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ORIGINAL ARTICLES

Regulation of Harvest-induced Senescence in Broccoli (*Brassica oleracea* var. *italica*) by Cytokinin, Ethylene, and Sucrose

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ABSTRACT

Broccoli (*Brassica oleracea* var. *italica*) deteriorates rapidly following harvest. The two plant hormones ethylene and cytokinin are known to act antagonistically on harvest-induced senescence in broccoli: ethylene by accelerating the process, and cytokinin by delaying it. To determine the level at which these hormones influenced senescence, we isolated and monitored the expression of genes normally associated with senescence in broccoli florets treated with exogenous 6-benzyl aminopurine (6-BAP), 1-aminocyclopropane-1-carboxylic acid (ACC), a combination of 6-BAP and ACC, and sucrose, in the five days following harvest. Exogenous 6-BAP caused both a reduction (Bo*ACO*) and an increase (*BoACS*) in ethylene biosynthetic gene expression. The expression of genes used as senescence markers, Bo*CP5* and Bo*MT1*, was reduced, whereas Bo*CAB1* levels were maintained after harvest in response to exogenous 6-BAP. In addition, the expression of genes encoding sucrose transporters (Bo*SUC1* and Bo*SUC2*) and carbohydrate metabolizing enzymes (Bo*INV1* and Bo*HK1*) was also reduced upon 6-BAP feeding. Interestingly, the addition of ACC prevented the 6-BAP-induced increase in expression of Bo*ACS*, but 6-BAP negated the ACC-induced increase in expression of Bo*ACO*. The culmination of these results indicates a significant role for cytokinin in the delay of senescence. The implication that cytokinin regulates postharvest senescence in broccoli by inhibiting ethylene perception and/or biosynthesis, thus regulating carbohydrate transport and metabolism, as well as senescence-associated gene expression, is discussed and a model presented.

Key words: Cytokinin; Ethylene; Sucrose; Senescence; Broccoli; *Brassica oleracea*; Postharvest.

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Introduction

The senescence-delaying effects of both cytokinin and sucrose, and the senescence-promoting effects of ethylene are not well characterized at the molecular level. Harvested broccoli (*Brassica oleracea* var. *italica*) heads are an ideal system in which to study these effects, as there is considerable physiological and biochemical knowledge of this commonly grown crop plant (King and Morris 1994; Pogson and Morris 1997). Broccoli is a member of the *Brassicaceae* family, so gene isolation experiments should be straightforward because of genome similarity with *Arabidopsis* (Coupe and others 2004). Broccoli is transformable, so isolated genes of interest can be ectopically expressed or silenced. Finally, broccoli is sensitive to both ethylene (Tian and others 1997) and cytokinin (Clarke and others 1994; Rushing 1990) during senescence and has been shown to respond to sucrose treatment after harvest (Irving and Joyce 1995).

Cytokinin and ethylene have antagonistic effects during the harvest-induced senescence of broccoli. Cytokinin, endogenously supplied via ectopic expression of a bacterial *ipt* gene (Chen and others 2001) or by exogenous feeding (Clarke and others 1994; Rushing 1990), causes a delay in postharvest senescence, whereas exogenously supplied ethylene accelerates the process (Tian and others 1994). Clarke and others (1994) proposed that cytokinin may inhibit the catalytic effects of ethylene on senescence at the level of its perception, as exogenous cytokinin had a stronger effect on the delay of senescence than did treatment with silver ions. However, although exogenous cytokinin was sufficient to prevent chlorophyll loss and reduce ammonia accumulation (Clarke and others 1994), this treatment was not sufficient to maintain ''at harvest'' sucrose, glucose, and fructose levels (Irving and Joyce 1995; Downs and others 1997). Sugars have been implicated in regulating senescence, as they are needed to provide a carbon source to maintain high respiration rates in harvested immature tissues (Irving and Joyce 1995). Simple sugars have also been implicated as signal transduction molecules (Smeekens 2000), and they have been identified as key compounds in the regulation of senescence (Coupe and others 2003a).

The level or extent to which cytokinin, sucrose, and ethylene interact during senescence is not well understood. It was the aim of this work to examine the expression profiles of genes associated with senescence, ethylene biosynthesis, carbohydrate transport and metabolism after harvest in broccoli florets, and to develop a model showing how cytokinin, ethylene, and carbohydrates interact during harvest-induced senescence in broccoli. To do this, gene expression profiles were monitored in wild-type plants following postharvest feeding with water, sucrose, 1-aminocyclopropane-1-carboxylic acid (ACC), and/or 6-benzyl amino purine (6-BAP).

A number of genes encoding proteins involved with ethylene biosynthesis and perception have been isolated and characterized from broccoli (Gonzalez and Botella 2003; Kato and others 2002; Pogson and others 1995a, b; Shaw and others 2002; Wang and others 2002; Yang and others 2003). Some of these provided probes for expression changes in ethylene biosynthetic genes in response to different hormone treatments. In addition, a chlorophyll a and b binding protein cDNA (Bo*CAB1*) and a metallothionein-like protein cDNA (Bo*MT1*) were cloned from broccoli and used as molecular markers of senescence, alongside a senescence-associated cysteine protease (Bo*CP5*) (Eason and others 2005). Furthermore, putative sucrose transporters (Bo*SUC1* and Bo*SUC2*), acid invertase (Bo*INV1*) (Coupe and others 2003a), and hexokinase probes (Bo*HK1* and Bo*HK2*) were used for the assessment of carbohydrate transport and metabolism gene expression following hormone treatment.

Materials and Methods

Plant Material and Treatments

Broccoli (*Brassica oleracea* var. *italica* cv. Marathon) was harvested from a commercially grown crop located at Aokatere near Palmerston North, New Zealand. The heads were placed on ice and returned to the laboratory within 1.5 h of harvest. Branchlets were selected randomly and subjected to postharvest treatments: vase fed in the water-based treatments or non-treated and held in air. The water-based treatments included water, sucrose (2% w/v), ACC (1 mM), 6-BAP (2.21×10^{-4} M), and 6-BAP (2.21×10^{-4} M) + ACC (1 mM). Branchlets were treated continuously through cut ends during postharvest storage at 20 °C in the dark. Floret tissue, containing immature florets, pedicel tissue, and stemlet tissue (Gapper 2003) was shaved from the branchlets at critical times following harvest and stored at –80 °C for later analyses. Rate of sepal yellowing following harvest was monitored by measuring color by hue angle. A total of three branchlets were individually measured for hue angle then pooled as one sample for molecular analyses.

Table 1. cDNA Clones Used

Clone name	Description of clone	Accession number	Reference
Bo*INV1*	Broccoli acid invertase 1	AF274298	Coupe and others (2003a)
Bo*SUC1*	Broccoli sucrose transporter 1	AY065840	(Unpublished)
Bo*SUC2*	Broccoli sucrose transporter 2	AY065839	(Unpublished)
Bo*HK1*	Broccoli hexokinase 1	AF454961	(Unpublished)
Bo*HK2*	Broccoli hexokinase 2	AF454962	(Unpublished)
Bo*ACO1*	Broccoli ACC oxidase 1	X81628	Pogson and others (1995a)
Bo*ACO2*	Broccoli ACC oxidase 2	X81629	Pogson and others (1995a)
Bo*ACS1*	Broccoli ACC synthase 1	X82273	Pogson and others (1995b)
Bo*ACS2*	Broccoli ACC synthase 2	AF338651	Gonzalez & Bo*tella (2003)*
Bo*ACS3*	Broccoli ACC synthase 3	AF338652	Gonzalez & Bo*tella (2003)*
Bo*MT1*	Broccoli metallothionein-like protein 1	AF458412	(Unpublished)
Bo*CP5*	Broccoli cysteine protease 5	AF454960	Eason and others (2005)
Bo*CAB1*	Broccoli chlorophyll a/b binding protein 1	AF458406	(Unpublished)
Bo*18S*	Broccoli 18S ribosomal RNA gene	AF513990	(Coupe and others 2004)

ACC, 1-aminocyclopropane-1-carboxylic acid.

Sepal Yellowing

Floret color was measured non-destructively by reflectance as described by King and Morris (1994), using a chromameter (model II; Minolta, Osaka, Japan) with an 8-mm measuring head and D-65 (6504K) illuminant. Reflectance was measured as hue angle (180° = green, 90° = yellow) on equally spaced sites around the circumference of each branchlet.

Chlorophyll Determination

Chlorophyll concentrations were determined as described by Lichtentaler (1987). Ground broccoli tissue (100 mg) was accurately weighed and pigments were extracted in 1 ml methanol at 4 °C for at least 24 h. Absorbances of 10-fold diluted samples were measured at 665 and 652 nm. Concentrations of pigments (mg ml^{-1}) were calculated using the following equations: Chla = $16.72 \times A_{665} - 9.16 \times A_{652}$; Chlb = $34.09 \times A_{652} - 15.28 \times A_{665}$; Chla + b = $1.44 \times A_{665} + 24.93 \times A_{652}$.

Endoprotease Activity

Endoprotease activity was measured in broccoli floret tissue as previously described by Coupe and others (2003b). Proteases were extracted from ground floret tissue (100 mg FW) in phosphate buffer (1 ml) (100 mM sodium phosphate, 30 mM cysteine, 30 mM EDTA, pH 7.0) on ice for 2 h with periodic vortexing. After centrifugation (14,000 × *g* for 30 min at 4 °C), 0.2 ml of supernatant was incubated with an equal volume of 2% azo-casein for 20 h at 40 °C. Proteins were then precipitated by the addition of 0.6 ml 10% (v/v) trichloroacetic acid, and the absorbance of the resulting supernatant was measured at 440 nm in an ELISA plate reader. Protease activity was calculated by reference to a papain regression equation. One protease unit is defined as the amount of enzyme required to hydrolyze (and TCA solubilize) 1 mol of tyrosine equivalents min^{-1} from soluble casein under standard assay conditions (pH 7.0, 40 °C).

RNA Extraction and Northern Analyses

Total RNA was extracted from 0.5 g broccoli floret tissue, previously ground in liquid nitrogen, using TRIzol (Invitrogen) according to the manufacturer's instructions. Northern analysis was carried out as described by Coupe and others (2003b), although 10–40 μg total RNA was used. RNA was transferred in 10 × SSC to nylon membranes (Hybond N+ or Hybond XL, Amersham) by downward capillary transfer (Chomczynski 1992). After blotting, membranes were washed in 2 × SSC, and the RNA was fixed to membranes by UV cross-linking (Hoefer UVC 500 UV Cross Linker). Double-stranded DNA probes (Table 1) were prepared using the High Prime DNA Labelling Kit (Roche) according to the manufacturer's instructions. Membranes were bathed in hybridization solution (Church and Gilbert 1984) and hybridized with [^{32}P]dCTP-radiolabeled probes at 65 °C for 16 h. Membranes were washed for 20 min in 3 × SSC, 1% SDS (w/v);

20 min in 2 × SSC, 1% SDS (w/v); 20 min in 1 × SSC, 1% SDS (w/v); 20 min in 0.5 × SSC, 1% SDS (w/v); and 20 min in 0.1 × SSC, 1% SDS (w/v), at 65 °C. After washing, Kodak Biomax MR or MS film was exposed to membranes at –80 °C.

All northern blots were repeated at least twice, with similar results. All membranes were re-probed with a partial cDNA clone encoding a broccoli ribosomal RNA (Bo*18S*) to assess loading equality. All autoradiographs were scanned with a phosphorimager (FLA5100, Fuji Film), and transcript hybridization was measured using the software package Multi Gauge v 2.3 (Fuji Film) and then compared with the measured rRNA hybridization and plotted as relative intensity.

Gene Isolation

The cDNA library was constructed as previously described (Pogson and others 1995a). Differential screening was performed using single-stranded cDNA probes synthesized from 1 μg poly $(A)^+$ RNA, isolated from 0 h and 48 h total RNA, as previously described (Coupe and others 1993). Differentially hybridizing plaques were cored out and subjected to a second and third round of screening.

The cDNA library was also screened with a heterologous probe from *B. napus*. The cDNA, LSC54 (Buchannan-Wollaston 1994) encoded a leaf senescence–associated, metallothionein-like protein. In addition, a number of *Arabidopsis* cDNAs were used to heterologously screen the cDNA library (Table 1). The probes were hybridized as described previously for heterologous broccoli library probes (Coupe and others 2003b), and several strongly hybridizing plaques came through several rounds of screening and were sequenced.

DNA Sequencing

DNA sequencing was performed using the Applied Biosystems (Foster City, CA) Dye Primer Cycle Sequencing kits using the chain termination method (Sanger and others 1977) in conjunction with an Applied Biosystems 373A DNA Sequencer at the University of Waikato's DNA Sequencing Facility. The programs BLASTN and BLASTP (Altschul and others 1990) at the NCBI (Bethesda, MD) Web site were used to search the computer databases.

Statistical Analysis

Statistical analysis was carried out using the Genstat Fifth Edition software package (Lawes Agricultural Trust, Rothamsted Experimental Station, VSN International Ltd, UK). Least significant differences (LSD) were generated by fitting a mixed model statistical analysis using Genstats REML procedure.

Results

Identification of Genes Expressed During Postharvest Senescence of Broccoli

Differential screening of 150,000 plaques of the broccoli 48 h postharvest cDNA library (Pogson and others 1995a) led to the identification of two cDNAs that hybridized with radiolabeled 0 h mRNA but not with radiolabeled 48 h mRNA used to screen duplicate lifts. Seven cDNAs were isolated because they hybridized with the 48 h probes but not with the 0 h probes. One of the two mRNAs present in 0 h RNA, but not in 48 h RNA populations, was identified as a chlorophyll a/b binding protein (Bo*CAB1*) following sequence analysis. The predicted protein sequence of Bo*CAB1* aligned with 99% identity to CAB from *Sinapsis alba* (Gauly and others 1992).When the library was screened with a heterologous cDNA from *B. napus* (*LSC54*) for a metallothionein-like protein, five strongly hybridizing plaques were identified. All five plaques that hybridized with the LSC54 probe were identified to encode a metallothionein-like protein (Bo*MT1*) with an observed 100% identity to the *B. napus* LSC54 predicted protein. Two putative hexokinase cDNAs (Bo*HK1* and Bo*HK2*) were isolated that were 90% identical at their predicted amino acid level and 87% identical to their *Arabidopsis* orthologs.

Color Change and Protease Activity

Sepal yellowing, measured as hue angle during postharvest storage, is shown in Figure 1A. Initial hue angle readings at harvest were around 130 and declined to around 110 in the following four days (96 h) for non-treated branchlets held in air. All branchlets that underwent the water-based treatments maintained a high hue angle until 3 days (72 h) after harvest. At this point during storage, sepals from branchlets treated with water, sucrose, and ACC started to yellow, and by 5 days after harvest, these sepals were a similar color to sepals from non-treated branchlets. Sepals from branchlets treated with 6-BAP, either with or without added ACC, remained green (hue angle ∼ 130) throughout the 5-day (120 h) postharvest period.

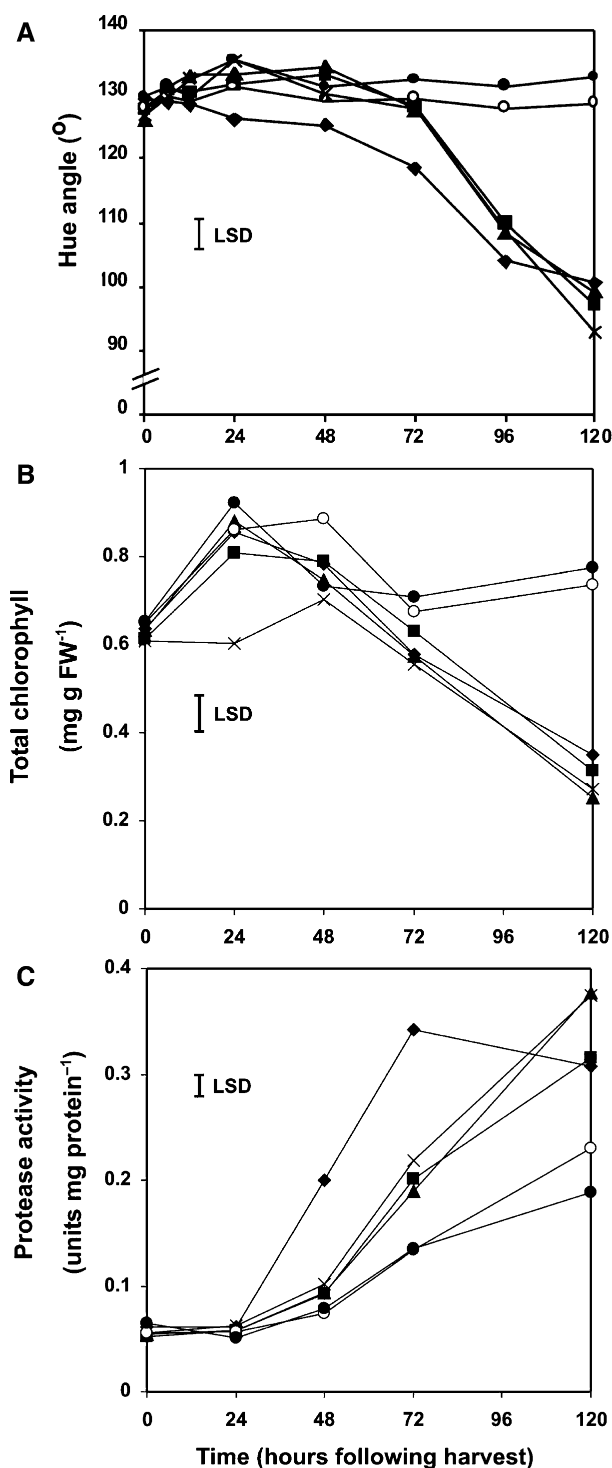

Figure 1. Biochemical changes of broccoli branchlets treated in air (♦), water (■), 6-BAP (2.21×10^{-4} M) (○), sucrose (2% w/v) (▲), ACC (1 mM) (×), 6-BAP (2.21×10^{-4} M) + ACC (1 mM) (●), and held at 20 °C in the dark following harvest. **A**. Hue angle. **B**. Total chlorophyll concentration, **C**. Endoprotease activity. A mixed-model statistical analysis was carried out for hue angle, total chlorophyll, and protease activity. Total chlorophyll data were log transformed to stabilize variance. Least significant differences (LSD) ($p = 0.05$) are indicated by single bars on all graphs. Each data point represents the mean of three individual measurements ($n = 3$).

As well as measuring the color of florets (as hue angle) in postharvest treated branchlets, chorophyll content was measured and the results are shown in Figure 1B. In general terms, the changes in chlorophyll mirrored the changes observed in color with a few subtle differences. There was an increase in total chlorophyll content in the 24 h following harvest for all florets except those treated with ACC. By 48 h after harvest, all florets had begun to lose chlorophyll, except those treated with 6-BAP alone. There was little difference at this time point in chlorophyll content for the other five treatments. From this point on during storage, floret chlorophyll levels continued to decline steadily for the 120 h of storage for all treatments, with the exception of those florets treated with 6-BAP. Even in the presence of ACC, florets treated with 6-BAP retained ''at harvest'' levels of chlorophyll until 120 h following harvest.

Protease activity was also measured as a biochemical marker of senescence (Figure 1C). Protease levels remained at basal levels in florets until 24 h for all treated branchlets. A marked increase in protease level occurred in florets from the non-treated air control branchlets, which continued until 72 h then leveled off out to 120 h postharvest. Protease activity increased in florets in all of the wet treatments after 48 h of storage, although both the rate and the final level of protease activity were lower in florets treated with 6-BAP, even in the presence of ACC. Florets from branchlets treated in water, sucrose, and ACC had protease activity levels at least as high as the non-treated florets maintained in air for 120 h following harvest.

Transcript Assay for Ethylene Biosynthetic Genes

Bo*ACO1* transcript levels were detected at low levels at harvest (Figure 2A). In florets from non-treated branchlets held in air, Bo*ACO1* transcript levels increased within the first 24 h of harvest, levelled off, but had increased again by 72 h. Bo*ACO1* levels were relatively high in florets at 24 h when treated with water alone, followed by a reduction at 48 h then an increase by 72 h.

Treatment with both sucrose and ACC resulted in a similar profile of Bo*ACO1* transcript as for air-

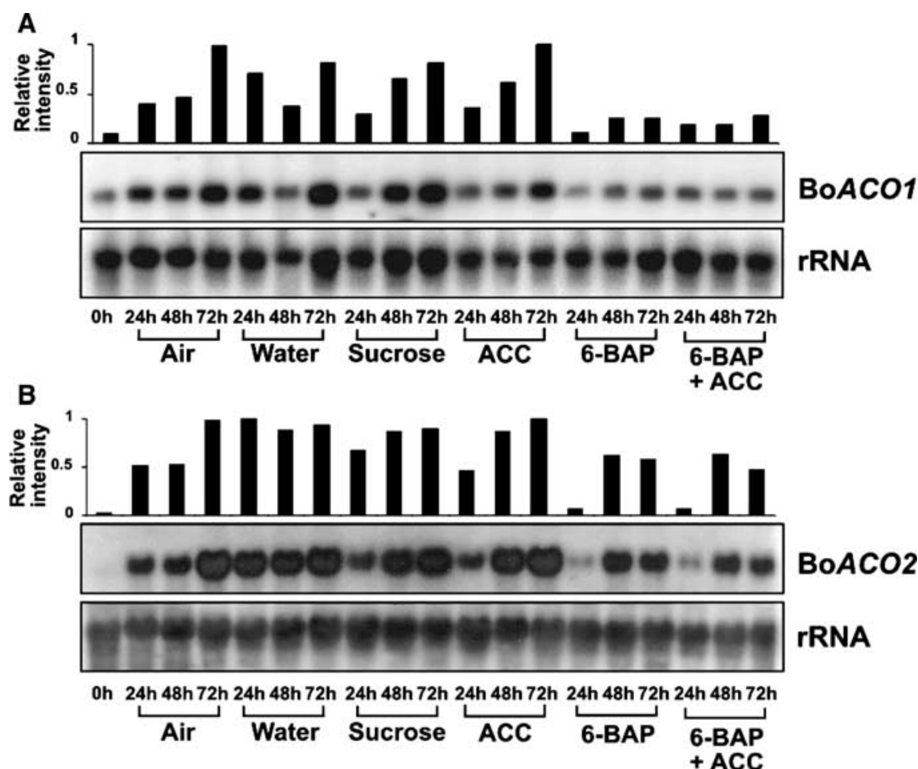

Figure 2. Northern hybridization of a 1.3-kb ^{32}P-labeled Bo*ACO1* cDNA fragment, a 1.3-kb ^{32}P-labeled Bo*ACO2* cDNA fragment, and a 400-bp ^{32}P-labeled rRNA (Bo*18S*) fragment, with total RNA from broccoli following harvest. ACC, 1-aminocyclopropane-1-carboxylic acid; 6-BAP, 6-benzyl aminopurine.

treated branchlets, although there was an increase at 48 h for both these treatments. Notably, exogenous 6-benzylaminopurine (6-BAP), even in the presence of added ACC, led to a reduction of Bo*ACO1* transcript accumulation in florets after harvest compared to all other treatments at 24, 48, and 72 h.

In contrast to Bo*ACO1*, Bo*ACO2* transcript was barely detectable at harvest (Figure 2B). In florets from non-treated branchlets held in air, Bo*ACO2* transcript levels increased at 24 h, leveled off at 48 h, and increased again by 72 h following harvest. As with Bo*ACO1*, a marked increase in Bo*ACO2* transcript levels at 24 h after harvest occurred in the water, compared to the air-treated, samples. This level was maintained to 72 h postharvest. The treatments with ACC and with sucrose had similar effects on Bo*ACO2* transcript levels, with a reduced level of transcript at 24 h compared to water, but subsequent levels were similar. However, exogenous 6-BAP, even in the presence of ACC, resulted in a reduction of Bo*ACO2* transcript accumulation compared to all other water-based treatments.

Bo*ACS1* transcript levels were undetectable at harvest and only visible 72 h following harvest in florets of non-treated broccoli material (Figure 3A). Transcript levels increased in florets when branchlets were treated with water 48 and 72 h following harvest compared to the non-treated branchlets held in air. Sucrose and ACC appeared to reduce the levels of Bo*ACS1* transcript after harvest in comparison to water-treated florets. Exogenously fed 6-BAP resulted in the greatest increase of Bo*ACS1* transcript, but not in the presence of ACC.

Partial length reverse transcriptase polymerase chain reaction (RT-PCR)-generated cDNAs for Bo*ACS2* and Bo*ACS3* (Gonzalez and Botella 2003) were used to further explore Bo*ACS* gene expression. Both Bo*ACS2* and Bo*ACS3* transcript levels increased in florets after harvest from non-treated branchlets (Figure 3B and 3C). The increase was reduced for all wet treatments, with the exception that Bo*ACS2* transcript levels increased in response to exogenous 6-BAP.

Transcript Assay for Senescence Marker Genes

Chlorophyll a/b binding protein (Bo*CAB1*) transcript levels in florets were high at harvest and declined rapidly following harvest for non-treated branchlets held in air (Figure 4A). Bo*CAB1* transcript levels were maintained to at-harvest levels in

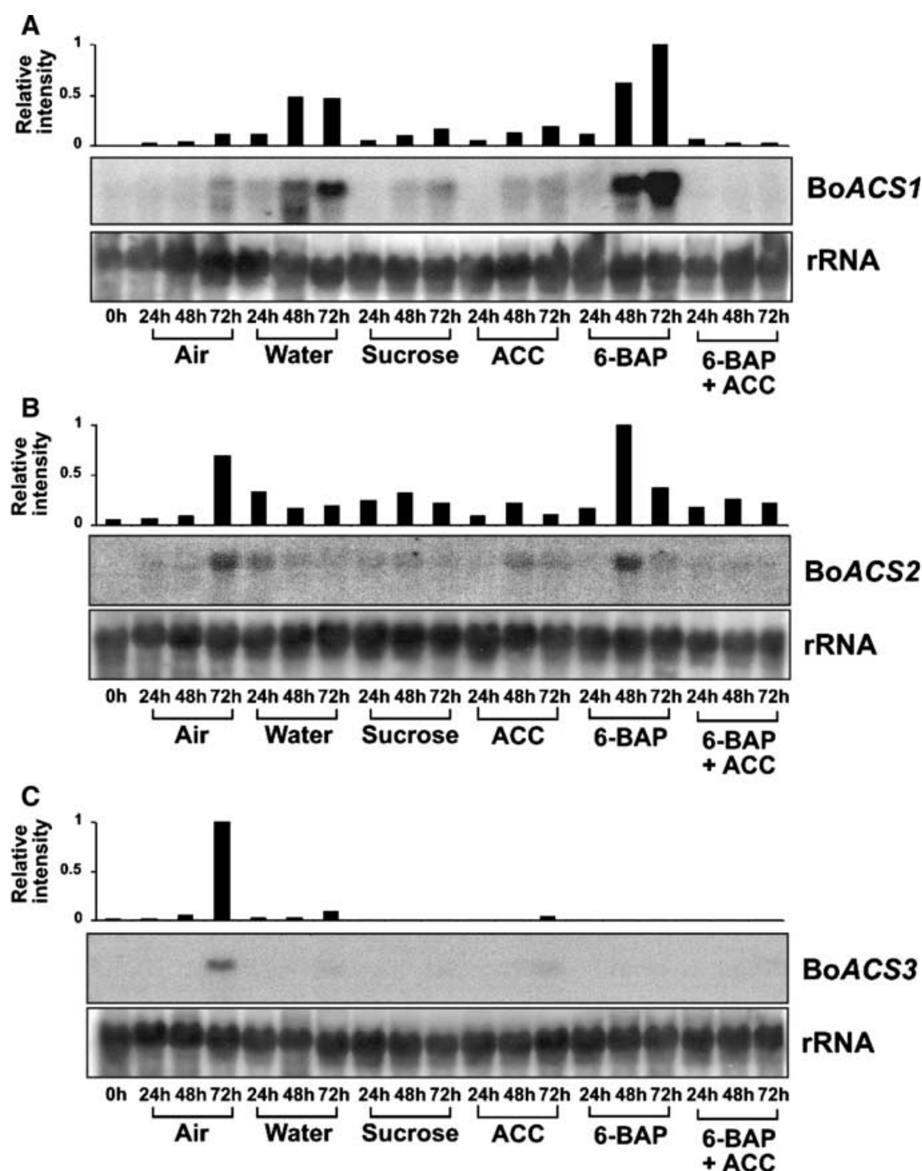

Figure 3. Northern hybridization of a 1.7-kb ^{32}P-labeled Bo*ACS1* cDNA fragment, a 1.1-kb ^{32}P-labeled Bo*ACS2* partial cDNA fragment, a 1.1-kb ^{32}P-labeled Bo*ACS3* partial cDNA fragment and a 400-bp ^{32}P-labeled rRNA (Bo*18S*) fragment, with total RNA from broccoli treated after harvest.

florets from branchlets treated with 6-BAP or ACC at 24 h following harvest. However, Bo*CAB1* transcript levels reduced subsequently for both 6-BAP treated samples, but the decline was less rapid when ACC was present.

Metallothionein-like protein (Bo*MT1*) transcript levels were undetectable in florets at harvest and increased steadily following harvest to peak at 72 h in air-treated branchlets (Figure 4B). Branchlets treated with water showed an increase in Bo*MT1* transcript levels in florets at 24 and 48 h compared to air-treated samples, but at 72 h levels were similar. Hormone-based treatments resulted in a reduction of Bo*MT1* transcript levels at 24 h following harvest compared to florets held in water. Bo*MT1* transcript levels in florets treated with 6-BAP were also reduced at 48 and 72 h following harvest compared to all other treatments, even in the presence of added ACC.

Cysteine protease (Bo*CP5*) transcript levels in florets were undetectable at harvest and increased within 24 h following harvest in florets from non-treated branchlets held in air (Figure 4C). This level steadily increased until 72 h postharvest. Treatment of branchlets with water caused an increase of Bo*CP5* transcript at 24 and 48 h compared to non-treated samples. Bo*CP5* transcript levels also increased to higher levels when treated with ACC

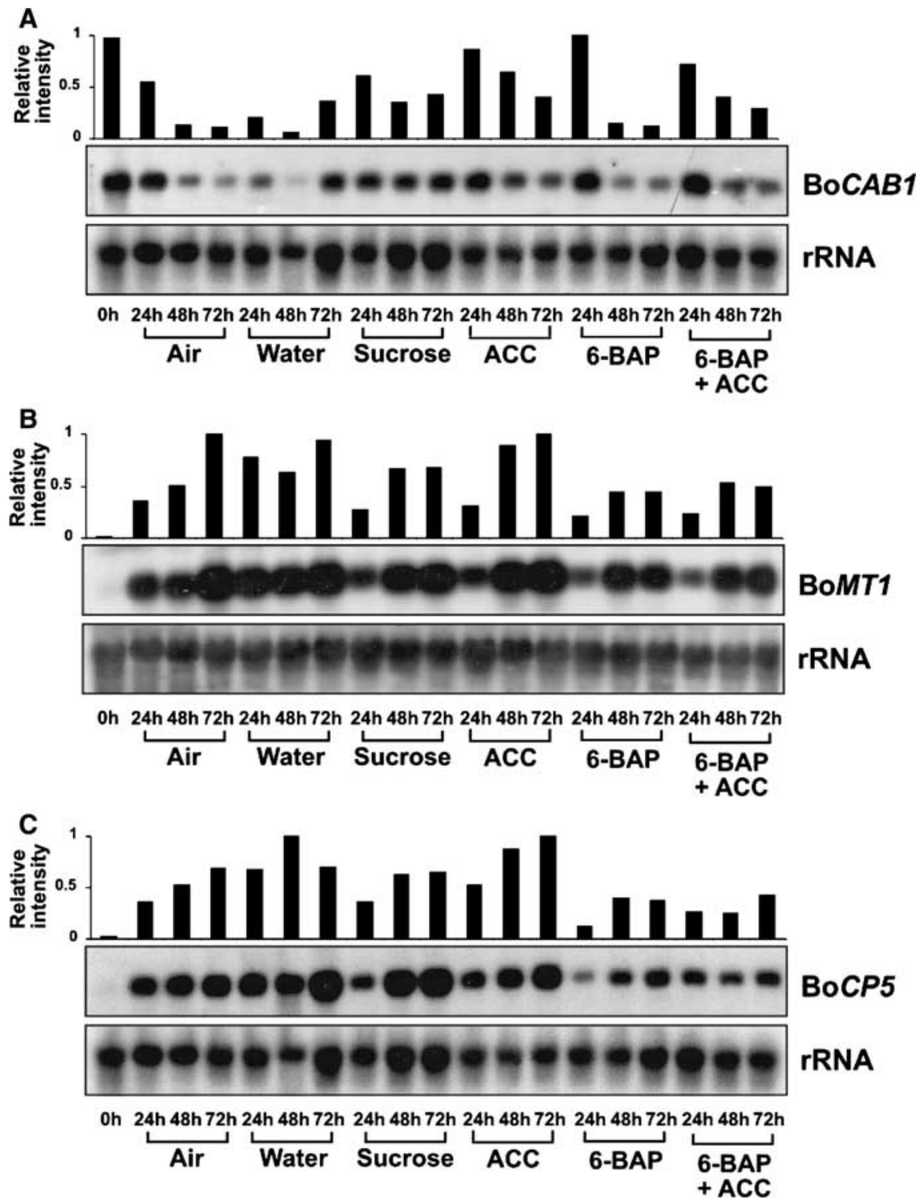

Figure 4. Northern hybridization of a 1.1-kb ^{32}P-labeled Bo*CAB1* cDNA fragment, a 500-bp ^{32}P-labeled Bo*MT1* cDNA fragment, a 900-bp ^{32}P-labeled Bo*CP5* cDNA fragment, and a 400-bp ^{32}P-labeled rRNA (*Bo18S*) fragment, with total RNA from broccoli treated after harvest.

than for non-treated air samples. Sucrose treatment caused a reduction in transcript compared to water and ACC. Even in the presence of ACC, Bo*CP5* transcript levels were lower when treated with 6-BAP than other treatments.

Transcript Assay for Sucrose Transport and Carbohydrate Metabolic Genes

Two full-length cDNAs for Bo*SUC1* and Bo*SUC2* were isolated by heterologous screening of the cDNA library (Coupe SA, Sinclair BK, Watson LM, Gapper NE, Pinkney TT, Eason JR, Greer LA and Heyes JA, unpublished data). The influence of exogenous feeding on the expression of sucrose transporter (Bo*SUC*) genes in broccoli florets following harvest is shown in Figure 5. Bo*SUC1* transcript was low at harvest. The level increased in the following 48 h before dropping 72 h after harvest (Figure 5A). Water and sucrose feeding caused transcript levels in florets to increase at 72 h compared to air-treated branchlets. Exogenous treatment with ACC and 6-BAP (both separately and in

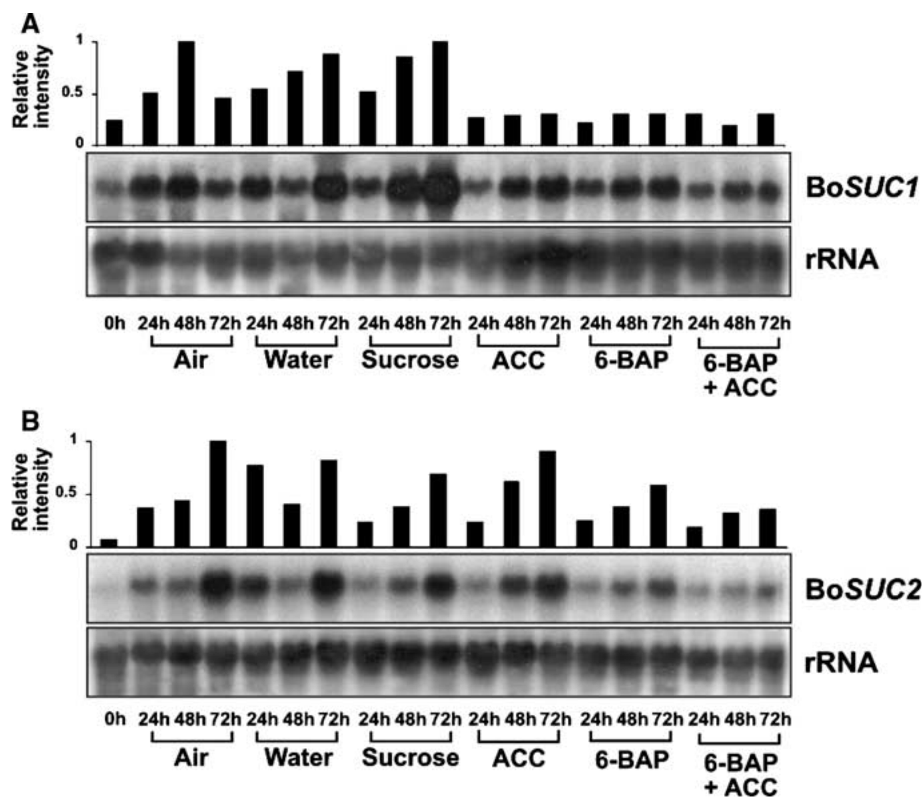

Figure 5. Northern hybridization of a 1.8-kb ^{32}P-labeled Bo*SUC1* cDNA fragment, a 1.7-kb ^{32}P-labeled Bo*SUC2* cDNA fragment, and a 400-bp ^{32}P-labeled rRNA (Bo*18S*) fragment with total RNA from broccoli treated after harvest.

combination) caused a reduction in Bo*SUC1* transcript levels in florets 24, 48, and 72 h after harvest compared to the other treatments. Bo*SUC2* levels were barely detectable at harvest, increasing in florets within 24 h and rising to high levels 72 h following harvest in non-treated branchlets held in air (Figure 5B). Most water-based treatments had a similar expression profile. However, water caused Bo*SUC2* transcript levels to be relatively high at 24 h compared to other treatments, and, even in the presence of ACC, Bo*SUC2* transcript levels were lower in florets treated with 6-BAP than in all other treatments, particularly 72 h postharvest.

Acid invertase (Bo*INV1*) transcript levels in florets were detectable at harvest and had significantly increased by 72 h in non-treated samples (Figure 6A). Otherwise, the most notable effect was that 6-BAP, even in the presence of ACC, caused greater reduction of Bo*INV1* transcript levels in florets after harvest compared to all other water-based treated samples.

The expression profiles of the putative hexokinase genes, Bo*HK1* and Bo*HK2*, were very similar in broccoli florets after harvest. However, hormone-induced changes were only characterized for Bo*HK1*. Bo*HK1* levels in florets were low at harvest and increased in the 72 h following in non-treated branchlets held in air (Figure 6B). This profile was similar for other treatments, with the exception, again, of 6-BAP. Bo*HK1* transcript levels were the lowest for samples treated with 6-BAP compared to all other treatments, even in the presence of added ACC.

Discussion

Bo*CAB1* encodes a putative chlorophyll a/b-binding precursor protein of 266 amino acids that is highly homologous to other published CAB genes of the *Brassicaceae* family (Gauly and others 1992). CAB proteins are major components of the light-harvesting photosystem of the chloroplast thylakoid membrane. These proteins bind chlorophyll and are the primary acceptors of light energy (McGrath and others 1992). Given that one of the first visual changes observed during broccoli postharvest senescence is loss of chlorophyll (Figure 1B), it is not surprising that the mRNA encoding the binding protein of this molecule was downregulated rapidly following harvest (Figure 4).

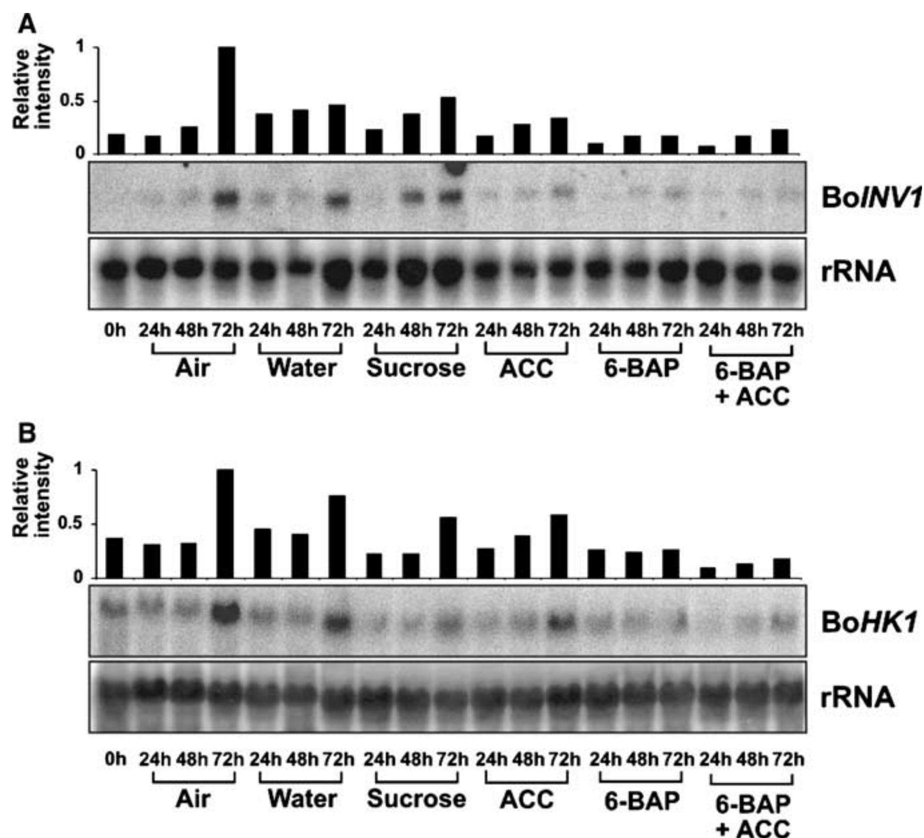

Figure 6. Northern hybridization of an 800-bp ^{32}P-labeled Bo*INV1* cDNA fragment, a 1.6-kb ^{32}P-labeled Bo*HK1* cDNA fragment, and a 400-bp ^{32}P-labeled rRNA (Bo*18S*) fragment with total RNA from broccoli treated after harvest.

Bo*MT1* encodes a putative metallothionein (MT)-like protein. This class of protein has been isolated previously from senescing leaves of *B. napus* (Buchannan-Wollaston 1994), from *B. oleracea* florets (Yang and others 2000), and during other developmental processes such as leaf abscission (Coupe and others 1995). Bo*MT1* is very similar at the nucleotide level and identical at the amino acid level to the *B. napus* MT-like protein, LSC54, used to isolate it. As expected, Bo*MT1* transcript levels increased following harvest; however, the function of these proteins during senescence remains to be fully explained. It is likely that the original suggestion of an antioxidant role is still appropriate (Buchannan-Wollaston 1994).

Bo*CP5* encodes a senescence-associated cysteine protease. The expression of Bo*CP5* was upregulated after harvest (Figure 4C) as shown recently by Eason and others (2005). Bo*CP5* is most closely related to At*SAG2*, a senescence-associated gene from *Arabidopsis thaliana* thought to encode a cysteine protease. Senescence-associated genes, or SAGs, are well characterized and often are used as molecular markers to assess senescence (Weaver and others 1998). Comparison of Bo*CAB1*, Bo*MT1*, and Bo*CP5* transcript levels provided suitable molecular markers of senescence for this study.

In agreement with the observations of Clarke and others (1994), exogenous application of 6-BAP caused a delay in sepal yellowing and chlorophyll loss, even in the presence of applied ACC (Figure 1). Furthermore, protease activity in florets was reduced when 6-BAP was exogenously fed. Applied 6-BAP also caused a reduction in the accumulation of transcripts normally upregulated during senescence (Bo*MT1*, a metallothionein-like protein; Bo*CP5*, a cysteine protease), and maintained transcript levels for chlorophyll a/b binding protein (Bo*CAB1*), which is normally reduced rapidly following harvest (Figure 4). Furthermore, even though Irving and Joyce (1995) showed that exogenous cytokinin was insufficient to prevent the depletion of carbohydrates in florets following harvest, exogenous cytokinin caused a reduction, during the later stages of postharvest storage, in the expression of genes involved with carbohydrate transport (Bo*SUC1* and Bo*SUC2*) and metabolism (Bo*INV1* and Bo*HK1*) (Figures 5 and 6, respec-

tively). This discrepancy may occur because transport of exogenously fed cytokinin is not sufficiently rapid through the branchlets to influence the very early changes in expression of genes involved in carbohydrate regulation during senescence. However, taken together, our observations implicate cytokinin as having a major role in regulating postharvest senescence in broccoli.

Genes encoding the enzymes responsible for the final stage of ethylene biosynthesis, namely Bo*ACO1* and Bo*ACO2*, were downregulated following exogenous treatment with cytokinin (Figure 2). These results led us to our initial hypothesis that cytokinin retards broccoli senescence by directly inhibiting ethylene biosynthesis. However, application of exogenous cytokinin caused an increase in the level of both Bo*ACS1* and Bo*ACS2* transcripts compared to all other wet treatments (Figure 3). As ACC synthase catalyzes what is generally regarded as the first dedicated and rate-limiting step of ethylene biosynthesis (Yang and Hoffman 1984; Kende 1993), 6-BAP appears to both negatively and positively regulate ethylene biosynthesis.

Ethylene biosynthesis has been reported to be regulated by both positive and negative feedback by ethylene itself. For example, in mung bean hypocotyls, exogenous ethylene increased ACC synthase transcript abundance, whereas ACC oxidase transcript abundance was reduced (Kim and others 2001). This feedback regulation by ethylene was reversed by the addition of the ethylene biosynthesis inhibitor aminooxyacetic acid (AOA). The application of 6-BAP also abolished ethylene responsiveness with respect to the expression of ACC oxidase and synthase genes (Kim and others 2001). Pogson and others (1995a) showed that the expression of a broccoli ACC oxidase gene (Bo*ACO2*) was positively regulated by ethylene during senescence in broccoli florets. It is possible that broccoli ACC synthase (Bo*ACS*) gene expression is under negative feedback control by ethylene during senescence, and that 6-BAP treatment nullifies the potential suppression by ethylene of Bo*ACS1* and Bo*ACS2* gene expression (Figure 3). Treatment with ACC, the precursor of ethylene, and with sucrose caused a reduction in Bo*ACS1* transcript abundance when compared to water treatment alone (Figure 3), and the effect of applied ACC was sufficient to override the effects of exogenous 6-BAP. It appears, then, that ACC might act as a negative regulator of ethylene biosynthesis at Bo*ACS* in broccoli florets during senescence.

It is clear from the work presented here that exogenous application of cytokinin directly or indirectly alters the expression of genes involved in ethylene biosynthesis. However, Clarke and others (1994) suggested that cytokinins exerted their inhibitory effect on senescence in broccoli by desensitizing the tissue to ethylene. In addition, Kim and others (2001) showed that exogenous application of 6-BAP also had an inhibitory effect on ethylene action, apart from the ability to inhibit ethylene biosynthesis. Furthermore, Hall and others (2001) suggested that cytokinins were involved in the early regulation of ethylene signal transduction. In pea epicotyls, treatment with ethylene led to the activation of monomeric GTP-binding proteins responsible for the activation of mitogen-activated protein kinase (MAPK) cascades (Novikova and others 1997). Also, the receptor-directed inhibitor 1-methylcyclopropene (MCP) antagonized the activation of these GTP-binding proteins. In leaves of *Arabidopsis*, a similar effect was shown by the application of cytokinin: GTP-binding protein activation was antagonized, and leaf senescence was delayed (Novikova and others 1999). One might then hypothesize that cytokinins regulate ethylene action at both the level of biosynthesis and the level of perception during senescence in broccoli. Recently, Chang and others (2003) ectopically expressed a bacterial *ipt* gene in petunia, decreasing corolla senescence and sensitivity to ethylene. Therefore, we suggest that 6-BAP may cause the effect on ethylene biosynthesis by inhibiting the feedback regulation of ethylene, rather than by direct action on the biosynthetic genes themselves (Figure 7). This, then, would support the hypothesis that cytokinin nullifies the feedback regulation of ethylene by desensitization of the receptors to the hormone (if ethylene is not perceived, ACS is upregulated and ACO is downregulated).

Broccoli is an immature tissue that has a very high respiratory rate (King and Morris 1994). Once harvested, this immature tissue, instead of functioning as a sink for carbon, becomes a source providing carbohydrate to maintain the high levels of respiration. Coupe and others (2003a) have shown that harvest triggers the upregulation of genes encoding enzymes involved in sucrose metabolism. Further, Irving and Joyce (1995) also concluded that sucrose supply could be a discrete senescence factor with an effect independent of its role as a respiratory substrate. Evidence is accumulating pointing toward simple carbohydrates being molecules involved with signal transduction (for review see Smeekens 2000). In contrast, exogenous cytokinin caused reduced expression of genes involved in carbohydrate transport (Bo*SUC1* and Bo*SUC2*) and metabolism (Bo*INV1* and Bo*HK1*) (Figures 5 and 6, respectively).

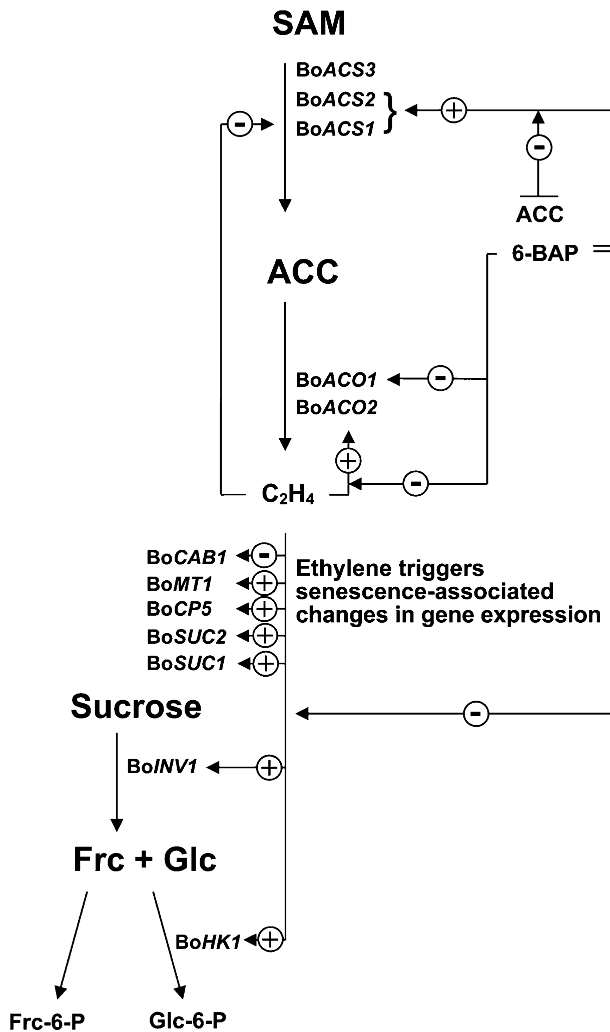

Figure 7. A schematic representation of the proposed regulation of ethylene biosynthesis and carbohydrate transport and metabolism by ethylene, ACC, and 6-BAP in broccoli florets following harvest. Ethylene induces the expression of Bo*ACO2* (Pogson and others 1995) and suppresses the expression of broccoli ACC synthase genes (Kim and others 2001). 6-BAP blocks the signaling pathway of ethylene, nullifying the induction of Bo*ACO2* gene expression by ethylene. 6-BAP also nullifies the suppression of Bo*ACS1* and Bo*ACS2* gene expression by ethylene, but this activation is reversed by ACC. 6-BAP also nullifies the trigger responsible for the downregulation of Bo*CAB1* and induction of Bo*MT1*, Bo*CP5*, Bo*SUC1*, Bo*SUC2*, Bo*INV1*, and Bo*HK1* gene expression following harvest. Arrow lines with plus or minus symbols represent the proposed signaling pathway. Plus and minus indicate the inductive and suppressive effects on the expression of genes, respectively.

In our proposed model we suggest that cytokinin has two key roles during senescence in broccoli: first, on nullifying the perception of ethylene and thus reducing the effect of ethylene on the upregulation of senescence-associated gene expression and, second, on carbohydrate transport and metabolism via nullification of ethylene perception. The effects of the ectopic expression of a cytokinin synthase gene in the immature floral organs on the retention of simple sugars in these tissues after harvest would be interesting. Comparison of such postharvest tissues with those from transgenic plants altered for reduced ethylene biosynthesis (Gapper and others 2002, 2005) or perception could provide a rigorous test for our proposed model.

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