Research Article

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Effects of ethylene, cytokinin and physical treatments on *BoPaO* gene expression of harvested broccoli

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Abstract

BACKGROUND: Broccoli is a highly perishable vegetable that shows enhanced postharvest senescence and intense de-greening caused by chlorophyll degradation. One of the key steps of chlorophyll catabolism is the opening of chlorophyll tetrapyrrole catalysed by pheophorbide a oxygenase (PaO). In this study the expression of a gene encoding a putative PaO was characterised under several chemical and physical treatments.

RESULTS: A fragment of a gene encoding a PaO from broccoli (*BoPaO*) was cloned. The expression of *BoPaO* showed an important increment during postharvest senescence, in correlation with chlorophyll degradation. Furthermore, broccoli heads were treated with the hormones cytokinin and ethylene. Cytokinin delayed the increment in *BoPaO* expression, while ethylene accelerated the process. Also, several postharvest treatments were applied in order to evaluate their effect on *BoPaO* expression. Samples treated with modified atmosphere, hot air, UV-C or white light showed a delay in chlorophyll degradation and de-greening. In most cases the treatments also delayed the increment in *BoPaO* expression during senescence.

CONCLUSION: A close correlation between chlorophyll degradation and *BoPaO* expression was found during broccoli senescence. This relationship was corroborated in samples treated with different hormonal and physical applications.

Keywords: broccoli; senescence; chlorophyll degradation; pheophorbide a oxygenase

INTRODUCTION

Broccoli (*Brassica oleracea* L. *italica*) is a floral vegetable with low caloric value that is rich in diverse nutrients such as vitamins A and C, antioxidants and fibre. In addition, recent studies have indicated that broccoli contains high levels of anticarcinogenic compounds such as glucosinolates.[1] Floral heads of broccoli are composed of hundreds of florets arranged in whorls on top of the stem. For consumption, they are harvested at an immature stage when male and female reproductive structures are still surrounded by petals and enclosed by chlorophyll-containing sepals. Harvesting causes the heads to experience disruption of energy, nutrition and hormone supplies, resulting in rapid senescence and chlorophyll degradation in the sepals.[2] Catabolism of chlorophylls leads to yellowing, which is the main sign of quality deterioration in harvested broccoli.[3]

A pathway of chlorophyll breakdown comprising several enzymatic reactions has been elucidated in recent years.[4] The pathway starts with the elimination of phytol by chlorophyllase[4] or pheophytinase[5,6] and the removal of Mg^{2+} by a metal-chelating substance.[7] Thereafter the porphyrin ring of the resulting intermediate, pheophorbide, is oxygenolytically opened by pheophorbide a oxygenase (PaO).[8] The product of this reaction is red chlorophyll catabolite (RCC), which is site-specifically reduced by RCC reductase to yield primary fluorescent chlorophyll catabolite (pFCC), which is exported to vacuoles.[9] After that, pFCCs are modified by diverse reactions and transformed into non-fluorescent chlorophyll catabolites (NCCs).[10,11] Structural analysis of NCCs from different plant species has revealed that they are all derived from chlorophyll a.[12] One reason for this is the exclusive specificity of PaO for pheophorbide a.[13] It has been proposed that chlorophyll b should then be converted into chlorophyll a to be degraded as a prerequisite for breakdown beyond the level of pheophorbide.[4,14]

PaO is a non-heme iron monooxygenase located in the inner envelope of maturing gerontoplast and catalyses the opening of the porphyrin macrocycle by adding two oxygen atoms.[15] This enzyme is considered to play an important regulatory role in chlorophyll catabolism, since PaO activity correlates positively with chlorophyll degradation.[16,17]

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The objective of the present work was to characterise the expression of a gene encoding a putative PaO (*BoPaO*) during postharvest senescence and to analyse the effect of several treatments that modify the senescence rate on *BoPaO* expression.

MATERIALS AND METHODS

Plant material

Broccoli (*B. oleracea* var. *italica*, cv. Cicco) heads and leaves were obtained from a local producer in La Plata, Argentina and immediately transported to the laboratory for processing.

Senescence treatments

Heads were stored together with leaves in darkness in a well-ventilated chamber at 22 °C for 96 h. Before (0 h) and after (96 h) incubation, samples were segmented into section 1 (inflorescences), section 2 (small branchlets) and section 3 (main floret stem), cut, frozen in liquid nitrogen and stored at −20 °C until analysis (about 3–4 weeks).

Another group of heads was placed upright in plastic cups with a small amount of distilled water, with stems touching the water to prevent dehydration, and stored as described above for 120 h. Heads were sampled periodically for colour analysis. After sampling, florets of five heads were separated from stems, randomly grouped, frozen in liquid nitrogen and stored at −20 °C until analysis (about 3–4 weeks).

Hormone treatments

Whole harvested broccoli heads were immersed for 10 min in solutions containing 0.1 g L^{-1} 6-benzylaminopurine (BAP) with 1.1 g L^{-1} dimethyl sulfoxide (DMSO), 0.1 g L^{-1} 2-chloroethylphosphonic acid (ethephon, an ethylene-releasing agent) with 1.1 g L^{-1} DMSO, or 1.1 g L^{-1} DMSO as control.[18] Thirty heads were utilised for each condition. After treatment, heads were placed in plastic cups containing a small amount of distilled water to prevent dehydration and stored as described above. Samples were collected at different times for colour measurements. After collection, six heads from each condition were chosen, florets were separated from stems, frozen in liquid nitrogen and stored at −20 °C until use (about 3–4 weeks).

Physical treatments

Forty-five broccoli heads were used to perform each physical treatment. In the case of hot air treatment, heads were placed in trays, covered with polyvinyl chloride (PVC) film to diminish water loss and treated with hot air at 42 °C for 3 h.[19] The same number of heads without heating were utilised as controls. After treatment the trays containing broccoli heads were loosely covered with PVC film to diminish water loss and stored at 22 °C. Samples were taken after 0 (initially for control samples and immediately following treatment for heat-treated samples), 72 and 120 h of storage. Then individual florets were removed, frozen in liquid nitrogen and stored at −20 °C until analysis.

For modified atmosphere treatment, heads were individually and hermetically covered with polyethylene film (40 μm thick, 20 cm × 30 cm) and stored in darkness at 22 °C for 120 h. The same number of heads were loosely covered with PVC film and utilised as controls. Samples were taken and processed as described above.

In the case of UV-C treatment, heads were placed vertically in plastic trays to ensure homogeneous irradiation of florets, put under a bank of four UV-C lamps (TUV G30T8, 30 W, Philips, Croydon, UK)[20] and irradiated at a distance of 30 cm to obtain a dose of 10 kJ m^{-2}. The flux intensity from the lamps was measured with a digital radiometer (Cole-Palmer Instrument Company, Vernon Hills, IL, USA). The same number of heads without irradiation were utilised as controls. After treatment, heads were loosely covered with PVC and stored in darkness at 22 °C for 120 h. Samples were taken and processed as described above.

For visible light treatment, stems of heads were placed in plastic cups containing a small amount of distilled water to prevent dehydration and transferred to a well-ventilated chamber isolated from external light sources. One half of the chamber was kept in complete darkness (<1 μmol m^{-2} s^{-1}) and the other half was exposed to 12 μmol m^{-2} s^{-1} continuous light intensity. Samples were collected and processed as described above.

Superficial colour measurement

Superficial colour was evaluated by measuring the parameters a and b at five positions on each broccoli head with a chromameter (CR-300, Minolta, Osaka, Japan). Hue angle ($h°$) was calculated as $h° = \tan^{-1}(b/a)$ when $a > 0$ and $b > 0$ or as $h° = 180° - \tan^{-1}(b/a)$ when $a < 0$ and $b > 0$.

Determination of chlorophyll content

Frozen broccoli florets were ground in liquid nitrogen, and 0.5 g of the resulting powder was mixed with 5 mL of acetone/water (8 : 2 v/v) solution and centrifuged at 10 000 × g for 10 min at 4 °C. The chlorophyll content in the supernatant was measured by spectrophotometry and results were expressed as mg total chlorophyll g^{-1} tissue fresh weight (FW).[21] All measurements were performed in quintuplicate.

Cloning of *BoPaO* from broccoli florets

Broccoli florets were ground in liquid nitrogen, and total RNA was extracted by the hot borate method[22] and quantified by UV spectrophotometry. Approximately 4 μg of total RNA was employed as template for cDNA synthesis using MML-V reverse transcriptase (Promega, Madison, WI, USA) and random hexamers as primers. The resulting cDNA was employed as template for polymerase chain reaction (PCR) with gene-specific primers. The primers for *BoPaO* cloning (forward: 5′-CTCTTCTTCCTCTTCCTTC-3′; reverse: 5′-GACCCTTCTTCTTCCTTATC-3′) were designed based on the expressed sequence tag (EST) of *B. oleracea* (accession number AM388844.1). The amplification protocol was as follows: (a) 4 min at 94 °C (one cycle); (b) 45 s at 94 °C, 1 min at 59 °C, 1 min at 72 °C (34 cycles); (c) 7 min at 72 °C (one cycle). Amplified DNA fragments of approximate size 315 bp were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chalfont St. Giles, UK) and subsequently cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Cloned DNAs from multiple colonies were sequenced from both directions with internal primers M13-RV and T7-FW to check their identity with *BoPaO*.

RNA extraction and real-time PCR

Broccoli florets were ground in liquid nitrogen, and total RNA was extracted by the hot borate method.[22] Approximately 6 μg of total RNA was treated with RQ1 DNAse (Promega), purified with chloroform/1-octanol (24 : 1 v/v) and precipitated with 3 mol L^{-1} sodium acetate. The purified RNA was quantified

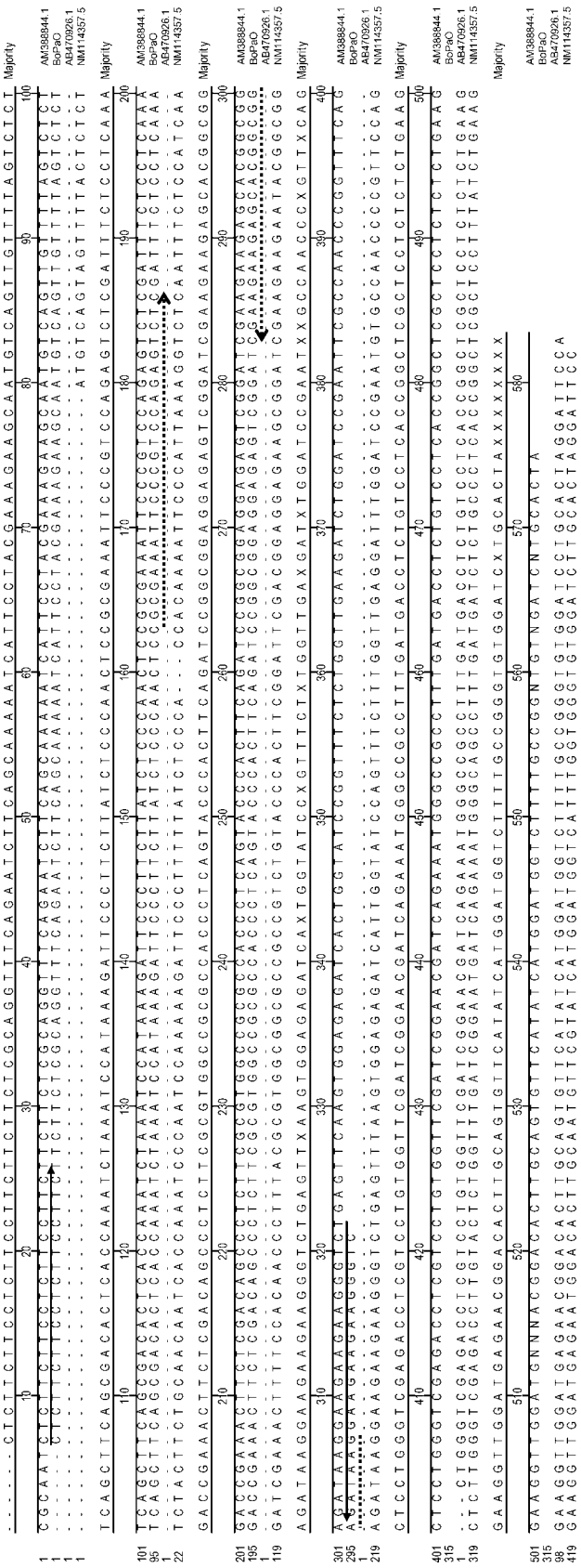

Figure 1. Nucleotide sequence alignment of EST from broccoli found through database search (AM388844.1), fragment cloned in this work (*BoPaO*), another fragment of *BoPaO* published in database (AB470926.1) and *AtPaO* from *Arabidopsis thaliana* (NM1143575). Solid arrows indicate sites chosen to clone the fragment of *BoPaO*, while dotted arrows indicate sites chosen for qPCR experiments.

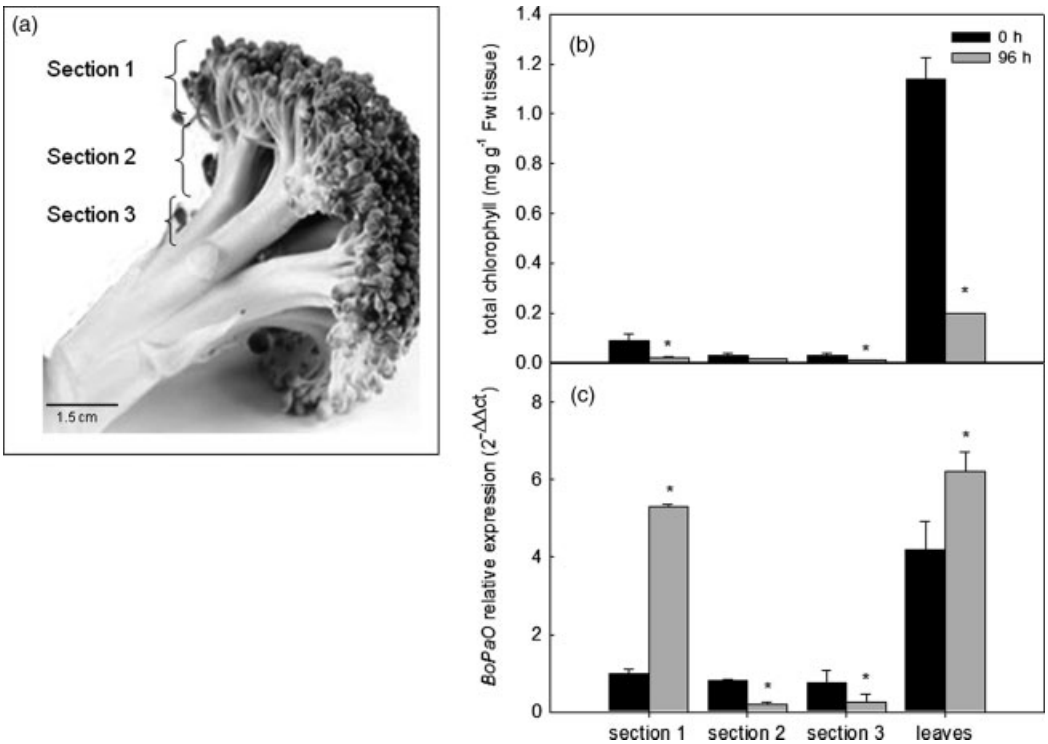

Figure 2. (a) Sectioning of broccoli florets. (b) Total chlorophyll content in different sections of broccoli florets at days 0 and 4 of induced senescence. (c) Relative gene expression assessment of *BoPaO* during senescence of different sections of broccoli florets. Asterisks indicate statistically significant differences between 0 and 96 h in the same section ($P < 0.05$).

again, and approximately 4 µg was employed as template for cDNA synthesis using MML-V reverse transcriptase (Promega) and random hexamers as primers. 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 normaliser, with forward primer 5′-CCAGAGGTCTTGTTCCAGCCATC-3′ and reverse primer 5′-GTTCCACCACTGAGCACAATGTTAC-3′. Specific primers (forward: 5′-GCGAAATTCCCGTCCAGAGTCTC-3′; reverse: 5′-TTATCTCCGCCGTGCTCTTCTTC-3′) were designed to amplify a fragment of 143 bp from the cloned fragment of *BoPaO*. Each measurement was performed in triplicate.

Statistical analysis

Experiments were performed according to a factorial design. Data were subjected to analysis of variance, and means were compared by the least significant difference test at a significance level of 0.05.

RESULTS

Cloning of *BoPaO* in broccoli

We performed a search by comparing the published sequence of PaO for *Arabidopsis*[16] against public EST databases of *B. oleracea* using current web-based tools.[23] We identified a 575 bp sequence (AM388844.1) from a *B. oleracea* EST database (library obtained from *B. oleracea* var. *alboglabra* LIBEST_020 118 AAFC_WHRI_BoE02a, submitted to GeneBank by Barker GC, WHRI Lab, Warwick University, UK) that matched with a sequence of PaO from *Arabidopsis thaliana* (*AtPaO*).[16] Using specific primers for this sequence, a 315 bp cDNA was isolated from broccoli and sequenced, and, after its identity with *AtPaO* (69.4%) and *BnPaO* from *Brassica napus*[17] (41%) was verified, it was named *BoPaO* (Fig. 1).

BoPaO expression and chlorophyll content in different sections of broccoli florets

We analysed the levels of chlorophylls and the expression of *BoPaO* during senescence of different parts of broccoli florets (Fig. 2a) and during senescence of leaves. Higher chlorophyll content was detected in section 1 (inflorescences) than in the other two sections (2 and 3), which had similar chlorophyll levels (Fig. 2b). Taken together, chlorophyll content decreased during senescence. Chlorophyll content was much higher in leaves than in florets and also showed a decline after 4 days of senescence (Fig. 2b).

The expression of *BoPaO* was approximately four times higher in pre-senescent leaves than in pre-senescent inflorescences (Fig. 2c). After 96 h in darkness the expression of *BoPaO* increased in both section 1 and leaves. In contrast, the expression of this gene decreased in sections 2 and 3 during the same period.

Changes in BoPaO expression, superficial colour (hue angle) and chlorophyll content in broccoli under different postharvest treatments

Ethephon and BAP

Senescence is mainly regulated by the hormones cytokinin and ethylene, which respectively inhibit and promote the process. We decided to analyse the effect of these hormones on *BoPaO* expression. To do that, broccoli florets were treated with BAP or ethephon (an ethylene-releasing agent) and stored in darkness at 22 °C for 120 h to accelerate senescence. Superficial colour remained unchanged up to 48 h of storage (Table 1). Thereafter,

Table 1. Hue angle and total chlorophyll content in control and hormone-treated broccoli florets during postharvest senescence at 22 °C

	Hue angle (°)			Total chlorophyll content (mg g^{-1} tissue FW)		
Time (h)	Control	Ethephon	BAP	Control	Ethephon	BAP
0	125.75 ± 2.89	125.75 ± 2.89	125.75 ± 2.89	0.160 ± 0.011	0.160 ± 0.011	0.160 ± 0.011
24	125.18 ± 3.29	125.71 ± 3.17	127.17 ± 3.29	0.152 ± 0.020	0.118 ± 0.007*	0.161 ± 0.010
48	125.91 ± 2.92	126.07 ± 2.73	127.20 ± 2.85	0.140 ± 0.011	0.122 ± 0.007*	0.173 ± 0.008*
72	123.91 ± 3.86	120.00 ± 3.84*	127.37 ± 2.65*	0.093 ± 0.010	0.080 ± 0.012*	0.110 ± 0.007*
120	102.63 ± 8.09	98.79 ± 2.44*	127.06 ± 2.51*	0.056 ± 0.008	0.034 ± 0.002*	0.094 ± 0.014*

Asterisks indicate statistically significant differences between treated samples and controls at the same time ($P < 0.05$).

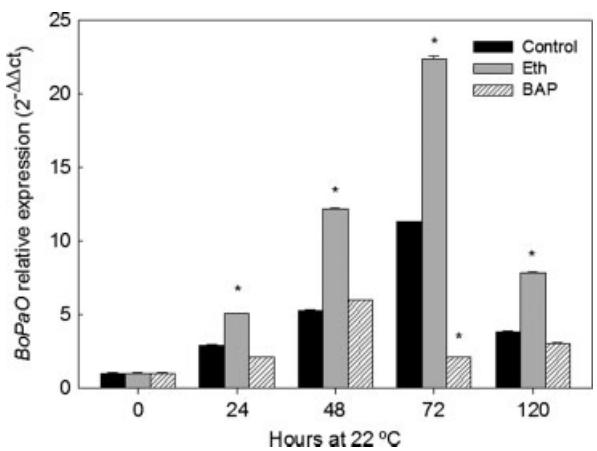

Figure 3. Relative *BoPaO* expression of hormone-treated broccoli heads during 5 days of induced senescence at 22 °C. Asterisks indicate statistically significant differences between treated samples and controls at the same time ($P < 0.05$).

control and ethephon-treated samples showed progressive yellowing and significant decreases in hue angle, the changes being more pronounced in samples treated with ethephon. In contrast, treatment with BAP inhibited colour changes over the whole incubation period.

In all samples, total chlorophylls started to decrease after 48 h and diminished continuously thereafter (Table 1). After 120 h of incubation the amount of total chlorophylls in controls was approximately 67% lower than that observed in pre-senescent tissues. Chlorophyll degradation started earlier (24 h) in ethephon-treated samples, whose chlorophyll levels were generally lower than those in control samples throughout incubation. In contrast, BAP-treated samples showed delayed chlorophyll degradation, with the decline in chlorophyll content starting after 72 h.

The expression of *BoPaO* in controls increased continuously up to 72 h and then decreased at 120 h (Fig. 3). In BAP-treated samples a similar increment in expression was detected until 48 h. However, the peak of expression at 72 h was not detected and mRNA levels were approximately six times lower than those in controls. In contrast, treatment with ethephon induced a larger increment in *BoPaO* expression. The pattern of expression was similar to that in controls, with a peak at 72 h, but values were significantly higher than those in controls throughout the incubation period.

Hot air and modified atmosphere

Broccoli florets were subjected to different postharvest physical treatments to evaluate their effect on *BoPaO* expression. As described above, *BoPaO* expression peaked at 72 h and then decreased at 120 h. In the physical treatment experiments we decided to choose these times for sampling, when changes in expression were more important. Previous studies have demonstrated that treatments with hot air[19,24] and modified atmosphere[25] can delay senescence and chlorophyll degradation in broccoli. Here we performed these treatments as described above and stored the samples at 22 °C. Hue angles were approximately 124° initially and decreased in all samples during storage (Table 2). Control florets showed the largest hue angle decline after 120 h, in correlation with the enhanced yellowing observed in florets, while hot air and modified atmosphere treatments caused smaller hue angle changes.

Chlorophyll levels decreased during storage at 22 °C (Table 2). However, control samples showed a larger decrease after 120 h in comparison with treated samples. Significantly, florets maintained in modified atmosphere retained their chlorophylls almost completely. Also, application of heat decreased chlorophyll degradation, but to a lesser extent than modified atmosphere did.

As in the hormone treatment experiments, the expression of *BoPaO* in controls increased at 72 h and decreased at 120 h (Fig 4). Samples treated with hot air or modified atmosphere also showed an increment in *BoPaO* expression at 72 h, but it was lower than that detected in controls. In contrast, *BoPaO* expression after 120 h was higher in samples treated with hot air or modified atmosphere than in controls.

UV-C and visible light

Broccoli florets were also treated with different kinds of radiation to evaluate the expression of *BoPaO*. A previous study has demonstrated that UV-C treatment can delay broccoli senescence.[20] It has also been reported that leaves of Chinese kale showed delayed postharvest senescence when stored under visible light.[26] Our results showed that superficial colour in control and UV-C-treated samples remained almost unchanged during storage up to 72 h (Table 3). Thereafter, hue angles decreased significantly but yellowing was less evident in samples treated with UV-C. In contrast, treatment with light resulted in almost complete inhibition of yellowing over the whole period of incubation, and samples maintained their green colour. In all samples, chlorophyll levels decreased during storage at 22 °C (Table 3). However, control samples showed a larger decrease after 120 h in comparison with treated samples.

As in other cases, *BoPaO* expression increased at 72 h and decreased at 120 h in non-treated samples (Fig. 5). Samples treated with UV-C showed a smaller increment at 72 h, but *BoPaO* expression remained high at 120 h. Samples treated with visible

Table 2. Hue angle and total chlorophyll content in control, modified atmosphere (MA)-treated and heat-treated (HT) broccoli florets during postharvest senescence at 22 °C

	Hue angle (°)			Total chlorophyll content (mg g^{-1} tissue FW)		
Time (h)	Control	MA	HT	Control	MA	HT
0	123.83 ± 3.05	123.83 ± 3.05	123.83 ± 3.05	0.226 ± 0.050	0.226 ± 0.050	0.226 ± 0.050
72	120.55 ± 3.41	123.49 ± 2.92*	122.04 ± 2.12*	0.120 ± 0.031	0.221 ± 0.023*	0.114 ± 0.010*
120	102.42 ± 7.94	115.11 ± 7.92*	119.85 ± 2.00*	0.030 ± 0.002	0.223 ± 0.017*	0.054 ± 0.001*

Asterisks indicate statistically significant differences between treated samples and controls at the same time ($P < 0.05$).

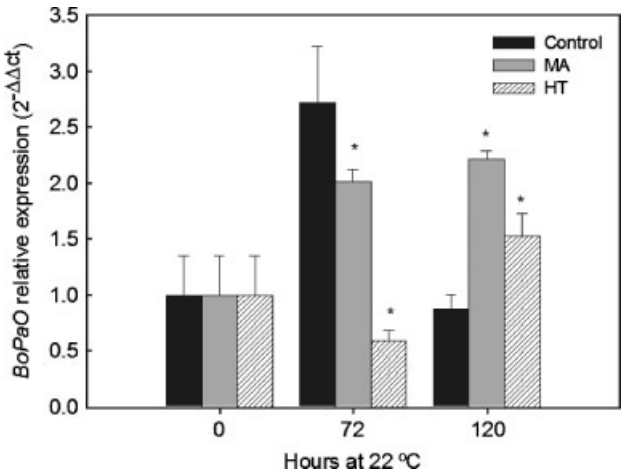

Figure 4. Relative *BoPaO* expression of broccoli florets subjected to heat or modified atmosphere treatment during 5 days of induced senescence at 22 °C. Asterisks indicate statistically significant differences between treated samples and controls at the same time ($P < 0.05$).

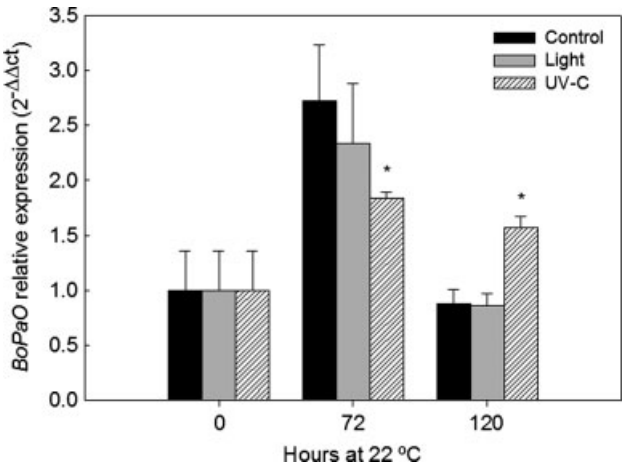

Figure 5. Relative *BoPaO* expression of broccoli florets subjected to visible light or UV-C treatment during 5 days of induced senescence at 22 °C. Asterisks indicate statistically significant differences between treated samples and controls at the same time ($P < 0.05$).

light did not show any differences in *BoPaO* expression relative to controls.

DISCUSSION

De-greening and yellowing detected during senescence are caused by chlorophyll degradation. A review published recently has stated that one of the main enzymes involved in such catabolism is PaO.[4] The conversion of pheophorbide a to pFCC catalysed by PaO and RCC reductase is an important step in chlorophyll catabolism, because it is responsible for the loss of green colour. The enzyme PaO is considered to play a key regulatory role, since its activity correlates positively with chlorophyll degradation.[17,27]

The phenotypic changes, mainly yellowing, that occur in broccoli during postharvest senescence are the major determinant of its commercial quality. For this reason, analysis and characterisation of genes and enzymes involved in chlorophyll catabolism are of great importance in this system.

In this study we performed a search of public databases and found a sequence belonging to *B. oleracea* of 575 bp (AM388844.1) with similarities to previously reported sequences of genes encoding PaO. In a previous study by Fukasawa *et al.*,[3] another fragment that also matched with part of the EST (AM388844.1) had been cloned and named *BoPaO* (Fig 1), so we decided to conserve the denomination even though the cloned fragments were different. Until now, only 17 genes encoding putative PaOs have been cloned, some of which were aligned and are shown in Fig. 1. In *Arabidopsis, AtPAO* is a single copy gene that encodes a protein belonging to a small family of Rieske-type iron–sulfur oxygenases.[4] Broccoli and *Arabidopsis* belong to the same plant family, Brassicaceae, which suggests the possibility that *BoPaO* is also a single gene in broccoli and that fragments cloned by Fukasawa *et al.*[3] and us correspond to the same gene.

An increment in PaO activity or expression of related genes was detected during senescence of *Arabidopsis*[16] and maize[28] and during ripening of fruits from *Capsicum annuum*[29] and orange,[30] indicating a close relationship between processes with enhanced chlorophyll catabolism and PaO. We found that the expression of *BoPaO* increased during postharvest senescence, simultaneously with chlorophyll degradation. At the end of the experiment, chlorophylls were almost completely degraded and, at the same time, *BoPaO* expression was reduced, probably owing to a regulatory mechanism that detects the absence of substrate. A similar increment in *BoPaO* expression was also shown previously in broccoli heads stored at 20 °C.[3] This increment was detected not only in senescent florets but also during leaf senescence. The decrement in *BoPaO* expression during senescence of stems could also be due to a lack of substrate.

A previous study has shown that chlorophyll degradation during postharvest senescence of broccoli is regulated positively by ethylene and negatively by cytokinin.[18] These hormones also regulate the activity of catabolism-related enzymes. For example, 1-methylcyclopropene (an inhibitor of ethylene action)

Table 3. Hue angle and total chlorophyll content in control, visible light-treated and UV-C-treated broccoli florets during postharvest senescence at 22 °C

	Hue angle (°)			Total chlorophyll content (mg g^{-1} tissue FW)		
Time (h)	Control	Light	UV-C	Control	Light	UV-C
0	125.44 ± 3.99	125.44 ± 3.99	125.44 ± 3.99	0.226 ± 0.050	0.226 ± 0.050	0.226 ± 0.050
72	122.55 ± 3.41	126.22 ± 1.61*	121.62 ± 1.54	0.120 ± 0.013	0.160 ± 0.033*	0.117 ± 0.020
120	102.42 ± 7.94	122.90 ± 7.68*	110.23 ± 3.80*	0.030 ± 0.002	0.050 ± 0.003*	0.054 ± 0.010*

Asterisks indicate statistically significant differences between treated samples and controls at the same time ($P < 0.05$).

delayed broccoli yellowing and decreased chlorophyll-degrading peroxidase and chlorophyllase activities.[31] We found that ethylene greatly enhanced *BoPaO* transcript levels, while BAP diminished the expression peak detected at 72 h. Other researchers have also reported that expressions of PaO-related genes are modulated by hormone treatments. In *Citrus*, treatment with gibberellins reduced de-greening as well as the increment in the expression of a PaO gene during ripening.[30] Similarly, PaO activity has been correlated with chlorophyll loss in barley leaves both during natural senescence and after hormonal treatments.[32]

Several studies have focused on the use of postharvest or preharvest treatments to maintain the green colour of broccoli and reduce chlorophyll degradation during senescence.[24,33–35] One of the most successful methods to delay chlorophyll degradation is the utilisation of films to induce modified atmospheres. Hermetic enclosure of broccoli heads in polyethylene bags strongly reduced chlorophyll degradation during storage at 22 °C, as shown by the present results and in accordance with a previous report.[24] However, ours is the first study to show an effect of modified atmosphere on the expression of a gene related to chlorophyll catabolism. Although chlorophyll degradation was almost completely blocked by modified atmosphere, we did not find complete inhibition of *BoPaO* expression, suggesting that the expression of other genes associated with chlorophyll catabolism could also be inhibited.

It has been shown that heat treatment can reduce chlorophyll degradation by delaying the increment in chlorophyll-catabolic enzyme activities, including those of chlorophyllase, Mg-dechelatase, chlorophyll-degrading peroxidase and chlorophyll oxidase.[24,36] We found that heat treatment also delayed the increment in *BoPaO* expression at 72 h, which recovered after 120 h.

UV-C treatment also provokes a delay in chlorophyll degradation, probably via a reduction in chlorophyll-degrading enzyme activities.[20] In the present study, UV-C-treated samples showed lower expression of *BoPaO* at 72 h but higher expression after 120 h relative to controls.

Postharvest application of heat shocks or low doses of UV-C causes a transitory inhibition of gene expression or decrease in enzymatic activities, which recover when the tissue is returned to non-stressed conditions.[37,38] In the present study, treatment with heat or UV-C irradiation also delayed the increment in *BoPaO* expression at 72 h, which recovered after 120 h, and probably led to a lower rate of chlorophyll catabolism.

In the case of visible light treatment, treated samples showed an important maintenance of green colour even though chlorophyll catabolism was not completely blocked. In that sense, *BoPaO* expression in samples treated with light showed a similar behaviour to that in controls. It could be that light prevented degradation of chlorophyll on the surface of heads but that catabolism continued inside the tissue where light could not penetrate.

Recently, it was shown that postharvest treatment with ethanol also reduced chlorophyll degradation during senescence of broccoli as well as delaying the increment in *BoPaO* expression.[3]

In conclusion, we cloned a fragment of a gene encoding a putative PaO (*BoPaO*) from broccoli, a horticultural crop with enhanced postharvest senescence, and characterised the expression of the gene in different parts of the inflorescence during senescence. *BoPaO* expression was also analysed in samples exposed to different hormonal and physical treatments that affect chlorophyll degradation. The results support a close relationship between chlorophyll degradation and *BoPaO* expression.

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