures within the storage atmosphere did not decisively change this loss. Results obtained by various researchers on the impact of various keeping conditions on water soluble antioxidants were contradictory. Connor et al. (2002) found that the antioxidant activity of imma-

Table 1
Domains structure of the *Vaccinium corymbosum MDAR* protein.

Pfam-A	Description	Entry type	Sequence		HMM		Bits score	*E*-value
			Start	End	From	To		
Pyr_redox_2	Pyridine nucleotide-disulphide oxidoreductase	Domain	7	302	1	272	143.7	5.1e−40
FAE_3-kCoA_syn1	Fatty acid elongase 3-ketoacycl-CoA synthase	Family	12	25	316	329	2.2	0.82
Biotin_lipoyl	Biotin-requiring enzyme	Domain	200	258	1	75	−23.7	0.80
Cas_Cas5a	CRISPR-associated protein (Cas_Cas5a)	Family	268	287	168	188	6.8	0.28

ture harvested blueberries cv. Elliott, demonstrated an increase in antioxidant activity, total phenolic and anthocyanin content during the first 3 weeks of storage. Moreover, Kalt et al. (1999) found that there were no ascorbate losses in strawberries or highbush blueberries during 8 days of storage at the various temperatures, with a significant increase in anthocyanin content measured after 8 days at 20 °C. On the contrary, Remberg et al. (2003) found, with the blueberry cultivars 'Bluecrop', 'Hardyblue', 'Patriot', 'Putte' and 'Aron', that the total antioxidant capacity decreased considerably during refrigerated storage as well as controlled atmosphere (10% O_2 + 10% CO_2) storage. Further, Gil et al. (1997) found that strawberries stored under elevated CO_2 exhibited degraded internal color, while air-treated fruit remained red, and that internal and external tissue differed in composition and concentration of phenolic compounds. Their major finding was that CO_2 had a minimal effect on the anthocyanin (water soluble pigments) content of external tissue, but induced a significant decrease in anthocyanin content of internal tissue. Furthermore, Lin et al. (1989) reported that very high CO_2 concentrations as a result of modified atmosphere packaging (MAP) destabilized cyanidin derivatives in the skin of cv. Starkrimson apples. Similar results were reported on delphinidin derivatives in pomegranate stored under MAP (Gil et al., 1996). Agar et al. (1991) also found that high CO_2 conditions stimulated the degradation of AA of red and black currants. That same trend was also observed with highbush blueberries wrapped in polyethylene or tight (laminate PE/PET) packages at 10 °C (Haffner et al., 1993).

Reasons beyond this trend are difficult to find, and are highly speculative. Stewart et al. (2000) stated that the antioxidant capacity declined with prolonged storage, possibly due to O_2-promoted oxidation of the main antioxidants including anthocyanins and other phenolic compounds. This may explain the decline of water soluble antioxidants, including AA, of cold-stored blueberries in our experiment. Holcroft and Kader (1999) also stated that treatment with CO_2 inhibits the increase in anthocyanin concentration by affecting its biosynthesis, degradation, or both. On the other hand, Xuan (2003) found that storage of pears cv. 'Conference' under high CO_2 levels resulted in rapid loss of AA, which was in agreement with the finding of Bangerth (1977). Xuan (2003) suggested that high CO_2 concentration around fruit may stimulate the oxidation of AA by ascorbate peroxidase, although no literature is available to support this hypothesis. Further, Xuan (2003) considered high CO_2 as stress factor, and indicated that under high CO_2 stress, the available antioxidants will be consumed to protect cell membranes, but the regeneration of antioxidants is disturbed due to reduced respiration rate; it was hypothesized that the influence of a high CO_2 atmosphere lies with its impact on the ascorbate–glutathione-cycle. Holcroft and Kader (1999) found that anthocyanin concentrations increased in both external and internal tissue of 'Selva' strawberries stored in air at 5 °C for 10 days. But the increase was lower in fruit stored in air enriched with 10 or 20 kPa CO_2. The activity of phenylalanine ammonia lyase (PAL) and UDP glucose: flavonoid glucosyltransferase (GT) also decreased during storage, mainly in the external and internal tissue of strawberry fruit stored in 20 kPa CO_2. A further explanation may be related to the biosynthesis of AA; it is known that ascorbate peroxidase catalyzes the reduction of H_2O_2 to water using the reducing power of ascorbate. In the process monodehydroascorbate is produced, which can spontaneously disproportionate to form AA and dehydroascorbate, the latter being converted back to AA using glutathione as the electron donor (Lurie, 2003). According to our results, we hypothesize that a decrease in water soluble antioxidants, including glutathione which is a major water soluble antioxidant in all aerobic organisms, during storage will affect negatively the biosynthesis of AA. This is reflected by a decrease in AA content in fruits. Further possible reason for the decrease in water soluble antioxidants may be related also to the biosynthesis of anthocyanins. As cited by Holcroft and Kader (1999) and according to Holton and Cornish (1995), it is well known that the first step of anthocyanin synthesis is the production of cinnamic acid, which converted to coumaric acid that is then modified to CoA form. A further step, in anthocyanin biosynthesis, is the combination of malonyl CoA molecules with ρ-coumaroyl-CoA. Harb (1994) found that fatty acid biosynthesis was highly reduced in 'Golden Delicious' apples stored under depressed O_2 and elevated CO_2 concentrations. We suggest, consequently, that a reduction in fatty acid biosynthesis may reduce the availability of malonyl CoA, which in turn reduces anthocyanin biosynthesis. An investigation to assess changes in fatty acids of blueberries stored under various conditions is currently running in our laboratories. Furthermore, Harb and Streif (2004) found with gooseberries cv. Achilles that increasing CO_2 level and/or decreasing O_2 level resulted in a significant reduction in respiration rate that also persisted even after the 24 or 48 h conditioning period at 20 °C. These changes in respiration activity may also affect anthocyanin biosynthesis.

Table 2
Changes in ascorbic acid concentration and antioxidative capacity of the water soluble substances (ACW) of blueberries (cv. Bluecrop) following storage periods of 3 and 6 weeks under various conditions (%CO_2 + %O_2) at 1 ± 0.5 °C.

(%CO_2 + %O_2)	00 + 18	06 + 18	12 + 18	18 + 18	24 + 18	06 + 02	12 + 02	18 + 02	24 + 02
Ascorbic acid content (mg g^{-1} fresh weight)									
At harvest						6.3			
3 weeks in store	3.2 ab[a]	2.5 b	2.4 b	2.9 ab	2.3 b	3.2 ab	3.5 ab	3.9 a	3.1 ab
6 weeks in store	2.2 bc	2.2 bc	2.2 bc	2.3 bc	1.0 d	2.9 ab	–	3.3 a	1.6 cd
Antioxidative capacity of the water soluble compounds (ACW) (mg g^{-1} fresh weight)									
At harvest					5.373.9				
3 weeks in store	3.85 cd	4.55 abc	4.67 ab	3.79 d	4.58 abc	3.64 d	3.94 bcd	4.91 a	4.64 ab
6 weeks in store	3.21 a	4.53 a	4.61 a	3.40 a	3.12 a	3.52 a	3.78 a	3.11 a	2.70 a

[a] Mean values in the same row that are not followed by the same letter are significantly different using Duncan's Multiple Range Test at 5%.

The loss in AA and ACW during the storage clearly indicated that consumption of freshly harvested blueberries is healthier, and storage, if needed, should be limited to few weeks only. However, further work is needed, to elucidate the impact of elevating CO_2 and/or decreasing O_2 storage conditions on the biosynthesis, degradation, and stabilization of antioxidants and genes responsible for their biosynthesis in stored blueberries.

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