# Ethylene biosynthetic genes in *Actinidia chinensis*

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# Abstract

*Actinidia chinensis*, a diploid relative of kiwifruit, has valuable fruit characteristics, and varieties with superior flavour and marketable size have recently been selected in a classical breeding programme. However, the marketability of the fruit of *A. chinensis* and many other species of *Actinidia* is limited by poor fruit storage properties. Pioneering work in tomato has demonstrated that fruit ripening and senescence can be very effectively delayed by down-regulating genes required for biosynthesis of the phytohormone ethylene. The goal of this work was to isolate genes for ethylene biosynthesis in *A. chinensis*, characterise their expression, and to generate transgenic plants containing T-DNA constructs designed for ethylene downregulation.

A small cDNA library was constructed from RNA isolated from the ripe fruit of *A. chinensis*. The library was screened for genes encoding each of the enzymes in the ethylene biosynthetic pathway, by probing with PCR products amplified from kiwifruit cDNA and with a cDNA clone previously isolated from kiwifruit. Three distinct cDNA clones encoding *S*-adenosyl-L-methionine (SAM) synthetase (ACSAM1, ACSAM2 and ACSAM3) were isolated from the library, together with two distinct 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase cDNA clones (ACAO1 and ACAO2). No ACC synthase cDNAs were detected in the library, indicating low transcript abundance. However, a partial ACC synthase cDNA (ACAS1) was amplified from ripe fruit using PCR techniques, and subsequently cloned in a plasmid vector. Phylogenetic analysis of SAM synthetase protein sequences from *A. chinensis* and other plant species indicates bifurcation of angiosperm SAM synthetase sequences into two main branches; ACSAM3 was assigned to a different branch from ACSAM1 and ACSAM2. The peptide sequence of ACAS1 shows higher homology to several auxin-inducible ACC synthase peptides than the product of the ethylene-inducible ACC synthase gene which is predominantly transcribed in ripening tomato fruit.

RNAse protection assays were employed to estimate the relative transcript levels of each of the ethylene biosynthetic genes isolated from *A. chinensis* during ethylene-induced, post-harvest fruit ripening, and in immature fruit and floral samples. The response of the mature fruit to exogenous ethylene indicated a clear separation of ethylene sensitivity and ethylene production in *A. chinensis*. The application of exogenous ethylene correlated with increased transcript levels for all three SAM synthetase genes (ACSAM1, ACSAM2 and ACSAM3) and for the ACC oxidase gene family. Transcription of the ACC synthase gene ACAS1 was not affected by exogenous ethylene, but transcript levels increased during subsequent ethylene biosynthesis, consistent with this being a controlling step for the onset of ethylene production. One or more ACC oxidase transcripts increased significantly both prior to and during ethylene production. Only one of the SAM synthetase transcripts (ACSAM3) was induced during the late ethylene burst, and these transcripts were also

abundant in floral tissues and young fruit. A role for SAM synthetase genes in the methionine salvage pathway is discussed.

The expression patterns for ACAS1 and the ACC oxidase gene family are consistent with the consensus view that the rate of ethylene biosynthesis in plant tissues is dependent on both ACC synthase and ACC oxidase activity levels. Therefore, with the aim of down-regulating ethylene biosynthesis in *A. chinensis*, expression cassettes containing ACAS1 and ACAO1 cDNAs, each controlled by a d35S promoter, were inserted in tandem into the *Agrobacterium* binary vector pCGN1549, in both the sense and antisense orientations. Leaf tissue from the 'Earligold' variety of *A. chinensis* was transformed with the resulting binary vectors, and transgenic plants were regenerated. PCR and Southern analysis indicated intact T-DNAs were integrated in at least half of the transformed plants, and Northern analysis detected mRNAs from one of the transgenes transcribed from both the sense and antisense constructs. No decrease in wound-induced ethylene biosynthesis was detected in the leaves of a small sample of these transgenic plants, and a larger number of transformants are now being grown for phenotypic screening. Down-regulation of ethylene biosynthesis may improve the storage properties and/or the shelf life of transgenic *A. chinensis* plants and may provide insights into the roles of ethylene in fruit ripening.

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# Abbreviations

| | |
|---|---|
| $^0$C | degrees Celsius |
| 35S | CaMV 35S promoter |
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| ADP | adenosine diphosphate |
| AOA | aminooxyacetic acid |
| ATP | adenosine triphosphate |
| AVG | aminoethoxyvinylglycine |
| bp | base pairs |
| BRL | Bethesda Research Laboratories |
| CaMV | cauliflower mosaic virus |
| cDNA | complementary DNA |
| CIP | calf intestinal phosphatase |
| cfu | colony forming units |
| cpm | counts per minute |
| CTAB | cetyl-trimethylammonium bromide |
| cv. | cultivar |
| d35S | modified CaMV 35S promoter |
| dATP | 2'-deoxyadenosine triphosphate |
| dCTP | 2'-deoxycytadine triphosphate |
| dGTP | 2'-deoxyguanosine triphosphate |
| DMDC | dimethyl-dicarbonate |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dNTP | unspecified 2'-deoxynucleotide triphosphate(s) |
| DTT | dithiothreitol |
| dTTP | 2'-deoxythymadine triphosphate |
| EDTA | ethylene diamine tetra-acetic acid |
| EtBr | ethidium bromide |
| *g* | acceleration of gravity |
| h | hour(s) |
| IBA | indole-3-butyric acid |
| IPTG | isopropyl-β-D-thiogalactoside |
| kb | kilobase(s) |
| kDa | kilodaltons |
| KMB | 2-keto-4-methylthiobutyric acid |
| MACC | malonyl ACC [1-(malonylamino)cyclopropane-1-carboxylic acid] |

| | |
|---|---|
| min | minute(s) |
| MOPS | 3-(N-morpholino) propanesulfonic acid |
| mRNA | messenger RNA |
| MTA | 5'-methylthioadenosine |
| MTR | 5'-methylthioribose |
| MTR-1-P | 5'-methylthioribose-1-phosphate |
| N-terminal | amino terminal |
| NAA | α-naphthalene acetic acid |
| NaOAc | sodium acetate |
| *nptII* | neomycin phosphotransferase |
| PAUP | phylogenetic analysis using parsimony |
| PCR | polymerase chain reaction |
| PE | pectin methylesterase |
| PEG | polyethylene glycol |
| PG | polygalacturonase |
| $P_i$ | inorganic phosphate |
| $PP_i$ | pyrophosphate |
| ppm | parts per million |
| rATP | adenosine triphosphate |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| SAM | *S*-adenosyl-L-methionine |
| SAMase | *S*-adenosyl-L-methionine hydrolase |
| SDS | sodium dodecyl sulphate |
| T-DNA | transferred DNA |
| TEMED | NNN'N'-tetramethylethylenediamine |
| Tris | 2-amino-2-hydroxymethylpropane-1,3-diol |
| USA | United States of America |
| UTP | uridine-5'-triphosphate |
| UV | ultraviolet |
| UWGCG | University of Wisconsin Genetics Computer Group |
| v/v | volume per volume |
| var. | variety |
| X-Gal | 5-bromo-4-chloro-3-indolyl-β-D-thiogalactoside |
| XET | xyloglucan endotransglycosylase |
| YN | yeast nutrient |