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Time of day at harvest affects the expression of chlorophyll degrading genes during postharvest storage of broccoli

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ABSTRACT

Broccoli is an important component of the human diet with a high nutritional value. This vegetable is harvested when its development is not complete, triggering a quick senescence that is accompanied by chlorophyll degradation and a shortening of shelf life. Recently it was shown that the time of the day at which the broccoli is harvested can influence in the rate of chlorophyll degradation. This report describes changes of expression of chlorophyll degrading related genes during postharvest storage of heads harvested at different moments of day. At harvest, the content of chlorophyll did not change among samples but the expression of many genes associated with chlorophyll degradation did. The level of chlorophyll diminished during storage, but samples harvested at 18:00 h had a lower rate of degradation in comparison with heads harvested at 08:00 h. Most of genes that were previously associated with chlorophyll degradation during senescence such as *BoSGR*, *BoCLH2*, *BoPPH* and *BoPaO* showed a lower expression or a delay in their mRNA level increments in samples harvested at 18:00 h. Other genes related to chlorophyll degradation during senescence, like *BoNYC* or *BoRCCR*, showed an increased expression during senescence but there was no positive correlation between chlorophyll catabolism and expression of these genes. In conclusion, changes in the metabolism due to time of day at harvest not only influence the expression of genes during the day but also may cause different patterns of expression during postharvest.

1. Introduction

Broccoli is an important vegetable of high nutritional value and a common component of the human diet. Vegetables like broccoli are harvested when their development is not complete or their growing has not ended. In addition, the stress generated due to the sudden disruption of energy, nutrients and hormones by the harvest triggers the senescence process. Chlorophyll degradation is the first visible symptom of senescence exhibited by a loss of green color and shortening of shelf life (Aubry et al., 2008).

In the pre-senescent stage, chlorophyll molecules interact with diverse proteins forming light harvesting complexes (LHC). As a prerequisite for the subsequent chlorophyll degradation, complexes must be dismantled. Several reports assumed that a protein named SGR is involved in destabilizing Chl-protein complexes (Hörtensteiner and Kräutler, 2011).

For many years, dephytilation of chlorophyll by Chlorophyllase (CHL) was considered to be the first step in chlorophyll degradation (Matile et al., 1999; Takamiya et al., 2000; Benedetti and Arruda, 2002). However, other recent studies have suggested that CHL would not be involved in chlorophyll degradation during leaf senescence but its action would be more relevant during pathogen attack or fruit ripening (Chen et al., 2008; Schenk et al., 2007). In addition, Schelbert et al. (2009) proposed that a new enzyme, pheophytinase (PPH), would be involved in dephytilation of pheophytine to generate pheophorbide, proposing a new model for the early steps of chlorophyll degradation. Pheophorbide is taken by the pheophorbide *a* oxygenase (PaO), which catalyzes the opening of porphyrin ring of pheophorbide and generates red chlorophyll catabolytes (RCC). This reaction is coupled to the following reduction of RCC by RCC reductase (RCCR), generating primary fluorescent chlorophyll catabolytes (pFCC) which are translocated to vacuoles. pFCC undergoes several modifications and products (non-fluorescent

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chlorophyll catabolites or NCCs) are finally stored inside the vacuole (Hörtensteiner, 2006).

The structures of chlorophyll catabolites from higher plants indicates a direct structural correlation with Chl a, and no analogous from Chl b could be identified (Sato et al., 2009). Transformation from Chl b to Chl a is catalyzed by a Chl(ide) b reductase encoded by a gene named *Non-Yellow Coloring1* (*NYC1*)) (Hörtensteiner and Kräutler, 2011).

In broccoli, techniques to maintain commercial and nutritional quality have focused on postharvest treatments such as refrigeration, thermal shock treatments (Costa et al., 2005, 2006a), modified atmospheres (Barth et al., 1993; Toivonen and DeEll, 2001), UV-C (Costa et al., 2006b; Lemoine et al., 2007) and 1-MCP (Gong and Mattheis, 2003; Ku and Wills, 1999). However, recently it has been shown that the time of the day at which the broccoli heads are harvested can influence in the rate of degreening during postharvest life (Hasperué et al., 2011).

The objective of the present study was to investigate the effect of time of harvest during the day on changes of expression of chlorophyll degrading related genes during postharvest of broccoli.

2. Materials and methods

2.1. Plant material

Broccoli (*Brassica oleracea* var. *Italica* cv. Iron) heads were obtained from a local producer in La Plata, Argentina (34°59′ S and 58°3′ W) during autumn of the southern hemisphere (May–June) with a 10 h average length of day. Fifty broccoli heads were harvested at different hours of the day: 08:00, 13:00 and 18:00 and carried immediately to the laboratory. Heads were placed in plastic trays, wrapped with perforated PVC, and stored in darkness at 20 °C with 95% humidity and no air movement for a period of 96 h. Samples (approximately 15–16 heads) were taken periodically, analyzed fresh or processed by separating stems from florets. The latter were frozen in liquid nitrogen and stored at −20 °C until used.

2.2. Superficial color measurement

Superficial color, i.e., the color of the outermost florets, was determined daily in all samples by measuring a^* and b^* parameters at six different positions on each head with a Minolta CR-400 chromameter (Osaka, Japan). Hue angle (h°) was calculated as $h^\circ = \tan^{-1}(-(b/a))$ when a and $b > 0$ or $h^\circ = 180 + \tan^{-1}(b/a)$ when $a < 0$ and $b > 0$, and was used to follow the degreening of heads.

2.3. Chlorophyll content

Frozen broccoli florets were ground in liquid nitrogen and 0.5 g of the resulting powder was mixed with 5 mL 80% (v/v) acetone and centrifuged at 10,000 × *g* for 10 min at 4 °C. The chlorophyll content in the supernatant was measured by a Hitachi U-1900 spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan) and results were expressed as g Kg^{-1}. All measurements were performed in triplicate.

2.4. RNA extraction and real-time PCR

Broccoli florets were ground in liquid nitrogen, and total RNA was extracted by the RNAeasy plant mini kit (Qiagen), according to the manufacturer's instructions. Approximately 6 μg of total RNA was treated with RQ1 DNAse (Promega), purified with chloroform:1-octanol (24:1) and precipitated with 3 mol L^{-1} sodium acetate. The purified RNA was quantified again, and approximately 4 μg was employed as template for cDNA synthesis using MML-V reverse transcriptase (Promega) and random primers hexamers. The resulting cDNA was employed as template for two-step qPCR using an Mx3005P real-time PCR system (Stratagene, La Jolla, CA, USA) and FastStart Universal SYBR Green Master (Roche). Actin (AF044573) was used as normalizer, with forward primer 5′-CCAGAGGTCTTGTTCCAGCCATC-3′ and reverse primer 5′-GTTCCACCACTGAGCACAATGTTAC-3′. The following primers were utilized in order to assess relative expression by RT-qPCR: stay-green protein (*BoSGR*): 5′-TTCCGACAACCGAAGTAA-3′ and 5′-GTGAGCGTATAAGTTCTTGG-3′, amplifying a fragment of 198 bp; chlorophyll b reductase (*BoNYC*): 5′-TTACATCTCGCAGTTCTGA-3′ and 5′-GCAATACCAACTACCTTAGC-3′, amplifying a fragment of 130 bp; chlorophyllase 1 (*BoCLH1*; AF337544): 5′-AGACCCATCCATCAAGTTTTCAGC-3′ and 5′-AGATTTCGGGATCGGTTCTTATGC-3′, amplifying a fragment of 85 bp; chlorophyllase 2 (*BoCLH2*; AF337545): 5′-AGATGCCTGTTCTAGTTATTGG-3′ and 5′-CACGCTGGACCTTGACATTC-3′, amplifying a fragment of 125 bp; pheophorbide *a* oxygenase (*BoPaO*; AM388844.1): 5′-GCGAAATTCCCGTCCAGAGTCTC-3′ and 5′-TTATCTCCGCCG TGCTCTTCTTC-3′ amplifying a fragment of 143 bp; pheophytinase (*BoPPH*; OL386R): 5′-AGAGGTTATCGGTGAGCCA-3′ and 5′-GACGAGATGAGGATGGG-3′, amplifying a fragment of 90 bp; and red catabolite chlorophyll reductase (*BoRCCR*; AB470927) forward: 5′-CCTCCCTCATCGCAAAGACCTAG-3′ and reverse: 5′-AGCAGAGCGGACAAAGAGAGAC-3′, amplifying a fragment of 164 bp. Three independent extracts of RNA were done and each measurement was performed by quintuplicate.

2.5. Experimental design and statistical analysis

The whole experiment was repeated twice and performed according to a factorial design. Data were subjected to analysis of variance (ANOVA), and means were compared by the least significant difference (LSD) test at a significance level of $P < 0.05$ by using InfoStat 2012 Version (Di Rienzo et al., 2012).

3. Results and discussion

Superficial color of broccoli is one of the most important attributes that influences the perception of quality by consumers (Francis, 1995), and that perception changes when the senescence process begins. In this work, broccoli samples showed the same dark green color and similar *Hue* values at any time of harvest. The green color intensity diminished throughout the storage in all samples. However, after 96 h, heads harvested at 18:00 h showed higher *Hue* values, followed by 13:00 h samples (Table 1). With respect to chlorophyll content, at harvest, samples obtained at different times of the day had similar levels of both chlorophyll *a* and *b*. The chlorophyll content diminished during storage, but after 48 h the decrease was higher in samples picked at 08:00 h. At the end of storage, samples harvested at 18:00 h had the highest chlorophyll values, while samples harvested at 08:00 h presented the lowest values (Table 1). These results suggest that heads harvested at 18:00 h had a delayed senescence compared to those harvested at 08:00 h. It was suggested that this delay could be due to a higher accumulation of starch during the day in samples harvested at 18:00 h (Hasperué et al., 2011).

3.1. Cloning of BoSGR and BoNYC from broccoli

Genes encoding SGR and NYC proteins have not been described previously in broccoli. Consequently, a search was performed by comparing the published sequences of SGR and NYC for *Arabidopsis* against public EST databases with *Brassica oleracea* specificity using current web-based tools (Altschul and Lipman, 1990). As a result, two sequences of *Brassica oleracea* of 872 bp (DK463677)

Table 1
Changes in Hue values and Chlorophyll a and b contents (g Kg^{-1}) in broccoli florets harvested at different hours and stored for 4 d at 20 °C.

	Hue			Chlorophyll a			Chlorophyll b		
	8 h	13 h	18 h	8 h	13 h	18 h	8 h	13 h	18 h
0 h	130.2 ± 2.8 a	131.3 ± 3.8 a	129.9 ± 2.1 a	0.210 ± 0.012 a	0.206 ± 0.013 a	0.202 ± 0.011 a	0.089 ± 0.012 a	0.086 ± 0.010 a	0.091 ± 0.008 a
48 h	124.2 ± 1.7 a	128.3 ± 2.2 a,b	129.2 ± 2.4 b	0.165 ± 0.005 a	0.183 ± 0.011 b	0.199 ± 0.011 c	0.042 ± 0.004 a	0.067 ± 0.006 b	0.072 ± 0.008 c
96 h	110.1 ± 1.5 a	116.7 ± 2.3 b	122.4 ± 1.6 c	0.082 ± 0.007 a	0.099 ± 0.006 b	0.124 ± 0.006 c	0.019 ± 0.009 a	0.030 ± 0.008 b	0.046 ± 0.005 c

Different letters indicate significant differences ($P < 0.05$) among samples at the same time.

and 682 bp (EH428873) that matched respectively with the corresponding genes of SGR and NYC from *Arabidopsis* were detected. Genes were re-named as *BoSGR* and *BoNYC*. Specific primers were designed from these sequences to amplify fragments from both ESTs found. The fragments obtained were cloned and sequenced to confirm their identities. The same primers were utilized to quantify mRNA levels from these genes by real-time PCR.

3.2. Changes of expression during daytime

Expression of *BoSGR* showed significant variations according to the time of day at harvest. It was detected an increment of the expression through the day from 08:00 to 18:00 h. In the case of *BoCLH1*, an increased expression at 13:00 h and a decrease toward 18:00 h were detected. By contrast, expression of *BoPPH*, *BoNYC* and *BoCLH2* showed no changes during the day. Finally, in the case of *BoPaO* and *BoRCCR*, a similar behavior to that of *BoCLH1* was detected, with an increase toward midday and a decrease at 18:00 h. In the particular case of *BoRCCR*, the detected increment was approximately 4 times the value of that observed at 08:00 h (Fig. 1).

A comparison with in-silico data of the web-based tool from Mockler Lab Diurnal (Mockler et al., 2007) was also performed. It is assumed that the lighting conditions and the type of sampling carried out in this work resemble the long-day experiences used in that web tool. "In silico" analysis rendered an increased expression of *AtSGR*, *AtCLH1* and *AtPPH* toward the end of the light period, while the expression of *AtNYC* showed an increment toward the midday and then decreased. Finally, *AtCLH2* remained unchanged under these conditions. Changes in expression of *AtSGR*, *AtCLH2*, *AtPaO* and *AtRCCR* are quite similar to that observed in this study for broccoli. Similarly, in rice, Tang et al. (2011) described changes in the expression of orthologs genes encoding PaO (*OsPaO*) and RCCR (*OsRCCR1*) during the day.

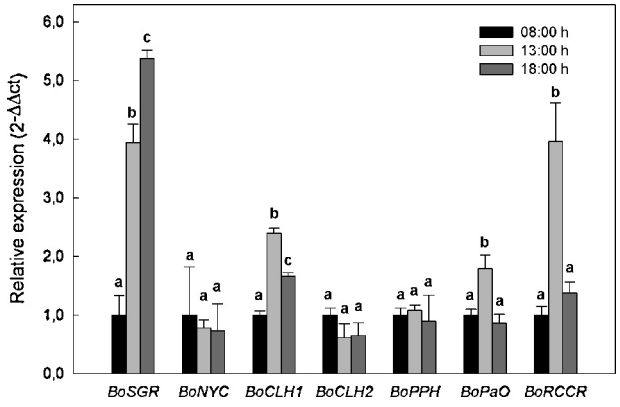

Fig. 1. Relative expression as measured by Q-RT-PCR of *BoSGR* (stay-green protein), *BoNYC* (chlorophyll b reductase), *BoCLH1* (chlorophyllase 1), *BoCLH2* (chlorophyllase 2), *BoPPH* (pheophytinase), *BoPaO* (pheophorbide *a* oxygenase) and *BoRCCR* (RCC reductase) in broccoli florets harvested at different hours of the day. Different letters indicate significant differences for the same gene ($n = 15$; $P < 0.05$).

3.3. Changes of expression during postharvest

Expression of *BoSGR* increased after 48 h of storage in all samples (Fig. 2, panel a). However, the increase was approximately of 12 times in samples harvested at 08:00 h and only 2 times in samples obtained at 18:00 h. Consequently, 08:00 h samples had the highest expression of *BoSGR*. The level of mRNA decreased after 96 h in all samples but the highest values were measured in samples harvested at 08:00 h. In several species, it was shown that orthologs of SGR have an increased expression during senescence (Aubry et al., 2008; Park et al., 2007). In addition, recently, Sakuraba et al. (2012) showed that SGR and chlorophyll catabolic enzymes interact at the light-harvesting complex II contributing to its dismantling and for chlorophyll detoxification during senescence.

Expression of *BoNYC* increased after 48 h of storage and then decreased, but no differences were detected among samples harvested at different moments of the day (Fig. 2, panel b). Similar changes of expression of genes encoding NYC during senescence have been described in other systems (Kusaba et al., 2007; Yang et al., 2009).

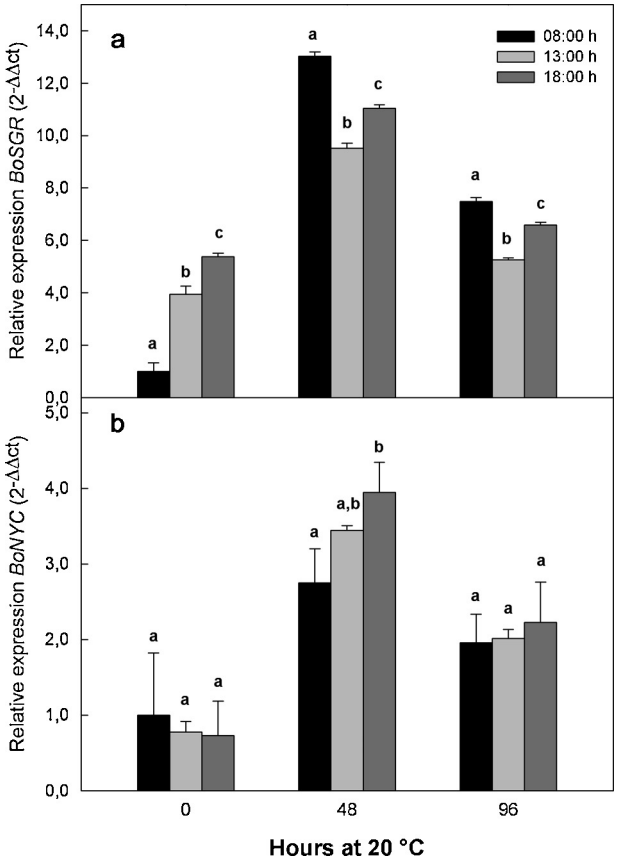

Fig. 2. Relative expression as measured by RT-PCR of (a) *BoSGR* (stay-green protein) and (b) *BoNYC* (chlorophyll b reductase) in broccoli florets harvested at different hours of the day and stored for 4 d at 20 °C. Different letters indicate significant differences at the same storage time ($n = 15$; $P < 0.05$).

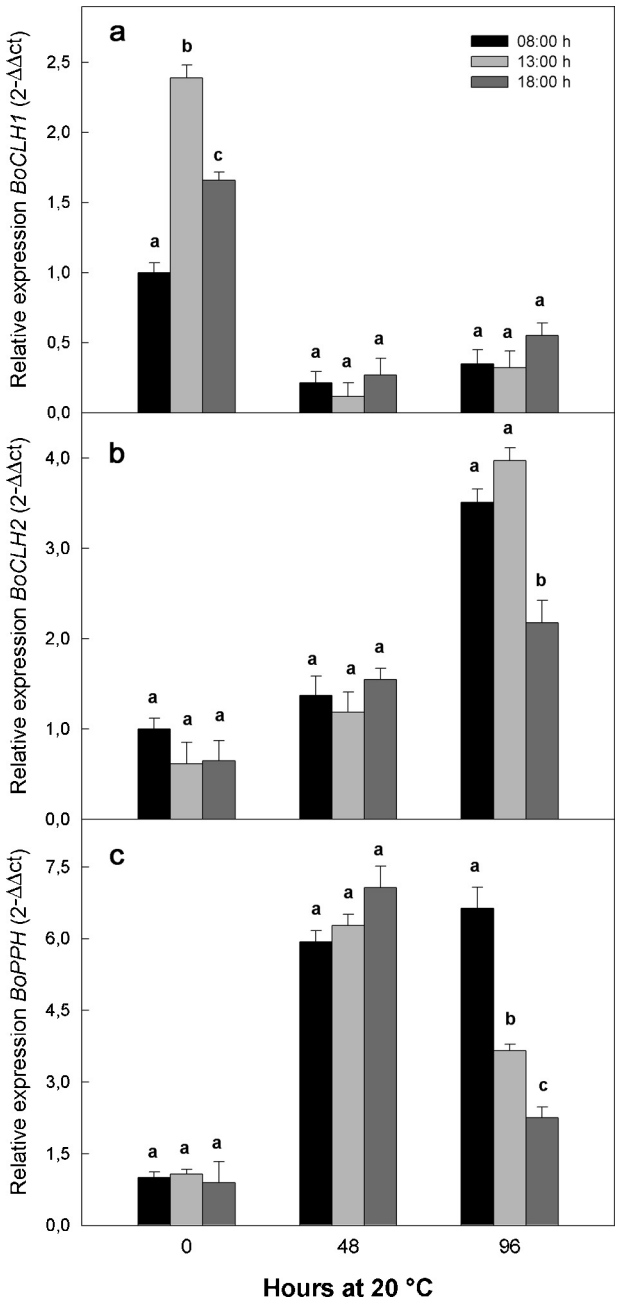

Fig. 3. Relative expression as measured by RT-PCR of (a) *BoCLH1* (chlorophyllase 1), (b) *BoCLH2* (chlorophyllase 2) and *BoPPH* (pheophytinase) in broccoli florets harvested at different hours of the day and stored for 4 d at 20 °C. Different letters indicate significant differences at the same storage time ($n = 15$; $P < 0.05$).

Expression of *BoCLH1* greatly decreased during postharvest storage, but there was no difference among the samples harvested at different times of day (Fig. 3, panel a). In the case of *BoCLH2*, there was an increase in the level of transcripts in samples harvested at 08:00 h and 13:00 h after 96 h. Differently, expression of *BoCLH2* in samples harvested at 18:00 h slightly increased (Fig. 3, panel b) at the end of storage, reaching lower values than those detected in the other samples.

There are contradictory reports in literature about the relation of chlorophyllase activity and chlorophyll degradation during senescence. Early studies suggested a primary role of chlorophyllase (Benedetti and Arruda, 2002; Tsuchiya et al., 1999), while later studies demonstrated a lower importance of these enzymes in chlorophyll catabolism. In *Arabidopsis*, it has been postulated that *AtCLH2* and *AtCLH1* have different temporal and spatial expression patterns and physiological properties (Liao et al., 2007), being *AtCLH2* partially involved in chlorophyll degradation during senescence, excluding in this process the requirement of *AtCLH1* (Schenk et al., 2007). Similarly, Zhang et al. (2011) showed that there was not a correlation between chlorophyll degradation and the expression of chlorophyllase genes during senescence of cabbage leaves. It has been suggested that chlorophyllases would be activated and related to chlorophyll degradation during fruit ripening (Shemer et al., 2008) and pathogen attack (Kariola et al., 2005). In this work, expression of both *BoCLH1* and *BoCLH2* was different during senescence. These results are similar to those published previously by Büchert et al. (2011a, 2011b), who found a reduced expression in *BoCLH1* and an increase in *BoCLH2* throughout the storage period, suggesting that *BoCLH2* could be involved in chlorophyll degradation. In this sense, the lower chlorophyll degradation detected in samples harvested at 18:00 h correlates with decreased expression of *BoCLH2* at 96 h.

The expression of *BoPPH* showed a marked increase of up to 6 times from the harvest to 48 h of storage in all samples. Samples harvested at 08:00 h maintained high levels of mRNA from the 48 to 96 h, but those harvested at 13:00 h and 18:00 h showed an important decrease of *BoPPH* expression at 96 h. Heads harvested at 13:00 h showed intermediate levels among the samples of 08:00 h and 18:00 h (Fig. 3, panel c). Schelbert et al. (2009) demonstrated that loss of phytol from chlorophylls during senescence is strongly associated with the action of a pheophytinase (encoding by *AtPPH*) that specifically catalyzes the hydrolysis of phytol from pheophytin without acting on chlorophylls. Moreover, in broccoli, expression of *BoPPH* increases by treatment with ethylene and decreases after treatments that delay senescence, such as cytokinins, heat treatments, UV-B, and UV-C light (Aiamla-or et al., 2012; Büchert et al., 2011a, 2011b. In this sense, in this study, samples harvested at 18:00 h were those that showed a delay in senescence and also a decreased expression of *BoPPH*.

The expression of *BoPaO* increased markedly after 48 h, except for samples harvested at 13:00 h, in which the transcripts remained at levels similar to that observed at harvest. However, this increment was higher in samples harvested at 08:00 h, reaching values significantly higher than those detected in samples obtained at 13:00 h and 18:00 h (Fig. 4, panel a). After 96 h, expression decreased in all samples but the highest values were maintained in samples harvested at 08:00 h. PaO expression and activity were positively associated to senescence both during the normal chlorophyll degradation in green tissues (Pruzinská et al., 2003) and during senescence induced by ethylene (Zhang et al., 2011). In other studies on broccoli, similar increases in the expression of *BoPaO* were observed during postharvest senescence, as well as the occurrence of lower levels of expression in samples subjected to different treatments that delay senescence (Fukasawa et al., 2010; Gomez-Lobato et al., 2011). In this work, heads harvested at 13:00 h and 18:00 h showed lower chlorophyll degradation and *BoPaO* expression in comparison with those obtained at 08:00 h. PaO activity is coupled with the following enzyme of the catabolic pathway, RCCR, establishing a two-step reaction (Pruzinská et al., 2005; Wüthrich et al., 2000). Fukasawa et al. (2010) cloned a fragment that codified for a gene encoding RCCR from broccoli (named *BoRCCR*) and characterized its expression during senescence. They detected an increase in the expression after 48 h of postharvest and then a decrease. In the present study, a similar behavior was detected in samples harvested at 08:00 h (Fig. 4, panel b). Differently, samples picked at 18:00 h showed the same expression after 48 h and an increment only after 96 h. Finally, samples harvested at 13:00 h presented the same *BoRCCR* expression through the storage.

Taking all data together, it can be observed that most of genes that were previously associated with chlorophyll degradation

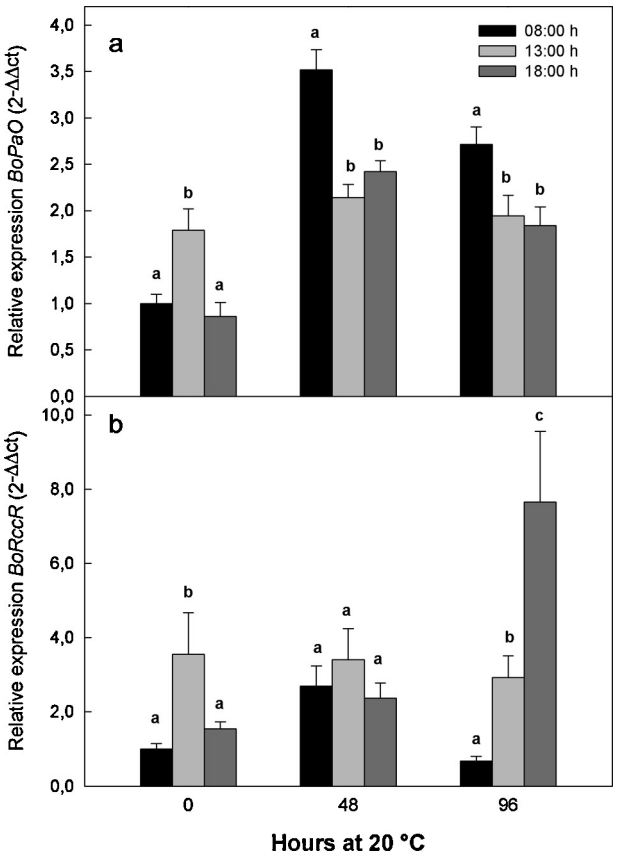

Fig. 4. Relative expression as measured by RT-PCR of (a) *BoPaO* (pheophorbide *a* oxygenase) and (b) *BoRCCR* (RCC reductase) in broccoli florets harvested at different hours of the day and stored for 4 d at 20 °C. Different letters indicate significant differences at the same storage time ($n = 15$; $P < 0.05$).

during senescence such as *BoSGR*, *BoCLH2*, *BoPPH* and *BoPaO* (Büchert et al., 2011a; Pruzinská et al., 2005; Schelbert et al., 2009) had a lower expression or a delay in their mRNA level increments in samples harvested at 18:00 h in comparison with those samples harvested at 08:00 h. In addition, in samples obtained at midday, a similar behavior to the samples of the 08:00 h for *BoCHL2* expression and to the samples of the 18:00 h for *BoSGR* and *BoPaO* expression was detected. In the case of *BoPPH*, the samples of 13:00 h showed an intermediate behavior compared to the others. These data correlated with chlorophyll degradation since the samples with the highest levels of expression were the ones that showed lower levels of chlorophyll. A change of the metabolism due to the time of day at harvest not only influences the expression of genes during the day but also may cause different patterns of expression during postharvest.

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