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<RESEARCH ARTICLE>

Characterisation of Three ACC Synthase Gene Family Members during Post-Harvest-Induced Senescence in Broccoli (*Brassica oleracea* L. var. italica)

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We investigated the gene expression profiles of different members of the 1-aminocyclopropane-1-carboxilic acid (ACC) synthase (EC 4.4.1.14) gene family in broccoli (*Brassica oleracea* L. var. italica) during the post-harvest-induced senescence process. Using RT-PCR, three different cDNAs coding for ACC synthase (*BROCACS1*, *BROCACS2* and *BROCACS3*) were amplified from floret tissue at the start of the senescence process. The three genes share relatively little homology, but have highly homologous sequences in *Arabidopsis thaliana*, and could be functionally related to these counterparts. Southern analyses suggest that *BROCACS1* and *BROCACS3* are present as single copy genes, while there are probably two copies of *BROCACS2*. All three genes showed different expression patterns: *BROCACS1* is likely to be either wound - or mechanical stress-induced showing high transcript levels after harvesting, but no detectable expression afterwards. *BROCACS2* shows steady expression throughout senescence, increasing at the latest stages, and *BROCACS3* is almost undetectable until the final stages. Our results suggest that *BROCACS1* could be required to initiate the senescence process, while *BROCACS2* would be the main ACC synthase gene involved throughout the post-harvest-induced senescence. *BROCACS3's* expression pattern indicates that it is not directly involved in the initial stages of senescence, but in the final remobilization of cellular resources.

Keywords: ACC synthase, *Brassica oleracea*, broccoli, ethylene, post-harvest, senescence

Senescence, the final stage in the life of a plant (or a particular organ) was once erroneously considered to be an unregulated process arising from the uncontrolled breakdown of cell structures. We know now that senescence is a well programmed developmental stage, involving highly coordinated cellular events that require the sequential action of many genes (Smart, 1994; Buchanan-Wollaston, 1997). Nevertheless, the biochemical and molecular mechanisms governing senescence are not yet fully understood. There are two kinds of senescence processes, a natural one occurring at the end of the useful life of an organ, and a stress- or environmentally- induced senescence. A leaf will develop and reach full maturity performing a genetically programmed function in the plant. Once the leaf has reached the end of its functional life, it will remobilize its resources exporting almost anything that can be useful to the rest of the plant before shriveling and falling. This is natural senescence and it is also a genetically programmed process. On the other hand, harvesting plant tissue for commercialization causes a series of stresses in the detached tissue that will inexorably trigger senescence (Pogson and Morris, 1997). Once a plant is harvested, it starts a process of accelerated decay that results in the death of the organism, and the deterioration of its organic matter. Natural and induced senescence do not always share similar mechanisms.

Broccoli is a floral vegetable that exhibits rapid floral differentiation and development. Broccoli is a member of the brassica vegetable group, and belongs to the same plant species as cauliflower (var. botrytis), cabbage (var. capitata) and Brussels sprouts (var. gemmifera). Fresh broccoli has a very limited life span with heads yellowing and becoming unmarketable within 1 to 3 days when held at ambient temperature (Forney, 1995). Refrigeration is needed immediately after harvest and modified atmosphere storage is the

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxilic acid (ACC); SAM , S-adenosylmethionine

primary means of maintaining broccoli quality, however such practice is costly and sub-optimal conditions are frequently experienced. Substantial product loses are experienced as a result of senescence and moisture loss. Before biotechnological solutions to the post-harvest problem can be attempted, the molecular mechanisms controlling the senescence process in broccoli need to be characterized.

Ethylene has been reported to play an important role in the onset and development of senescence in floral tissues (Borokov and Woodson, 1989; Brandt and Woodson, 1992). Even though ethylene has been clearly linked to the senescence process, the molecular mechanisms leading to its synthesis are complicated. The two most important enzymes controlling ethylene biosynthesis, ACC synthase and ACC oxidase, are encoded by multigene families (Botella et al., 1992; Botella et al., 1993; Tang et al., 1993). The different members of each family are tightly regulated and subjected to complex control mechanisms. Some genes are exclusively expressed in very specific tissues during well defined developmental processes, such as fruit ripening, while others are induced by a large variety of stimuli such as wounding, biotic and abiotic stresses. In addition, several members of a family can also be activated at different times during the same process (Liang et al., 1992).

Broccoli is harvested while the floral structures are still immature and undergoing rapid growth, therefore the post-harvest-induced deterioration of the tissue is not a typical senescence process but is combined with a strong disruption of the metabolic pathways (King and Morris, 1994). The most visible sign of senescence in harvested broccoli florets is the yellowing of the head caused by a loss of chlorophyll (Wang, 1977). A role for ethylene in the yellowing of broccoli after harvest has been established with reproductive structures within the flowers making a major contribution to the ethylene production with strong increases in ACC oxidase activity and mRNA levels (Tian et al., 1994; Pogson et al., 1995a; Pogson et al., 1995b). The effect of ethylene has also been attributed to an increase in ethylene sensitivity in the floret after harvesting (Tian et al., 1994).

In broccoli, an ACC synthase gene has been previously cloned from detached senescing floret tissue but its expression was reported to be stationary during senescence (Pogson et al., 1995b). We report here the cloning and characterization of three different ACC synthase genes with markedly different behavior during the post-harvest-induced senescence process. The distinct behavior of each gene suggests that they play different roles in the process.

MATERIALS AND METHODS

Plant Material

Broccoli heads (cv. Pacific) were harvested and kept in the dark at 25°C before floret tissue was collected from each head 0, 4, 24, 48, 72, 96 and 120 h after harvest. The floret tissue was then snap-frozen in liquid nitrogen and stored at −80°C until required. The 0 h samples were frozen within 1-2 minutes of collection.

Total RNA Extraction from Broccoli Floret Tissue

Broccoli total RNA was extracted from floret tissue as described by Etheridge et al. (1999) with minor modifications. Tissue (0.8 g) was ground to a fine powder in liquid nitrogen, and shaken for 15 min in a mixture composed of 5 mL of 100 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 5 mM EDTA, 0.5% SDS (w/v), plus 0.7% β-mercaptoethanol (v/v) and 2.5 ml of phenol prewarmed to 60°C. An additional 2.5 ml of chloroform/isoamyl alcohol (24/1, v/v) was then added and the mixture shaken for 15 min. The mixture was centrifuged and the aqueous phase repeatedly extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) until no interphase was observed. The nucleic acids were recovered by ethanol precipitation, the pellet resuspended in 5 ml of water, and the RNA selectively precipitated by adding 1/3 volume of 8 M LiCl and incubating overnight at 4°C. After centrifugation at 15,000g for 30 min the RNA pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), ethanol precipitated, redissolved in 50 mL of water and spectrophotometrically quantified.

PCR Amplification of ACC Synthase cDNA

Total RNA (1 μg) extracted from broccoli floret tissue 24 h after harvest was reverse transcribed using 2.5 U of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase in a reaction mixture of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 1 U RNase inhibitor and 0.75 mM of oligo d $(T)_{16}$ as primer. The reverse transcription reaction was allowed to proceed for 15min at 42°C before heating at 99°C for 5 min. ACC synthase cDNA was amplified using three degenerate primers (EZ-2: 5'-TAYTTYGAYGGNTGGAARGC-3; EZ-4: 5'-TCRTCCATRTTNGCRAARCA-3 and EZ-5: 5'-CARATGGGNYTNGCNGARAA-3) coding for highly conserved regions of ACC synthase proteins. PCR reactions were carried out using 2 μL of the reverse transcription reaction and 5 units of *Taq* DNA poly-

merase (QIAGEN) in a 25 µL reaction mixture containing 10 mM Tris-HCl pH 8.7, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.75 mM of each primer. The primary amplification was performed using primers EZ-2 and EZ-4 and the PCR parameters consisted of an initial denaturation step at 94°C for 2 min, followed by 45 cycles of 30 s template denaturation at 94°C, 30 s primer annealing at 52°C and 70 s primer extension at 72°C plus a final extension step at 72°C for 7 min. The products of this PCR reaction were further reamplified using a set of semi-nested oligonucleotide primers (EZ-5 and EZ-4). After an initial denaturation step of 2 min at 94°C, the PCR parameters were 30 s template denaturation at 94°C, 30 s primer annealing at 52°C and 70 s primer extension at 72°C for 30 cycles. PCR products were ligated into pGEM®-T Easy vector and sequenced using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according the manufacturers instruction.

Northern Analysis

Total RNA (10 mg) extracted from broccoli floret tissue 0, 4, 24, 48, 72, 96 and 120 h after harvest was fractionated using 1% agarose in 1x TBE gel electrophoresis. RNA was transferred onto a nylon membrane by capillary action in 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), and then covalently linked to the membrane by UV cross-linking. Membranes were prehybridized in Church buffer (Church and Gilbert, 1984) at 65°C for 1h. Hybridizations were performed overnight at 65°C using 1×10^6 cpm/mL of radiolabeled probe. Hybridized filters were washed at room temperature in 2x SSC, 0.1% SDS for 30 min, in 2x SSC, 0.1% SDS at 55°C for 15 min, and then twice in 0.2x SSC, 0.1% SDS at 55°C for 15 min. The blot was then exposed to a Molecular Dynamics phosphoimager. Blots were stripped by boiling in 0.1% SDS and rehybridized to a wheat ribosomal probe (Gerlach and Bedbrook, 1979) to ensure that equal amounts of RNA were present in each lane.

Southern Analysis

After pelleting the RNA by LiCl precipitation in the RNA extraction procedure described above, genomic DNA was extracted from the supernatant by adding two volumes of ethanol and centrifuging at 15,000g for 60 min. The DNA was washed in 70% ethanol and resuspended in TE buffer. Genomic DNA (10 mg) was digested with *Eco*RI, *Bam*HI, *Eco*RV, *Hin*dIII or *Xho*I and then fractionated using 0.8% agarose, 0.5x TBE gel electrophoresis. DNA fragments were transferred to a nylon membrane as described above for northern analysis. Probe hybridization and washes were performed as per northern membranes.

RESULTS AND DISCUSSION

Amplification and Cloning of Three Members of the ACC Synthase Gene Family in Broccoli

In order to isolate the ACC synthase gene -or genes- involved in the process of post-harvest-induced senescence in broccoli we performed RT-PCR using degenerate primers (EZ-2, EZ-4 and EZ-5) coding for highly conserved regions of the ACC synthase protein family (Cazzonelli et al., 1998). Total RNA from senescing broccoli floral tissue, (24 h after harvest), was reverse transcribed and the resulting cDNA amplified using the EZ-2 and EZ-4 primers. The products of the first PCR reaction were further amplified using semi-nested primers (EZ-5 and EZ-4) obtaining a single amplification product of approximately 1.1 kb in size that was subsequently cloned. Analysis of more than 80 colonies showed the presence of three DNA species with different restriction patterns. Partial sequencing identified these clones as three different ACC synthase cDNAs, named *BROCACS1*, *BROCACS2* and *BROCACS3*. *BROCACS2* and *BROCACS3* are novel genes, (GenBank accession numbers AF338651 and AF338652 respectively), while *BROCACS1* has been previously reported in broccoli by Pogson et al., (1995b). Additional RT-PCR experiments performed in the 4 h and the 72 h samples did not result in the amplification of new ACC synthase clones.

The partial-length cDNAs *BROCACS1* and *BROCACS3* are 1109 bp long, which encode 369 amino acids, while *BROCACS2* is 1115 bp long encoding 371 amino acids (Fig. 1). These represent approximately 77% of the open reading frame of other ACC synthase cDNAs and include the active site of the protein where the highly conserved lysine residue plays a crucial role in the transformation of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxilic acid (Yip et al., 1990). Also present in all sequences are 11 of the 12 invariant amino acids involved in the binding of the substrate and the coenzyme pyridoxal-5-phosphate (Huang et al., 1991) (Fig. 1). These amino acid residues are highly conserved in group I aminotransferases, which include alanine-, tyrosine-, histidinol-phosphate-, phenylalanine-, and aspartate- aminotransferases (Tarun and

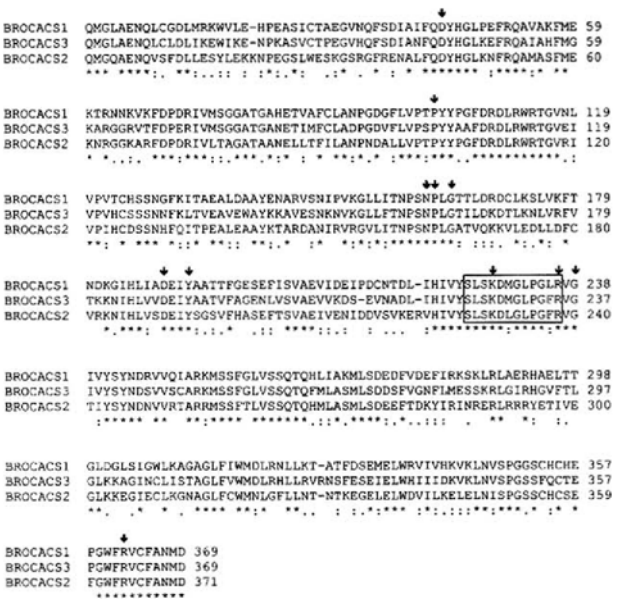

Figure 1. Multiple sequence alignment of BROCACS1, BROCACS2 and BROCACS3 proteins. The ACC synthase active site is boxed and the conserved amino acid residues found in aminotransferases are marked with an arrow. Identical residues in a column are marked with "*"; columns containing conservative amino acid substitutions are marked with ":"; semi-conserved substitutions are marked with ".". The alignment was performed using the ClustalW software located at the European Bioinformatics Institute (http://www.ebi.ac.uk/) using default parameters.

Theologis, 1997).

The level of homology among the three ACC synthase cDNAs and their encoded proteins is relatively low being 59% identity and 75% similarity between BROCACS1 and BROCACS2 proteins, 67% identity and 82% similarity between BROCACS1 and BROCACS3 and 58% identity and 75% similarity between BROCACS2 and BROCACS3 (Fig. 1). The *Brassica* genus is closely related to *Arabidopsis thaliana* as evidenced by an average of 87% sequence identity of homologous genes and an extensive conservation of synteny between large segments of the genomes (Cavell et al., 1998). It is therefore not surprising that all three broccoli genes are highly homologous to genes previously identified in *Arabidopsis*.

At the protein level BROCACS1 displays strong sequence homology to ATACS6 (Arteca and Arteca, 1999) with 93% identity and 96% similarity, while BROCACS3 shows highest homology to ATACS2 (Liang et al., 1992) with 88% identity and 93% similarity. BROCACS2 shows high homology to an ACC synthase-like protein first identified by the European Union *Arabidopsis* sequencing project with 90% identity and 92% similarity. This gene has not been characterized yet, but has been recently identified as a member of the ACC synthase family and named ATACS7 (GenBank accession # AF332390).

Southern analysis of broccoli genomic DNA using ^{32}P-labelled *BROCACS1*, *BROCACS2* or *BROCACS3*

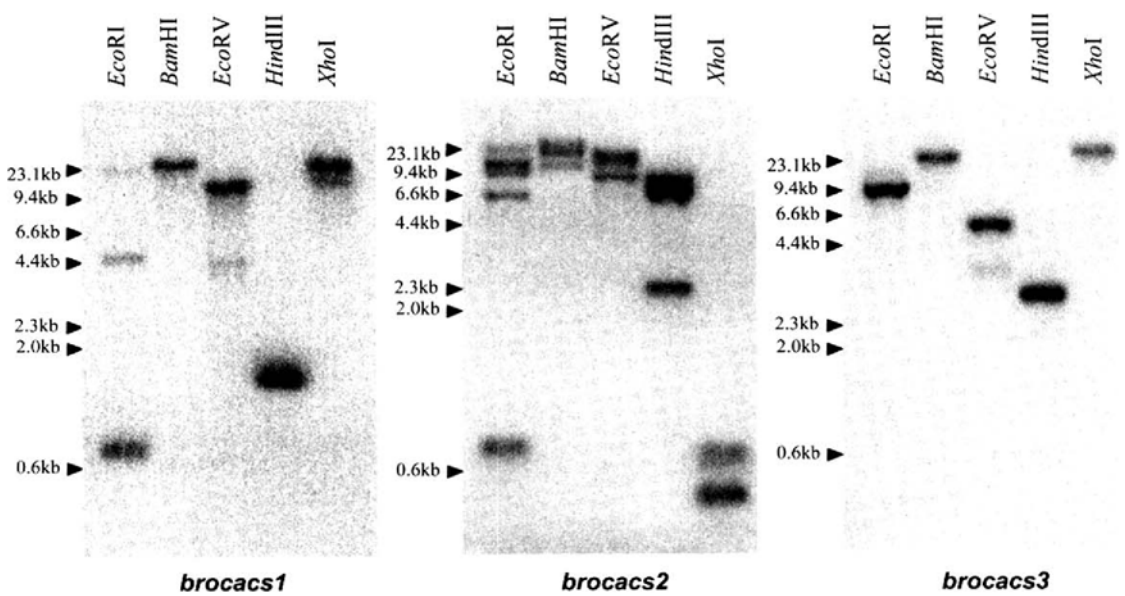

Figure 2. Southern analysis of *broacs1*, *broacs2* and *broacs3*. Broccoli genomic DNA (10 µg) was digested with *Eco*RI, *Bam*HI, *Eco*RV, *Hind*III or *Xho*I and the resulting fragments separated by electrophoresis. The gels were blotted onto nylon membranes and hybridized at high stringency with ^{32}P-labelled broacs1, broacs2 and broacs3 probes, respectively.

probes showed distinct hybridisation patterns confirming that each of the genes reside in different loci within the broccoli genome (Fig. 2). The restriction patterns observed in the Southern blots strongly suggest that *BROCACS1* and *BROCACS3* are single copy genes, while in the case of *BROCACS2* it appears that either two copies of the gene or highly homologous sequences are present in the broccoli genome.

Patterns of Gene Expression during Post-Harvest-Induced Senescence

Northern blots were carried out to determine the patterns of post-harvest gene expression for the three cloned ACC synthase genes. Tissues at various stages of senescence were collected (0, 4, 24, 48, 72, 96 and 120 h after harvest) and the transcript levels of each of the three genes determined. None of the three genes showed any detectable expression at harvest time but very distinct patterns of gene expression were observed during storage for all three genes (Fig. 3).

BROCACS1 was found to be highly expressed 4 h after harvest. Transcript levels were then observed to decrease sharply within 20 h and remained at very low, almost undetectable basal levels thereafter (Fig. 3). This pattern of gene expression follows very closely the peak production of ethylene usually observed 4 to 6 h after harvest (Tian et al., 1995; Pogson and Morris, 1997). *BROCACS1* has been previously identified in broccoli by Pogson et al. (1995b) who performed a preliminarily characterisation of the gene. In their study however, *BROCACS1* was referred to as *PBROC3* and its transcript abundance was reported to remain unchanged in floret tissue at 0, 24, 48 and 72 h after harvest. Comparable transcript levels in green and senescing leaves were also observed suggesting that the gene is not senescence-specific. The peak of *BROCACS1* expression detected in our study 4 h after harvest was missed by Pogson et al. (1995b) due to their sampling timing. The expression profile observed for *BROCACS1* could be a result of the rapid changes observed in broccoli after harvest including the loss of large amounts of sugars within 6 h of harvesting (King and Morris, 1994). Alternatively, the sharp induction observed in the *BROCACS1* transcript resembles the behaviour of other ACC synthase genes in response to mechanical stimulation (Botella et al., 1995). In our study, the broccoli heads were harvested and transported to our laboratory before freezing the tissues with the inevitable mechanical stimulation. *ATACS6*, the *Arabidopsis* homologue of *BROCACS1*, is highly inducible by mechanical stress (touch), and wounding (Arteca and Arteca, 1999).

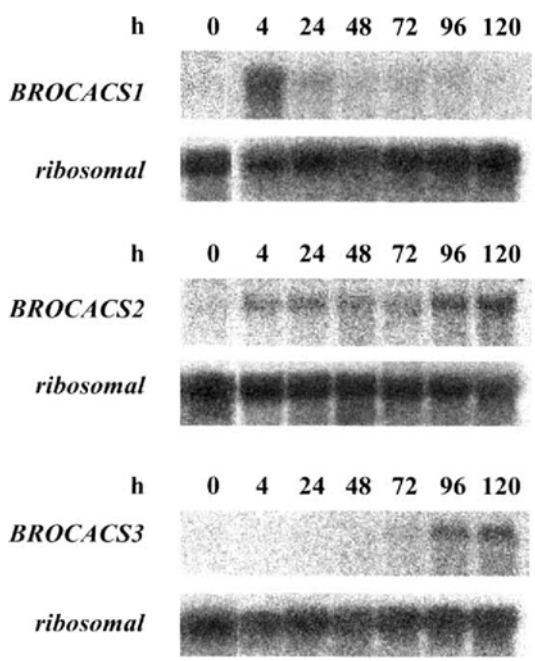

Figure 3. Expression profile analysis of *broacs1*, *broacs2* and *broacs3* during post-harvest-induced senescence of broccoli florets. Total RNA (10 µg) was isolated from broccoli floret tissues 0, 4, 24, 48, 72, 96 and 120 h after harvest. The RNA was electrophoresed, transferred to a nylon membrane and hybridized at high stringency with ^{32}P-labelled *broacs1*, *broacs2* and *broacs3* probes respectively.

The expression pattern observed for *BROCACS2* was quite different from *BROCACS1* with gene transcripts not present at harvest time but increasing 4 h after harvest and reaching stationary levels until 96 h in which a marked increase was again observed (Fig. 3). The broccoli florets used in this study showed some symptoms of yellowing 24 h after harvest with marked senescence at 48 h and almost complete yellow colour at 72 h. The expression pattern of *BROCACS2* resembles the visual assessment (yellowing due to chlorophyll degradation) of the senescence process and it is therefore likely that this gene plays an important role in the induction of the post-harvest senescence in broccoli. The sharp increase in expression observed at the late stages of senescence could be a by-product of the general tissue degradation and remobilisation of resources.

The levels of *BROCACS3* mRNA were almost undetectable until very late in the senescence process. It was only 96 h and 120 h after harvest, when the florets were showing clear symptoms of spoilage that high levels of gene expression were observed (Fig. 3).

Despite these high levels of expression it is very unlikely that *BROCACS3* plays any important role in the onset or development of senescence. It is probable that *BROCACS3* expression is induced by the production of endogenous ethylene. The *Arabidopsis* homologue of *BROCACS3*, *ATACS2*, has been reported to be induced by multiple factors, including ethylene exposure (Liang et al., 1992; Van der Straten et al., 1992).

A Possible Role for the Differentially Expressed ACC Synthase Genes during Post-Harvest-Induced Floral Senescence

Ethylene plays an important role in the onset and the regulation of the senescence process in many flowers such as carnations and petunias (Woodson et al., 1992; Tang et al., 1993). In floral vegetables such as broccoli, ethylene is therefore likely to play a similar role. It is nevertheless important to differentiate between the natural senescence process occurring in the flower while attached to the plant and the post-harvest-induced senescence that occurs after the floral tissue has been detached and stored. Some vegetables such as lettuce, broccoli, cauliflower and asparagus are harvested while they are still immature and undergoing a phase of rapid growth in the plant. In these vegetables there are very rapid changes after harvest (Irving and Hurst, 1993) with broccoli experiencing major losses of sugars, organic acids and proteins from floral, middle and base sections within the first 6 h after harvesting (King and Morris, 1994). Significant changes in gene expression are also observed with accumulation of different transcripts such as asparagine synthase that catalyses the synthesis of the amino acid asparagine (Downs et al., 1994). The metabolic parameters of immature vegetables after harvest undergo important changes with loss of proteins and lipids and accumulation of free amino acids and ammonia that ultimately lead to tissue breakdown. This data strongly resembles starvation responses and suggest that starvation might be a critical stress regulating the senescence process in harvested immature vegetables.

From our expression studies, it is clear that the three isolated ACC synthase genes have very different roles in the process of broccoli floral senescence after harvest. Changes in sensitivity to ethylene have been found to play an important role in broccoli yellowing (Tian et al., 1994). In addition, Payton et al. (1996) have shown that increased ethylene sensitivity during floral senescence is likely mediated by increased expression of ethylene receptors. Activation of ethylene receptor genes in response to ethylene has also been recently reported in tomato (Lashbrook et al., 1998). It is possible that the early and transient expression of *BROCACS1* is the trigger that initiates the senescence process by sensitising the harvested floral tissue resulting in the activation of early senescence genes, in particular those responsible for ethylene perception, thus kick-starting the whole senescence process.

The presence of *BROCACS2* throughout the entire senescence of the floret suggests that this gene is contributing towards the production of steady levels of ethylene. Tian et al., (1994) clearly demonstrated the importance of ethylene in the senescence of broccoli florets showing that higher levels of ethylene exposure lead to an increased rate of floral senescence, marked by accelerated sepal de-greening.

The possible involvement of *BROCACS3* in the senescence process is unclear. While *BROCACS3* may seem to be a senescence-specific gene expressed only during the late stages of floral senescence (when the floret is completely yellow and senescence is well underway), it most likely does not play an active role in the onset and control of senescence. As previously discussed, gene expression of *BROCACS3* during late senescence could be simply a by-product of the process due to the accumulation of toxic levels of ammonia in the final stages of tissue degradation.

An important consideration not explored in this research is the tissue specificity of the different ACC synthase genes. Two different broccoli ACC oxidase genes have been shown to be induced in the florets after harvest (Pogson et al., 1995a). Interestingly, the expression profiles of *accox1* and *accox2* have been found to be differentially regulated in a temporal and spatial pattern. *Accox1* is primarily expressed in sepals, while *accox2* is expressed exclusively within reproductive structures. It will be interesting to determine whether the ACC synthase genes behave in a similar way to the ACC oxidase genes being restricted to different parts of the flowers.

In view of our results, *BROCACS1* and *BROCACS2* appear to be the best ACC synthase targets for the biotechnological control of ethylene production in broccoli heads during post-harvest storage. ACC oxidase has already been targeted for down-regulation in broccoli by Henzi et al. (1999) who produced transgenic broccoli containing antisense copies of a tomato ACC oxidase gene. Preliminary agronomic evaluation has revealed some promising transgenic lines with significant improvements over the controls (Henzi et al., 2000).

It is clear from our results that there is a complex pattern of ACC synthase gene expression during the post-harvest-induced senescence of broccoli florets. The three cDNA shown to be active during this process in florets have highly homologous counterparts in *arabidopsis* showing the strong synteny between the two genomes and suggesting a conserved functional role of these genes in both plants. From a biotechnological perspective it is not clear whether the targeting of an individual gene will be effective in order to delay the onset of senescence in harvested broccoli florets and it seems possible that *BROCACS1* and *BROCACS2* might need to be simultaneously targeted.

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