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Chlorophyllase versus pheophytinase as candidates for chlorophyll dephytilation during senescence of broccoli

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ABSTRACT

Degradation of chlorophylls during senescence is a highly regulated process which requires the concerted action of several enzymes. Traditionally, it has been stated that the dismantling process of the chlorophyll molecule begins with a dephytilation step, followed by Mg^{2+} removal and other breakdown reactions. Recently, new evidence suggests the possibility of a rearrangement in the first two steps of this process, occurring Mg^{2+} removal prior to the loss of the phytol side chain. With the purpose of approximating to the real sequential order of these reactions and to assess if dephytilation occurs on intact (catalyzed by chlorophyllase) or Mg-free (catalyzed by pheophytinase) chlorophyll, expression of both genes was analyzed in broccoli tissue during senescence. Samples of broccoli florets treated with plant hormones, such as cytokinin and ethylene were utilized, as to assess the effect of such compounds on the expression of these genes. Results showed that chlorophyllase expression did not correlate to typical expression patterns for genes related to senescence, since a decrease in expression during senescence was found for one of the two chlorophyllase genes analyzed, and the hormonal-treatment effects on gene expression did not match those observed on chlorophyll content for both chlorophyllase genes. Pheophytinase expression patterns, on the other hand, displayed an increase in the first 3 days of induced senescence, followed by lower expression values towards the end of the experiment. Samples subjected to postharvest treatments mostly showed an inhibition of pheophytinase expression, especially in samples in which degradation of chlorophylls had been delayed. These results suggest that pheophytinase expression correlates to the visual manifestation of postharvest treatments, supporting the possibility that this enzyme is responsible for the dephytilation step in chlorophyll breakdown.

Introduction

Leaf senescence is accompanied by a rapid breakdown of chlorophylls (Chl) and a consequent loss of green color. The Chl degradation process is essential to leaf senescence and fruit ripening, due to the much needed elimination of Chl and their derivatives to avoid accumulation of phototoxic pigments (Matile et al., 1999; Hörtensteiner, 2006). A pathway has been established for chlorophyll degradation, involving the combined action of several enzymes on green pigments starting with the dephytilation of the Chl molecule, catalyzed by the enzyme chlorophyllase (CLH, EC 3.1.1.14), followed by removal of the central Mg^{2+} ion (Suzuki et al., 2005; Harpaz-Saad et al., 2007). CLH activity, producing chlorophyllide (Chlide) and phytol, has a preferential action towards Chl *a*, but it can also accept its *b* form and pheophytins as substrates (Hörtensteiner, 1999; Benedetti and Arruda, 2002). The direct intermediate resulting from Mg-dechelation, pheophorbide (Pheide), has its porphyrin ring oxygenolytically opened by Pheide *a* oxygenase, a Fe-depending monooxygenase located at the envelope membrane of gerontoplasts, resulting in loss of the green color of the molecule (Hörtensteiner et al., 1998; Matile et al., 1999). Afterwards, red chlorophyll catabolyte (RCC) is site-specifically reduced by RCC reductase, a soluble protein of chloroplasts, though it has also been associated with mitochondria (Rodoni et al., 1997b; Wüthrich et al., 2000; Mach et al., 2001). Further downstream products are transported to the vacuole and degraded by non-enzymatic tautomerization, induced by the acidic vacuolar pH (Hinder et al., 1996; Rodoni et al., 1997a; Oberhuber et al., 2003).

CLH catalyzes the hydrolysis of Chl to Chlide and phytol and has been considered the first enzyme in the Chl catabolic process

Abbreviations: 6-BAP, 6-benzylaminopurine; Chl, chlorophyll; Chlide, chlorophyllide; CLH, chlorophyllase; DMSO, dimethylsulfoxide; EST, expressed sequence tags; Pheide, pheophorbide; Phein, pheophytin; PPH, pheophytinase; RCC, red chlorophyll catabolyte; RT-qPCR, reverse transcription quantitative real-time PCR.

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(Takamiya et al., 2000; Harpaz-Saad et al., 2007). Several CLH genes have been isolated from different sources, such as *Citrus* species (Jacob-Wilk et al., 1999), wheat (Arkus et al., 2005), *Ginko biloba*, *Brassica oleracea* (Tang et al., 2004), *Chenopodium album* and arabidopsis (Tsuchiya et al., 1999). Phylogenetic analyses have shown that CLH genes cluster into two groups, being the first (arabidopsis *AtCLH1* and *Citrus sinensis CsCLH*) characterized by ethylene and methyl jasmonate-regulated expression; whereas the second group (arabidopsis *AtCLH2* and *C. album CaCLH*) are expressed at low, constitutive levels (Tsuchiya et al., 1999; Jacob-Wilk et al., 1999). Recently, the true involvement of CLH in Chl breakdown has been questioned, since not all isolated genes have a chloroplast transit peptide, suggesting alternative pathways occurring outside of the chloroplast or involvement of enzymes other than CLH (Takamiya et al., 2000; Hörtensteiner, 2006). Schenk et al. (2007) have shown that arabidopsis mutants with interrupted expression for both known CLH are still able to degrade Chl during senescence, indicating that these genes are not essential for this catabolic process. Based on these results, Schelbert et al. (2009) set out to reveal the true CLH responsible for Chl dephytilation in arabidopsis. Instead, their findings revealed the existence of a new enzyme, termed pheophytinase (PPH), which would act as a pheophytin (Phein, Mg-free Chl) hydrolase. Given the possibility that dephytilation occurs on Mg-free Chl, the whole degradation pathway must be revised, especially in the order of the early reactions. In the suggested model, Mg release seems to precede phytol cleavage, producing Phein, which is dephytilated by PPH to give Pheide (Schelbert et al., 2009). The possibility of a Chl degradation pathway involving an initial Mg-dechelation step and formation of Phein has been addressed previously, even before PPH was brought into the light. In cabbage, Heaton et al. (1996) indicated the existence of two Chl degradation pathways in green plant tissues, one through Phein to Pheide and other though Chlide to Pheide. Also, pathway differences have been suggested in both *Citrus* and parsley senescence (Amir-Shapira et al., 1987). In *Brassica napus*, Chl breakdown was also suggested to begin with Mg removal (Langmeier et al., 1993).

Fresh broccoli (*B. oleracea* L.) has become a popular vegetable in many parts of the world due to its high nutritional value, low caloric and high dietary fiber content, important levels of ascorbic acid and a wide range of anticarcinogenic and antioxidant compounds (King and Morris, 1994). This vegetable is harvested while its inflorescences are still immature, previous to sepal opening, which implies water loss and variations in nutrient and hormone content. These events, in addition to harvesting and handling, cause severe stress conditions, inducing an early onset of senescence and a significant increase in Chl degradation. As a consequence, a loss of the superficial green color is observed, which decreases the commercial approval of the broccoli florets. Moreover, senescence accelerates loss of sugars, proteins and lipid peroxidation conducting to a loss of nutritional quality (King and Morris, 1994; Page et al., 2001).

In the present work, we identified a putative PPH from broccoli, based on nucleotide sequence information available from arabidopsis, and compare its expression with that of known CLH genes in hormone-treated broccoli florets, in order to bring to light which enzyme would be responsible for dephytilation of Chl during senescence of broccoli.

Materials and methods

Plant material and induced senescence experiments

Broccoli (*Brassica oleracea* L. var. *Italica* cv. Iron) heads were obtained from a local producer in La Plata, Buenos Aires, Argentina, and immediately transported to the laboratory and processed.

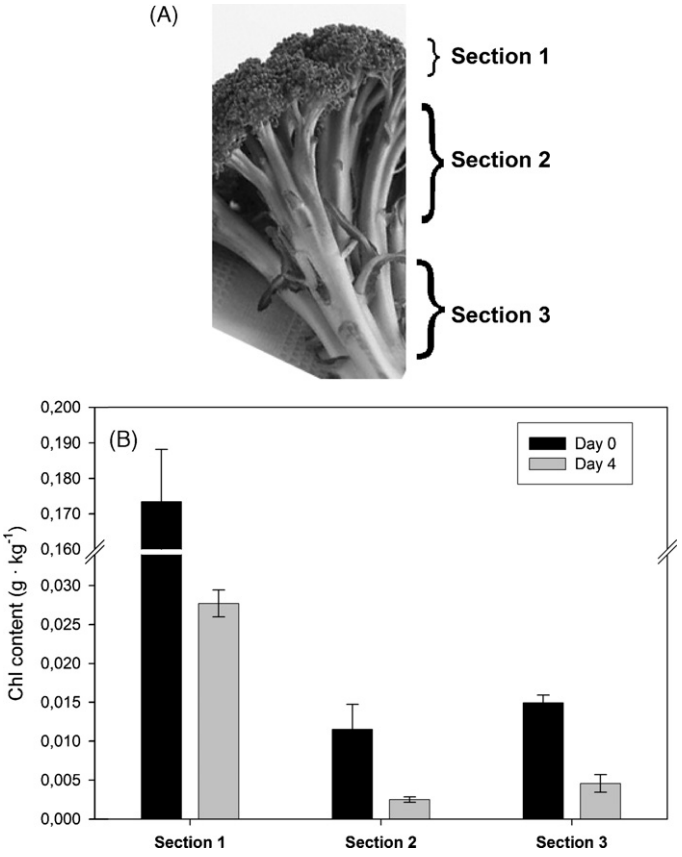

Fig. 1. Sectioning of broccoli florets (A). Total Chl content in different sections of broccoli florets at day 0 and day 4 of induced senescence (B).

Whole broccoli heads were stored in darkness at 22 °C to induce senescence. After incubation, heads were segmented into three sections, as depicted in Fig. 1A. For all experiments, except the floret sections experience, only inflorescence tissue (Section 1) was analyzed.

Hormone treatments

Whole harvested broccoli florets were treated as described by Costa et al. (2005). Samples were immersed for 10 min in solutions containing 100 ppm 6-benzylaminopurine (6-BAP, Sigma), 0.1% DMSO; 100 ppm 2-chloroethylphosphonic acid (Ethephon, an ethylene-releasing agent), 0.1% DMSO; or 0.1% DMSO as control. Twenty heads were employed for each condition. After treatments, samples were placed in plastic cups containing a small amount of distilled water to prevent dehydration and stored in a well ventilated chamber isolated from external light at 22 °C. Samples were collected daily for superficial color measurement. Afterwards, inflorescences were separated from stems, immediately frozen using liquid nitrogen and stored at −20 °C until use. Hormone treatments were repeated at least three times, using different samples harvested at different times.

Superficial color measurement

Superficial color was determined daily for all broccoli heads in all treatments by measuring L^*, a^* and b^* parameters at five different positions on each sample with a Minolta CR300 chromameter (Osaka, Japan). Hue angle (h°) was calculated as $h^\circ = \tan^{-1}(b^*/a^*)$ when $a^* > 0$ and $b^* > 0$, or as $h^\circ = 180^\circ - \tan^{-1}(b^*/a^*)$ when $a^* < 0$ and $b^* > 0$.

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. Chlorophyll content was measured in the supernatant according to Inskeep and Bloom (1985) and results were expressed as g total chlorophyll per kg fresh weight tissue. All measurements were performed by triplicate.

RNA extraction and qPCR assays

Total RNA was extracted from broccoli tissue using a modification of the phenol method (Kirby, 1968) and quantified by UV spectrophotometry. RNA integrity was evaluated by gel-electrophoresis. An amount corresponding to 4 μg of total RNA was treated with RQ1 DNAse (Promega) according to the manufacturer's protocol, purified with chlorophorm: 1-octanol (24:1), precipitated with 3 M sodium acetate and reverse-transcribed using MML-V reverse transcriptase (Promega) and random primers (Hexamers), according to the manufacturer's suggestions. Absence of DNA contamination was assessed by PCR and gel-electrophoresis. Resulting cDNA was stored at −20 °C and employed as template for two-step qPCR reactions using an Mx3005P qPCR system (Stratagene) and FastStart Universal SYBR Green Master Mix (Roche), using recommended conditions. Sequences of primers employed are described below. Every RT-qPCR measurement was performed at least four times.

Primer design

The following primers were designed in order to assess relative expression by RT-qPCR of two known broccoli CLH genes: *BoCLH1* (AF337544): 5′ agacccatccatcaagttttcagc 3′ and 5′ agatttcgggatcggttcttatgc 3′; *BoCLH2* (AF337545): 5′ agatgcctgttctagttattgg 3′ and 5′ cacgctggaccttgacattc 3′ (Chen et al., 2008). All qPCR assays employed β-actin (AF044573) as normalizer, with the following primers: 3′ ccagaggtcttgttccagccatc 5′ and 3′ gttccaccactgagcacaatgttac 5′. The following specific primers were designed to amplify and quantify the expression of a 91-bp fragment of a broccoli EST similar to pheophytinase (PPH) (from *B. oleracea* var. *alboglabra*, OL386R): 5′ agaggttatcggtgagcca 3′ and 5′ gacgagatgaggatggg 3′.

Results and discussion

Finding chlorophyllase and PPH sequences in broccoli

Recently it has been proposed that the Chl degradation pathway should be revised, suggesting that a new enzyme, termed PPH could be involved in Mg-free Chl dephytilation during leaf senescence. In this new scheme, CLH would no longer be primarily involved in this senescence process, as was previously assumed (Hörtensteiner, 2006; Schelbert et al., 2009). Given the potential importance of PPH on leaf senescence, we set out to compare expression patterns of CLH and PPH during postharvest senescence of broccoli. In the past few years, three chlorophyllase genes have been identified in broccoli (*BoCLH1*, *BoCLH2* and *BoCLH3*). However, it has been

Fig. 2. Nucleotide sequence alignment of PPH from arabidopsis (*AtPPH*, At5g13800) and the EST from broccoli found through database search, which we termed *BoPPH*. Arrows indicate sites chosen for primer design for RT-qPCR experiments.

reported that only mRNA transcripts of *BoCLH1* are accumulated in freshly harvested and senescent florets and leaves. Until this point, no mRNA transcripts of *BoCLH2* or *BoCLH3* have been detected during broccoli postharvest senescence (Chen et al., 2008). However, in this work we present RT-qPCR quantification data of *BoCLH1* as well as *BoCLH2* in both senescent and freshly harvested broccoli florets.

Two broccoli CLH sequences were retrieved from public databases, namely *BoCLH1* and *BoCLH2* (AF337544 and AF337545, respectively). Also, in order to assess PPH expression in broccoli, we compared the published sequence for arabidopsis PPH (*AtPPH*, At5g13800) from Schelbert et al. (2009) against an expressed sequence tags database with *B. oleracea* specificity using current web-based tools (Altschul et al., 1990). The BLASTN query results displayed a highly similar EST sequence from *B. oleracea* var. *alboglabra* (OL386R), which was used as a starting point for further experiments (Fig. 2). Specific primers were designed (see Section 2) to amplify a fragment of the EST found and employed for RT-qPCR. The fragment cloned was sequenced to confirm its identity, and the EST from broccoli was hereby named *BoPPH*. Fig. 2 shows an alignment of *AtPPH* and *BoPPH*, indicating the positions of the primers employed for RT-qPCR.

CLH and PPH expression in different section of broccoli florets

In order to assess expression changes in different sections of broccoli tissue, florets were segmented into Section 1 (inflorescences), Section 2 (small branchlets) and Section 3 (main floret stem), as depicted in Fig. 1A. Samples were taken for presenescent (day 0) and dark-induced senescent (day 4) tissue. Higher Chl content in inflorescences (Section 1) was detected in comparison to the other two sections, while values were very similar for Sections 2 and 3. Regardless the section analyzed, Chl content showed a decrease during senescence (Fig. 1B).

Expression of CLH genes was analyzed by comparative RT-qPCR. *BoCLH1* showed a higher expression in presenescent tissue (Fig. 3A). Also, when comparing expression in Section 3 with Section 1, main stem tissue values were almost half for presenescent tissue and one third for senescent tissue. In a previous work, it was found that *BoCLH1* was expressed at harvest and occasionally on days 4 and 5 of postharvest (Chen et al., 2008).

Expression pattern of *BoCLH2* showed an expression increase in senescent tissue (Fig. 3B), and was found to be higher in stem tissue (Section 2) than in inflorescences.

Chen et al. (2008) reported three putative chlorophyllase genes (*BoCLH1*, *BoCLH2*, and *BoCLH3*) but only *BoCLH1* expression was detected by RNA gel blot hybridization during the course of broccoli postharvest senescence. Differences in detection of *BoCLH2* among the mentioned work and ours could be due to methodology (RNA gel blot hybridization versus RT-qPCR) employed.

PPH expression (Fig. 3C) was considerably higher in senescent samples compared to presenescent for Sections 1 and 2, while in the case of Section 3 values were not statistically different between senescent and presenescent samples.

Chlorophyllase expression in hormone-treated broccoli florets

We performed hormone treatments with both cytokinin and ethylene, two of the most influential plant hormones in senescence regulation (Pyung et al., 2007). Superficial color and Chl content were employed as senescence parameters, as shown in Fig. 4. Values of Chl content diminished 10-fold during senescence in control tissue, while 6-BAP-treated florets showed delayed Chl degradation during the course of induced senescence. Ethylene-treated florets exhibited an increase in the rate of Chl degradation, reaching values lower to controls even at the end of the experiment (Fig. 4B).

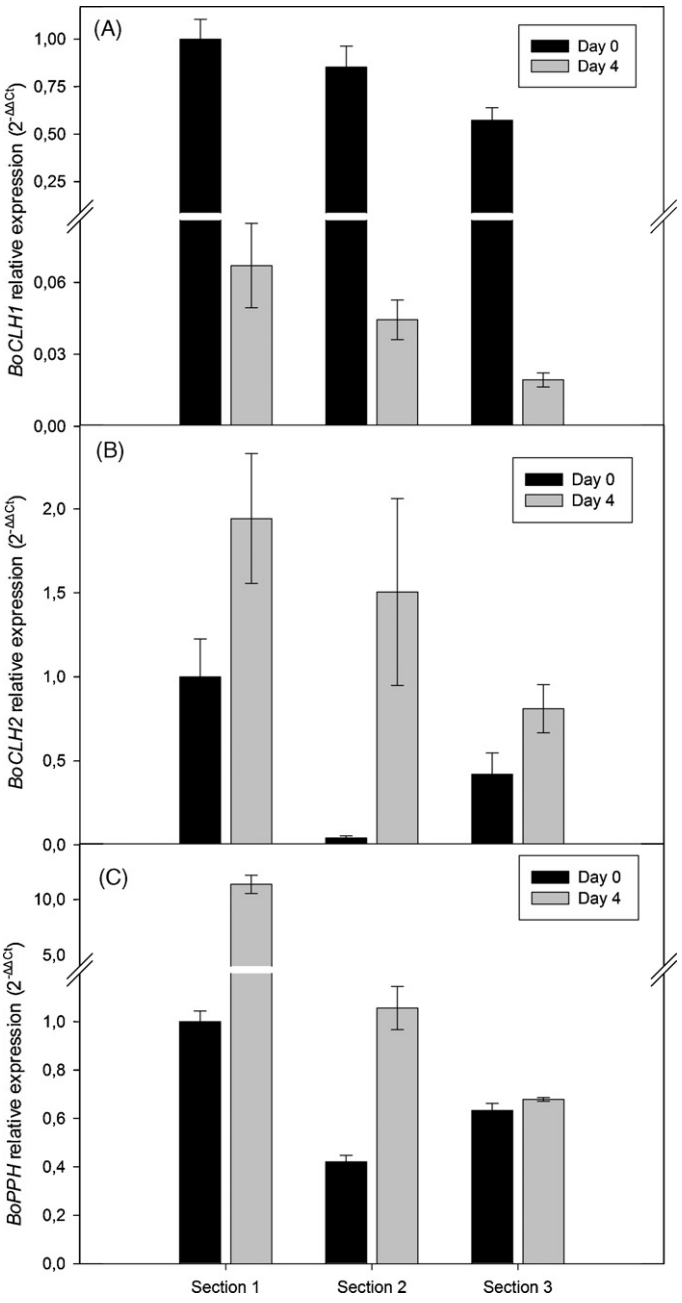

Fig. 3. Gene expression assessment during senescence of different sections of broccoli florets. Expression of *BoCLH1* (A); *BoCLH2* (B); *BoPPH* (C) was measured by RT-qPCR. Sectioning of samples is depicted in Fig. 1A.

Although changes in Chl content between controls and ethylene-treated samples are rather small compared to other publications, such as Wang et al. (2004), differences are significant and Chl content is lower in treated samples. Superficial color, as well as the visible aspect of florets, reflected the same results seen in Chl content (Fig. 4A). Except for all samples at day 0 and control versus cytokinin-treated samples at day 3, all data points shown in Fig. 4B are statistically different from each other ($P < 0.05$). Hue angle, as seen in Fig. 4A, shows values statistically different from controls in days 3 and 5 for both treatments ($P < 0.05$).

To compare levels of CLH gene expression, untreated broccoli florets at harvest (day 0) were used as calibrators. Treated as well as control florets were collected for RNA extraction after 3, 4 and 5 days of dark-induced senescence.

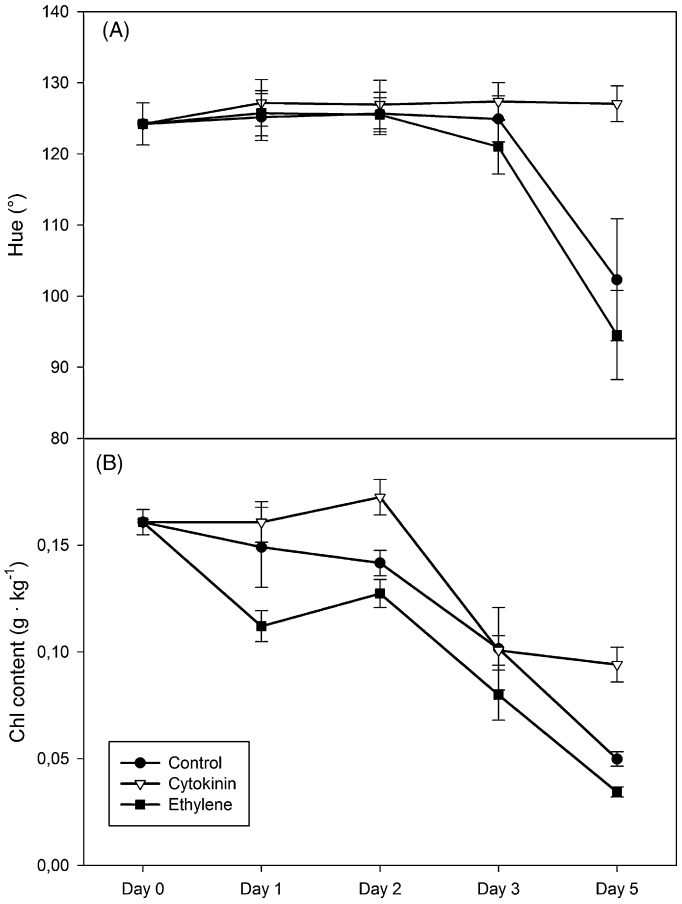

Fig. 4. Hue angle (A) and total Chl content (B) of hormone-treated broccoli florets during 5 days of induced senescence. Hue angle was calculated as described in "Materials and methods" and Chl content is expressed as grams total Chl per kilogram of fresh weight tissue.

Expression of CLH in hormone-treated broccoli florets showed distinct patterns. In the case of *BoCLH1* (Fig. 5A), an important decrease in expression was observed after day 0, since an over 50-fold drop was found from day 0 to day 3 of control florets, followed by a very slight increase at the final day of the experiment. Cytokinin-treated florets also showed a lower expression in senescent tissues in relation to initial values, but a gradual increase was seen with the course of senescence. However, a higher expression was detected in comparison to controls. Ethylene-treated samples displayed an opposite behavior, since expression of *BoCLH1* was higher than controls at day 3, followed by a decrease in expression. It is important to notice that regardless of the treatment, *BoCLH1* expression decreased by at least 80% after the initial day of the experiment, indicating that the marked decrease in expression of this gene is not greatly influenced by hormone treatment. On the other hand, overall expression patterns of *BoCLH2* displayed irregular results (Fig. 5B). Expression increased from day 0 to day 3 in control and hormone-treated samples, although no differences among treatments could be detected. In all cases, expression diminished in day 4, but this decrement was lower in cytokinin-treated florets. Finally, an increase in the expression was detected in control and cytokinin-treated samples at day 5, while for ethylene treatments it remained the same in days 4 and 5.

Results obtained for *BoCLH1* differ from expected patterns, since expression of relevant Chl-degrading enzymes are likely to be up-regulated during senescence, especially if regulated by hormonal treatments (Pyung et al., 2007). In the case of *BoCLH2*, although an increase of expression during senescence was detected, no clear correlation could be established with Chl degradation in hormone-treated samples.

Pheophytinase expression in hormone-treated broccoli florets

Expression patterns observed by RT-qPCR reactions using primers with specificity towards a putative broccoli PPH were different from the patterns observed for broccoli CLH. Expression in control florets increased during postharvest senescence (Fig. 6), exhibiting an expected behavior in relation to the decrease in Chl content. A 5-fold increase in PPH expression was found from day 0 to day 3 of the experiment, followed by a gradual decrease until day 5; which correlated with an important decrease in Chl content from day 2 to day 5. Cytokinin-treated material displayed lower PPH expression levels throughout the course of senescence. For example, after 3 days, BoPPH expression increased only twice in controls, while the increment was five times in treated heads indicating an inhibition in gene expression. On the contrary, ethylene-treated tissue showed an induction of PPH gene expression, since values at day 3 were nine times higher than controls at day 0, and over six times higher at the end of the experience.

Possible involvement of chlorophyllases and PPHs in chlorophyll degradation during postharvest broccoli senescence

Results reported in literature in relation to the evolution of CLH activity and Chl degradation during postharvest senescence of broccoli are contradictory. Funamoto et al. (2002) found no changes in

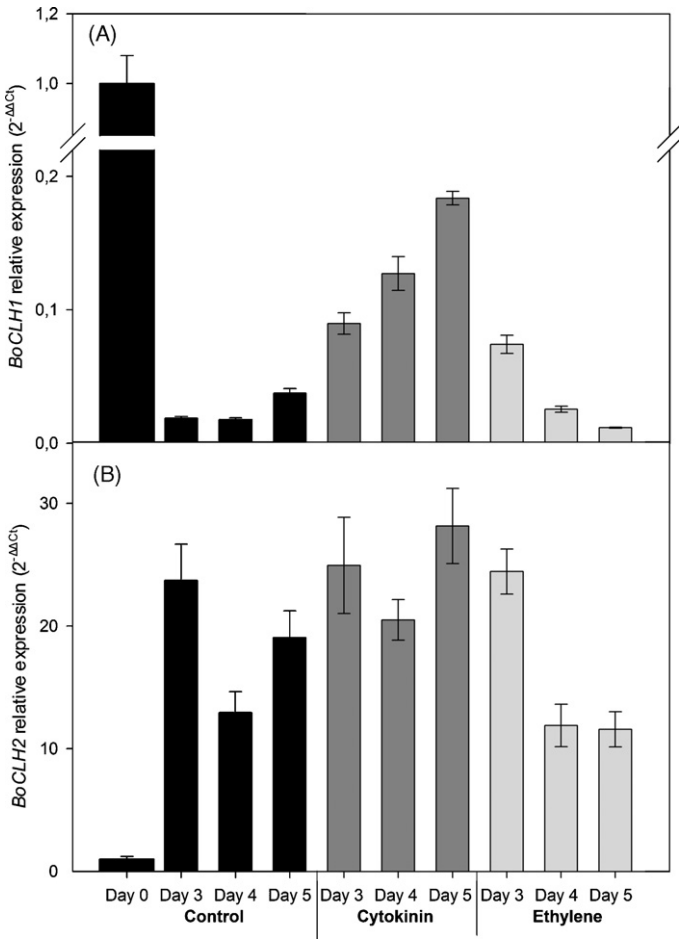

Fig. 5. Relative expression as measured by RT-qPCR of (A) *BoCLH1* and (B) *BoCLH2* in hormone-treated broccoli florets, during 5 days of induced senescence.

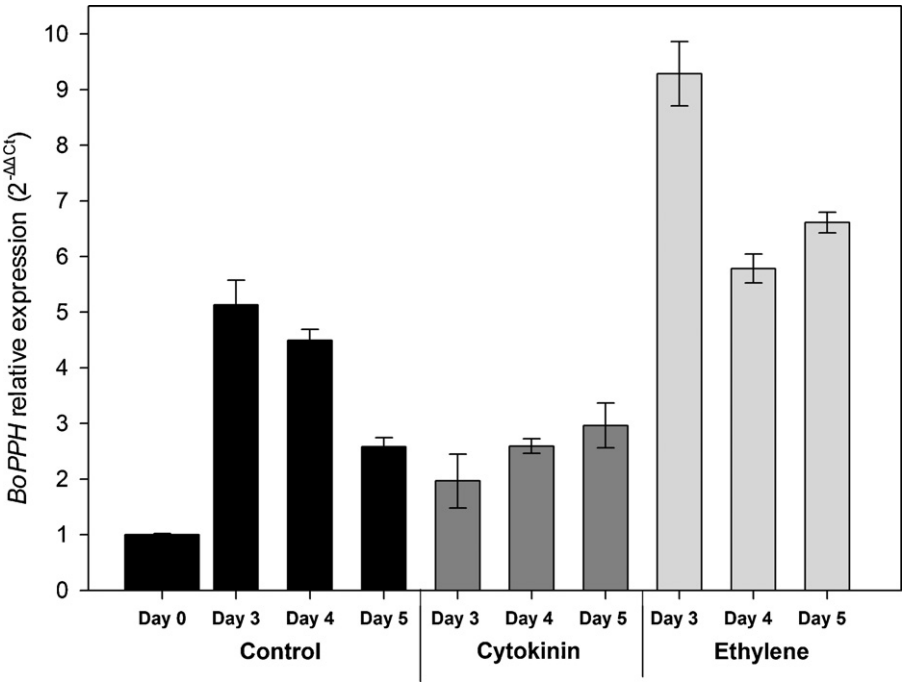

Fig. 6. Relative expression of broccoli PPH in hormone-treated broccoli florets during 5 days of induced senescence, measured by RT-qPCR.

CLH activity during senescence, although activity and Chl degradation were reduced by heat treatment. On the contrary, Costa et al. (2005) found an increment in CLH activity during postharvest senescence and a regulation by hormone (ethylene and cytokinin) treatments. Our results indicated that *BoCLH1* expression is negatively regulated during senescence, whereas *BoCLH2* expression is enhanced during the same physiological period. This suggests that *BoCLH1* would not be directly implicated in Chl breakdown while *BoCLH2* would be related with such process. Results obtained for CLH expression in hormone-treated tissues suggest that *BoCLH2* would play a minor role in Chl breakdown in senescent broccoli florets. Nevertheless, we cannot provide sufficient evidence to exclude the involvement of both enzymes in such physiological process.

It is noteworthy that phylogenetic analysis of identified plant CLH genes cluster into two groups, being *BoCLH1* grouped with *AtCLH1*, while *BoCLH2* clusters with *AtCLH2* (Tang et al., 2004). Both *AtCLH1* and *BoCLH1* do not have a chloroplast transit peptide, but their regulations are different: *AtCLH1* responds to hormonal regulation whereas *BoCLH1* does not, as we found. On the contrary, *AtCHL2* and *BoCLH2* have a typical transit peptide to chloroplast and they are believed to be involved in homeostasis (Tang et al., 2004). In both cases, no endoplasmic reticulum signals were found within broccoli CLH sequences.

The enhanced expression of *BoCLH2* with no regulation of senescence-related hormones (ethylene and cytokinin) could be related to tissue damage and a possible increment of methyl-jasmonate caused by harvest, although this is rather speculative and should be confirmed experimentally by jasmonate treatments. In this regard, it was reported that *AtCLH1* has an enhanced expression after methyl-jasmonate treatments (Tsuchiya et al., 1999)..

Transgenic broccoli expressing antisense *BoCLH1* exhibited only 1–2 days of delayed yellowing during postharvest senescence in comparison to non-transformed controls (Chen et al., 2008). Authors considered that genes other than *BoCLH1* might also be essential in the yellowing process. Our results suggest that *BoCLH2* may not directly relate to Chl degradation either. Although its expression is enhanced during senescence, it is not regulated by senescence-related hormones.

Altogether, there is a possible presence of a more complex regulation system for this enzyme activity and/or gene expression, as was suggested before (Harpaz-Saad et al., 2007). However, as CLH expression is regulated by ethylene in other species such as *Citrus* (Shemer et al., 2008), it is important to take into account that broccoli does not necessarily represent all plant systems, and important differences might be found in other species or biological processes, such as fruit ripening. Also, although *BoCLH2* expression appears not to correlate to Chl degradation, we cannot exclude the possibility that both *BoPPH* and *BoCLH2* are collectively involved in Chl breakdown in senescent chloroplasts, since *BoCLH2* expression is significantly enhanced during this process.

Sequential degradation of Chl by CLH and Mg-dechelatase implies formation of Chlide and Pheide. However, under this scheme, the possibility of Phein formation is excluded. In broccoli, several authors have detected Phein accumulation during postharvest senescence (Costa et al., 2006; Kaewsuksaeng et al., 2006), which suggests the presence of an enzyme that releases Mg^{2+} from Chl and thus provides substrates for PPH.

Differently to what was detected for broccoli CLH, PPH expression in broccoli seems to be strictly related to Chl breakdown during senescence. Postharvest treatments with plant growth regulators that delay or accelerate Chl degradation also regulate PPH expression, thus supporting the recently arisen theory of a new Chl degradation pathway, involving an initial Mg-dechelation step, by a still unknown catalyst, followed by Phein dephytilation, catalyzed by PPH. Further analysis still needs to be performed in order to establish if certain regulation mechanisms are at stake and to determine the responsible compound for Mg^{2+} elimination from intact Chl.

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