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Purified proteins, recombinant DNA sequences and processes for controlling the ripening of coffee plants

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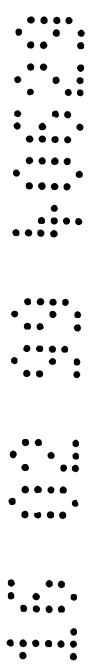
(54) Title: PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND PROCESSES FOR CONTROLLING THE RIPENING
OF COFFEE PLANTS

(57) Abstract

Purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith for transforming coffee plants to suppress the expression of enzymes necessary for ethylene synthesis. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for the enzymes ACC synthase or ACC oxidase that are elements of the pathway for ethylene biosynthesis in coffee plants. Coffee plants are transformed with vectors containing ACC synthase and/or with ACC oxidase DNA sequences that code on expression for the respective mRNA that is antisense to the mRNA for ACC synthase and/or ACC oxidase. The resulting antisense mRNA binds to XMI mRNA, thereby inactivating the mRNA encoding one or more enzymes in the pathway for ethylene synthesis. The described DNA sequences can also be used to block synthesis of ACC synthase or ACC oxidase using co-suppression. The result in either event is that the transformed plants are incapable of synthesizing ethylene, though other aspects of their metabolism is not affected.

ABSTRACT

The invention provides purified proteins, DNA sequences that code on expression therefore and recombinant DNA molecules, including hosts transformed therewith
5 for transforming coffee plants to suppress the expression of enzymes necessary for ethylene synthesis. The DNA sequences and recombinant DNA molecules are characterized in that they code on expression for the enzymes ACC synthase or ACC oxidase that are elements of the pathway for ethylene biosynthesis in coffee plants. Coffee plants are transformed with vectors containing ACC
10 synthase and/or with ACC oxidase DNA sequences that code on expression for the respective mRNA that is antisense to the mRNA for ACC synthase and/or ACC oxidase. The resulting antisense mRNA binds to the respective ACC synthase and/or ACC oxidase mRNA, thereby inactivating the mRNA encoding one or more enzymes in the pathway for ethylene synthesis. The described DNA
15 sequences can also be used to block synthesis of ACC synthase or ACC oxidase using co-suppression. The result in either event is that the transformed plants are incapable of synthesizing ethylene, though other aspects of their metabolism is not affected.



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PURIFIED PROTEINS, RECOMBINANT DNA SEQUENCES AND
PROCESSES FOR CONTROLLING THE RIPENING OF
COFFEE PLANTS

5 FIELD OF THE INVENTION

This application relates to purified proteins,
recombinant DNA sequences, hosts transformed
therewith and processes for controlling the ripening
of coffee plants. More particularly, this
10 application relates to purified proteins, and
recombinant DNA sequences that can be used to
suppress the expression of coffee fruit-specific 1-
aminocyclopropane-1-carboxylic acid (ACC) synthase
and ACC oxidase genes. This application further
15 relates to coffee plants transformed with such
sequences, thereby rendered incapable of
synthesizing ethylene necessary for ripening.
Application of exogenous ethylene to plants
transformed in accordance with this invention makes
20 it possible to synchronize and control fruit
ripening in coffee plants.

BACKGROUND OF THE INVENTION

Coffee is prepared from the roasted beans of
the plants of the genus *Coffea*, generally from the
25 species *C. arabica*. Beans are the seeds of the
coffee plant and are obtained by processing the
fruit, most ideally mature fruit which commands the

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best price due to its superior quality. In the past, high quality "gourmet" coffee was hand picked. This is necessary because the fruits of a coffee tree do not ripen uniformly and thus there are both
5 mature and immature fruit on the same tree. In the past, this was not a serious problem as most coffee is grown in areas of the world where labor is plentiful and not expensive. However, more recently lack of abundant and inexpensive labor has become a
10 major contributor to decreased productivity in coffee production. To increase productivity some regions of the world, such as the largest coffee producing country, Brazil, have resorted to strip harvesting where workers rapidly remove all fruit
15 from a branch whether ripe or unripe. This increases the speed of harvesting but decreases the yield of the highest quality beans as much of the fruit is immature (green).

Furthermore, the lack of uniform ripening has
20 seriously limited the effectiveness of mechanical harvesting. The force required to remove mature fruit (cherry) from the tree is similar to the force required to remove green fruit. Thus, mechanical harvesters do not distinguish well between green and
25 cherry and a large amount of immature fruit is harvested along with mature fruit. This greatly

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decreases the yield of mature fruit and limits productivity. If coffee fruit ripening could be controlled so that all fruit ripened at one time, both the strip method of hand harvesting and
5 mechanical harvesting would be much more efficient and a higher percentage of the harvested fruit would be in the higher quality grades. This would increase profitability of coffee production.

As is the case with many other fruit [Yang and
10 Hoffman, Ann. Rev. Plant Physiol. 35:155 (1984)], plant-produced ethylene plays an important role in the final stages of fruit ripening in coffee. Once coffee fruit reach a certain stage of maturity they can be induced to ripen by the exogenous application
15 of ethylene [Crisosto, C.H., P.C. Tausend, M.A. Nagao, L.H. Fuchigami and T.H.H. Chen, J. Haw. Pac. Agri. 3:13-17 (1991)]. This demonstrates the importance of ethylene for the final stages of fruit ripening in coffee.

20 Ethylene is synthesized in a two-step reaction from S-adenosylmethionine (SAM). The first step is the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM by ACC synthase. In most plants this is the rate limiting step. The final step is
25 the conversion of ACC to ethylene which is catalyzed by ACC oxidase (Yang and Hoffman, *supra*).

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Inhibition of ethylene biosynthesis by chemical (e.g., silver ions or carbon dioxide) or biotechnological means [Oeller et al., Science 254:437 (1991)] inhibits the final stages of ripening. This inhibition is reversible by the application of ethylene.

Accordingly, a strategy for controlling the ripening of coffee plants is to prevent synthesis of specific enzymes in the pathway for ethylene biosynthesis. In one embodiment this invention relates to genetic alteration of coffee plants to eliminate synthesis of ACC synthase; in another, ACC oxidase synthesis is suppressed. In the presently preferred embodiments, synthesis of one or both of these enzymes is suppressed by transforming coffee plants with a DNA sequence that codes on transcription for a messenger RNA (mRNA) that is antisense to the mRNA that codes on expression for the enzyme whose synthesis is to be suppressed. See Oeller et al., Science 254:437 (1991), who reported controlling ripening of tomatoes using a similar strategy.

Recombinant DNA technology has been used to isolate a number of ACC synthase and ACC oxidase genes. However, the genes for ACC synthase and ACC

oxidase in coffee have not been identified or sequence to date.

SUMMARY OF THE INVENTION

The present invention provides a substantially pure ACC synthase from a coffee plant comprising the amino acid sequence: (SEQ ID NO:10)

Also provided by the present invention is a substantially pure nucleic acid sequence that codes on expression for an ACC synthase produced by a coffee plant and comprising the nucleotide sequence set forth in (SEQ ID NO:11).

The present invention also provides a substantially pure nucleic acid sequence that codes on expression for an ACC synthase produced by a coffee plant, wherein the ACC synthase has the amino acid sequence (SEQ ID NO:10)

The present invention also provides a substantially pure ACC oxidase from a coffee plant comprising the amino acid sequence: (SEQ ID NO:12).

The present invention also provides a substantially pure nucleic acid sequence that codes on expression for an ACC oxidase produced by a coffee plant and comprising the nucleotide sequence set forth in (SEQ ID NO:13).

The present invention also provides a substantially pure nucleic acid sequence that codes on expression for an ACC oxidase produced by a coffee plant, wherein the ACC oxidase has the amino acid sequence; (SEQ ID NO:12).

The present invention also provides a coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10), wherein the RNA has a length sufficient to interfere with the expression of the ACC synthase.

The present invention also provides a coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12), wherein the RNA has a length sufficient to interfere with the expression of the ACC oxidase.



The present invention also provides a coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence (SEQ ID NO:10), wherein the RNA has a length sufficient to interfere with the expression of the ACC synthase.

The present invention also provides a coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12), wherein the RNA has a length sufficient to interfere with the expression of the ACC oxidase.

The present invention also provides a coffee plant transformed with an isolated nucleic acid sequence that codes on expression for the amino acid sequence; (SEQ ID NO: 10).

The present invention also provides a coffee plant of claim 19, wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation.

The present invention also provides a coffee plant transformed with a first isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10), and a second isolated nucleic acid comprising the nucleotide sequence that codes on expression for the amino acid sequence (SEQ ID NO:12).

The present invention also provides a transformed coffee plant produced by the process of inserting into the plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is antisense to the mRNA that codes on expression of an ACC synthase having the amino acid sequence (SEQ ID NO:10).

The present invention also provides a transformed coffee plant produced by the process of inserting into a plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription of RNA that is antisense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12).



The present invention also provides a transformed coffee plant produced by the process of inserting into the plant genome (i) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10), and (ii) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12).

The present invention also provides a transformed coffee plant produced by the process of inserting into the plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence; (SEQ ID NO:10).

The present invention also provides a transformed coffee plant produced by the process of inserting into the plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12).

The present invention also provides a transformed coffee plant produced by the process of inserting into the plant genome (i) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10), and (ii) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence; (SEQ ID NO:12).

The present invention also provides a transforming vector comprising a transcription promoter operably linked to:

- (a) the nucleic acid sequence: (SEQ ID NO:11); or
- (b) a nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10); or
- (c) the nucleic acid sequence: (SEQ ID NO:13); or
- (d) a nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).

The present invention also provides a coffee plant cell transformed with an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10).

5 The present invention also provides a coffee plant cell transformed with an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12)

The present invention also provides a coffee plant cell transformed with (i) an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10), and (ii) an isolated nucleic acid sequence that
10 codes on expression for the amino acid sequence: (SEQ ID NO:12).

The present invention also provides a transformed coffee plant cell produced by the process of inserting a transforming vector into a coffee plant cell, wherein the transforming vector comprises a transcription promoter operably linked to (i) an isolated nucleic acid sequence that codes on
15 expression of the amino acid sequence: (SEQ ID NO:10), or (ii) an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).

The present invention also provides a transformed coffee plant cell produced by the process of inserting a first transforming vector and a second
20 transforming vector into a coffee plant cell, wherein the first transforming vector comprises a transcription promoter operably linked to an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10), and the second transforming vector comprises a transcription promoter operably linked to an isolated nucleic acid sequence that codes on expression
25 for the amino acid sequence: (SEQ ID NO:12).

The present invention also provides a method for transforming a coffee plant cell with a nucleic acid sequence that codes on transcription for an RNA that is antisense to the mRNA that codes for an ACC synthase from a coffee plant, comprising the steps of:

30 Providing a transforming vector comprising a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC synthase having the amino acid sequence: (SEQ ID NO:10), wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation; and



Inserting the transforming vector into a coffee plant cell, wherein the nucleic acid sequence thereafter becomes inserted into the genome of the coffee plant cell.

The present invention also provides a method for transforming a coffee
5 plant cell with a nucleic acid sequence that codes on transcription for an RNA that is antisense to the mRNA that codes for an ACC oxidase from a coffee plant, comprising the steps of:

Providing a transforming vector comprising a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a
10 length sufficient to interfere with the expression of an ACC oxidase having the amino acid sequence: (SEQ ID NO:12);

Wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation; and

Inserting the transforming vector into a coffee plant cell, wherein the
15 nucleic acid sequence thereafter becomes inserted into the genome of the coffee plant cell.

The present invention also provides a method for transforming a coffee
plant cell with a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC synthase from a
20 coffee plant, comprising the steps of:

Providing a transforming vector comprising a transcription promoter operably linked to a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC synthase having the amino acid sequence: (SEQ ID
25 NO:10), wherein the nucleic acid sequence is operably linked to the promoter in a sense orientation; and

Inserting the transforming vector into a coffee plant cell, wherein the nucleic acid sequence thereafter become inserted into the genome of the coffee plant cell.

30 The present invention also provides a method for transforming a coffee plant cell with a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC oxidase from a coffee plant, comprising the steps of:



Providing a transforming vector comprising a transcription promoter operably linked to a nucleic acid sequence from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC oxidase having the amino acid sequence: (SEQ ID NO:12), wherein the nucleic acid sequence is operably linked to the transcription promoter in a sense orientation; and

Inserting the transforming vector into a coffee plant cell, wherein the nucleic acid sequence thereafter becomes inserted into the genome of the coffee plant cell.

The present invention also provides a method for transforming a coffee plant cell with (i) a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC synthase from a coffee plant, and (ii) a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC oxidase from a coffee plant, comprising the steps of:

Providing a first transforming vector comprising a first transcription promoter operably linked to a first nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC synthase having the amino acid sequence: (SEQ ID NO:10);

Providing a second transforming vector comprising a second transcription promoter operably linked to a second nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC oxidase having the amino acid sequence: (SEQ ID NO:12);

Wherein at least one of the nucleic acid sequences is operably linked to its transcription promoter in a sense orientation; and

Inserting each of the transforming vectors into a coffee plant, wherein each of the nucleic acid sequences thereafter becomes inserted into the genome of the coffee plant cell.



The invention provides purified proteins, DNA sequences that code on
expression therefore and recombinant DNA molecules,
including hosts transformed therewith, for
5 transforming coffee plants to suppress the
expression of enzymes necessary for ethylene
synthesis. The DNA sequences and recombinant DNA
molecules are characterized in that they code on
10 expression for the enzymes ACC synthase or ACC
oxidase that are elements of the pathway for
ethylene biosynthesis in coffee plants.

Coffee plants are transformed with vectors
containing ACC synthase and/or with ACC oxidase DNA
15 sequences inserted so that the transforming
sequences code on expression for the respective RNA
that is antisense to the mRNA for ACC synthase
and/or ACC oxidase. The resulting antisense RNA
20 binds to mRNA(s), thereby inactivating the mRNA
encoding one or more enzymes in the pathway for
ethylene synthesis. The described DNA sequences can
also be used to block synthesis of ACC synthase or
ACC oxidase using co-suppression. The result in
25 either event is that the transformed plants are

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incapable of synthesizing ethylene, though other aspects of their metabolism is not affected.

Ripening in the transformed plants can be regulated by exogenous ethylene. By application of ethylene to the entire plant, the entire plant will ripen at once, making mechanical harvesting of coffee more productive.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

SUMMARY OF THE DRAWINGS

FIGURE 1 is the complete sequence of the cDNA encoding coffee fruit expressed ACC synthase.

FIGURE 2 is the amino acid sequence of the coffee fruit ACC synthase deduced from the cDNA sequence shown in FIGURE 1.

FIGURE 3 is the sequence of the cDNA encoding coffee fruit expressed ACC oxidase.

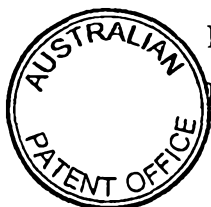
FIGURE 4 is the amino acid sequence of the coffee fruit ACC oxidase deduced from the cDNA sequence shown in FIGURE 3.

Figure 5 is a restriction map of coffee ACC synthase antisense cDNA in pKR1.

Figure 6 is a restriction map of coffee ACC synthase sense cDNA in pKR1.

Figure 7 is a restriction map of coffee ACC oxidase antisense cDNA in pKR1.

Figure 8 is a restriction map of coffee ACC oxidase sense cDNA in pKR1.



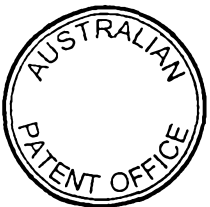
DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described
may be more fully understood, the following detailed
5 description is set forth. In the description the
following terms are employed:

Nucleotide -- A monomeric unit of DNA or RNA
consisting of a sugar moiety (pentose), a phosphate,
and a nitrogenous heterocyclic base. The base is

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linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four RNA bases are A, G, C, and uracil ("U").

DNA Sequence -- A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon -- A DNA sequence of three nucleotides (a triplet) which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, the nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal, which also encodes the amino acid methionine ("MET").

Polypeptide -- A linear array of amino acids connected one to the other by peptide bonds between the amino and carboxy groups of adjacent amino acids.

Genome -- The entire DNA of a cell or a virus. It includes inter alia the structural gene coding for the polypeptides of the substance, as well as

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promoter, transcription and translation initiation and termination sites.

Gene -- A DNA sequence which encodes through its template or messenger RNA ("mRNA") a sequence of amino acids characteristic of a specific polypeptide.

Transcription -- The process of producing mRNA from a gene or DNA sequence.

Translation -- The process of producing a polypeptide from mRNA.

Expression -- The process undergone by a gene or DNA sequence to produce a polypeptide. It is a combination of transcription and translation.

Plasmid -- A nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (TETR) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A cell transformed by a plasmid is called a "transformant."

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Phage or Bacteriophage -- Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

Cloning Vehicle -- A plasmid, phage DNA, cosmid
5 or other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological
10 function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites and which contain a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance. A
15 cloning vehicle is often called a vector.

Cloning -- The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

20 Recombinant DNA Molecule or Hybrid DNA - A molecule consisting of segments of DNA from different genomes which have been joined end-to-end outside of living cells and able to be maintained in living cells.

25 cDNA - A DNA strand complementary to an mRNA that codes for a particular polypeptide.

The strategy for controlling ethylene biosynthesis in coffee plants according to the present invention relates in the first instance to determination of the genes that code on expression
5 for two enzymes in the ethylene pathway: ACC synthase and ACC oxidase. Transformation of wild type coffee plants with constructs containing either or both genes in an orientation that is antisense to the normal genes is expected to block synthesis of
10 the respective enzymes. Messenger RNA transcribed under direction from the transforming sequence will bind to mRNA transcribed under direction from the normal sequence, thereby inactivating the normal message and precluding enzyme synthesis.

15 To isolate the DNA sequences that code on expression for ACC synthase and ACC oxidase in coffee, we screened a cDNA library produced from coffee plant tissue with synthetic DNA probes containing nucleotide sequences expected to occur.
20 These expected sequences were based on studies of nucleotide sequences that occur in genes that encode the respective enzymes, other climacteric plants and other plants.

In the present invention the cDNA corresponding to the gene encoding ACC
25 synthase or ACC oxidase is used to transform embryonic coffee plants. The plasmid pBI-121 is



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used as a transforming vector. The sequences corresponding to DNA that codes on expression for ACC synthase or ACC oxidase is inserted into the plasmid in an inverted orientation adjacent to a
5 cauliflower mosaic virus 35S promoter. RNA transcribed therefrom will be complementary to mRNA that encodes the amino acid sequence of the respective enzyme. Complete constructs are amplified in bacterial hosts. The hosts are
10 disrupted and the amplified vector is attached to colloidal gold particles. The gold particles with adherent vectors are inserted into coffee plant tissue by propelling the particles at high speed at the cells as described in U.S. patent 5,107,065.
15 Young plants successfully transformed are identified by antibiotic resistance. The transformed plants do not produce ACC synthase or ACC oxidase, depending on the gene used to transform the plants. Ripening of the transformed plants is initiated by
20 application of exogenous ethylene.

EXAMPLE 1**Isolation of Coffee Fruit-Specific ACC Synthase cDNA**

In order to isolate ACC synthase gene sequences involved in the ripening of coffee, a cDNA library
25 was prepared from a mixture of coffee fruit pericarp and mesocarp tissue at different stages of ripeness.

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This library was screened using a PCR product synthesized from first-strand cDNA made from the same mRNA used to construct the library and degenerate oligonucleotide primers corresponding to
5 consensus sequences derived from ACC synthase genes from other organisms. This example principally involved the isolation of mRNA, the construction of a cDNA library, and the subsequent steps involved in cloning the appropriate cDNA.

10 a) Isolation of mRNA

Total RNA was isolated from 66 g of pericarp and mesocarp tissue from several different developmental stages of coffee fruit (*C. arabica* L. cv Guatemalan) using the method of Levi et al.,
15 [Hort Science 27(12):1316-1318 (1992)']. Frozen coffee fruit pericarp and mesocarp tissue was powdered by grinding for about 2 minutes in a domestic coffee mill (Salton Model GC-5; Salton Maxam Housewares Group, Mt. Prospect, IL) with a
20 small piece of dry ice. The powdered fruit tissue was added to 200 μ L of 200 mM tris[hydroxymethyl]aminomethane hydrochloride (tris-HCl) (pH 8.5), 1.5% sodium dodecyl sulfate (SDS), 300 mM LiCl, 10 mM disodium
25 ethylenediaminetetraacetic acid (Na_2EDTA), 1.5% sodium deoxycholate (w:v), 1.5% Nonidet P-40 (Sigma

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Chemical Co.) (v:v), 0.5 mM thiourea, 1 mM
aurintricarboxylic acid, 10 mM dithiothreitol (DTT),
75 mM B-mercaptoethanol, 2% polyvinylpyrrolidone
(PVP) and 2% polyvinylpoly-pyrrolidone (PVPP) and
5 homogenized using a Polytron tissue homogenizer
(Tekmar, Cincinnati, OH). After 2 minutes of
homogenization, 200 μ L of chloroform was added and
homogenization continued for a further 3 minutes.
The homogenate was transferred to 250 μ L centrifuge
10 bottles (Nalgene) and centrifuged for 15 minutes at
2,500 x g. The upper aqueous phase was removed and
mixed with 12 μ L of 5 M NaCl, equally divided into
two centrifuge bottles, and 150 μ L of ethanol was
added to each bottle. The mixture was stored at -
15 20°C overnight. The RNA was collected by
centrifugation at 4,000 x g for 15 minutes at 4°C.
The RNA was dissolved in 50 μ L TE1 (50 mM tris-HCL
[pH 8.0], 10 mM Na₂EDTA) and clarified by
centrifugation at 12,000 x g for 10 minutes at 4°C.
20 The supernatant was transferred to a new centrifuge
bottle and 3 μ L of 5 M NaCl and 30 μ L of isopropanol
were added. The contents were mixed and stored at -
20°C overnight. The RNA was collected by
centrifugation at 14,000 x g for 10 minutes. The
25 RNA was washed with 20 μ L of 70% ice-cold ethanol
and collected by centrifugation as before. After

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drying under vacuum for 10 minutes, the RNA was resuspended in 50 μ L of TE1 buffer and 10 μ L of 12 M LiCl was added. The solution was incubated at 4°C for 48 hours and the RNA was collected by

5 centrifugation at 14,000 x g for 10 minutes and resuspended in 30 μ L TE1 buffer. After the addition of 15 μ L of 5 M potassium acetate, the RNA was incubated overnight at 0°C, recovered by

centrifugation at 14,000 x g for 10 minutes and

10 suspended in 50 μ L TE1 buffer. Three μ L of 5 M NaCl and 110 μ L of 95% ethanol were added and the RNA was incubated at -20°C overnight. The RNA was recovered by centrifugation at 14,000 x g for 10 minutes, washed with 20 μ L of 70% ice-cold ethanol, recovered

15 by centrifugation as above, dried under vacuum for 10 minutes and resuspended in 600 μ L of TE1 buffer. The RNA was transferred into a microcentrifuge tube and centrifuged at 14,000 rpm for 30 minutes at 4°C after which 300 μ L was removed to each of two new

20 microcentrifuge tubes. The original centrifuged tube was rinsed with an additional 300 μ L of TE1 buffer. Eighteen μ L of 5 M NaCl and 636 μ L of 100% ethanol were added to each of the three tubes. After mixing by inverting, the tubes were stored

25 overnight at -20°C. The RNA was collected by centrifugation at 14,000 rpm for 30 minutes and

-15-

washed with 1 μ L of 70% ice-cold ethanol. After centrifugation and drying as above, the RNA was resuspended in 400 μ L sterile H₂O. A total of 1.04 mg total RNA was obtained.

5 Messenger RNA (polyA⁺ RNA) was isolated using the PolyATtract[®] mRNA Isolation System IV (Promega Corporation, Madison, WI). A total of two isolations were done as follows. For each isolation, 0.48 mg total RNA was dissolved in 800 μ L
10 of RNase-free water. After heating at 65°C for 10 minutes, 3 μ L of 50 pmole/mL biotinylated oligo(dT) and 20.7 μ L of 20 X SSC (1 X SSC contains 150 mM NaCl and 15 mM sodium citrate) were added and the mixture was allowed to slowly cool to room
15 temperature over a period of approximately 30 minutes. An aliquot of streptavidin paramagnetic particles (provided in the PolyATtrack[®] mRNA Isolation System IV) was washed 3 times in 0.5 X SSC and resuspended in 0.1 mL of 0.5 X SSC. The RNA
20 solution containing the biotinylated oligo(dT) was added to the washed streptavidin paramagnetic particles. After a 10 minute incubation at room temperature, the paramagnetic particles containing the trapped mRNA were captured to the side of the
25 tube using a magnet.

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The supernatant was removed and the particles were washed four times with 0.3 mL of 0.1 X SSC. The mRNA was removed from the biotinylated oligo(dT) particles by suspending in 200 μ L RNase-free water.

5 An additional elution was carried out by adding 150 μ L of water sequentially to each of the two tubes. The elution fractions (550 μ L) were pooled and centrifuged at 14,000 rpm in a microcentrifuge for 30 minutes at 4°C. The supernatant was divided into

10 two microcentrifuge tubes and, after the addition of 1/10th volume of 3 M NaCl and 600 μ L of ethanol, the mRNA was recovered by incubating the tubes at -20°C overnight, followed by centrifugation as above. The mRNA was washed once with 1 mL of ice-cold 70%

15 ethanol, dried and resuspended in 20 μ L sterile H₂O. One μ L was added to 1 mL of water and a spectrum was obtained from 230 nm through 330 nm in a Shimadzu UV 160U spectrophotometer. Approximately 6 μ g of mRNA was recovered from 1.04 mg of total RNA.

20 b) Construction of a cDNA Library

First and second strand cDNA was synthesized using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). Six micrograms of mRNA in 20 μ L of water were incubated at 65°C for 5 minutes. Two

25 microliters of 100 mM methyl mercury were added and incubation was continued at room temperature for 10

-17-

minutes. Four microliters of 700 mM β -mercaptoethanol were added and the incubation was continued for an additional 5 minutes. To the denatured mRNA, 5 μ L of 10 X first strand buffer

5 (provided in the kit), 5 μ L of 100 mM DTT, 3 μ L nucleotide mixture (10 mM each dATP, dGTP, dTTP and 5-methyl-dCTP), 2 μ L of 1.4 μ g/ μ L linker-primer:

5'-GAGAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTTT-3'

10 (SEQ. ID NO. 1)

1 μ L RNase block and 5 μ L of water were added. The reaction was incubated at room temperature for 10 minutes to anneal the primer to the mRNA and then 3

15 μ L of 20 U/ μ L M-MuLV reverse transcriptase were added. Five microliters of this reaction mixture were removed to a tube containing 0.5 μ L (0.625 pmoles) of 800 Ci/mmol [α -³²P]dATP. Both reactions were incubated at 37°C for 1 hour. The

20 radioactively labeled reaction was frozen at -20° C for later gel analysis. To the 45 μ L main reaction, 40 μ L of second strand buffer, 15 μ L of 100 mM DTT, 6 μ L of nucleotide mixture (10 mM dATP, dGTP, dTTP and 26 mM dCTP), 268.3 μ L water and 2 μ L (2.5

25 pmoles) of 800 Ci/mmol [α -³²P]dATP were added. After mixing, 4.5 μ L of 1 U/ μ L RNase H and 19.2 μ L of 5.2 U/ μ L *E. coli* DNA polymerase I were added and the reaction was incubated at 16° C for 2.5 hours. The

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reaction was extracted with 400 μ L of phenol:chloroform (1:1). The phases were separated by centrifugation in a microcentrifuge for 5 min and the aqueous phase removed and re-extracted with 5 chloroform. The aqueous phase was recovered by centrifugation as before.

The double-stranded cDNA was precipitated by the addition of 33.3 μ L of 3M sodium acetate (pH 5.2) and 867 μ L of 100% ethanol and incubation 10 overnight at -20°C. The cDNA was recovered by centrifugation at 14,000 X g in a microcentrifuge at 4°C for 60 minutes. The cDNA was washed with 1 mL of 80% ethanol, recovered by centrifugation at room temperature in a microcentrifuge at 14,000 X g, 15 dried under vacuum and dissolved in 45 μ L of water. Three microliters of the resuspended double-stranded cDNA was removed and stored at -20°C for later analysis by gel electrophoresis.

To the remaining 42 ML of the double-stranded 20 cDNA, 5 μ L of 10 X Klenow buffer (buffer #3; supplied by Stratagene), 2.5 μ L of 2.5 mM nucleotides (dCTP, dGTP, dATP and dTTP), and 0.5 μ L of 5 U/ μ L *E. coli* DNA polymerase I Klenow fragment were added. After 30 minutes at 37°C, 50 μ L of 25 water were added and the reaction was extracted with an equal volume of phenol:chloroform (1:1) and then

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chloroform as described above. After the addition of 7 μ L of 3M sodium acetate (pH 5.2) and 226 μ L of 100% ethanol, the blunt-ended double-stranded cDNA was incubated on ice for 30 minutes and recovered by centrifuging at 14,000 rpm at 4°C for 60 minutes in a microcentrifuge. The cDNA was washed with 300 μ L of 70% ethanol, centrifuged and dried as before. Seven microliters of 0.4 μ g/ μ L EcoRI linkers were added to the dried cDNA. The structure of the EcoRI linkers are:

5' -AATTCGGCACGAG-3' (SEQ. ID NO. 2)

3' -GCCGTGCTC-5'

After vortexing to resuspend the cDNA, 1 μ L of 10 X ligation buffer, 1 μ L 10 mM ATP and 1 μ L of 4 Weiss U/ μ L T4 DNA ligase were added and the reaction was incubated over night at 8°C. The ligase was inactivated by heating at 70°C for 30 minutes. The 5' ends of the EcoRI linkers, that are now attached to the cDNA, were phosphorylated using polynucleotide kinase. One microliter of 10 X buffer #3 of the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA), 2 μ L of 10 mM ATP, 6 μ L of water and 1 μ L of 10 U/ μ L T4 polynucleotide kinase were added to the ligation reaction. After 30 minutes at 37°C the kinase reaction was stopped by heating the reaction at 70°C for 30 minutes. XhoI "sticky ends"

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were generated at the end of the cDNA corresponding to the 3' end of the mRNA by digestion of the *Xho*I site in the linker-primer. Twenty-eight μ L of *Xho*I buffer and 3 μ L of 40 U/ μ L *Xho*I were added to the
5 cDNA and the reaction was incubated at 37°C for 1.5 hours.

The cDNA, with *Eco*RI sticky ends at the 5' end and *Xho*I sticky ends at the 3' end (relative to the original mRNA), was size fractionated by passage
10 through a Sephacryl S-400 spin column prepared as follows. Five μ L of 10 X STE [100 mM Tris (pH 7.0), 5 mM EDTA and 100 mM NaCl] were added to the cDNA and the cDNA was applied to the top of a 1 mL syringe containing Sephacryl S-400 (Pharmacia
15 Biotech, Piscataway, NJ). A 500 μ L microcentrifuge tube was placed on the bottom of the syringe and the column was placed in a centrifuge tube and centrifuged at about 400 X g for 2 minutes. Sixty μ L of 1 X STE were added to the top of the syringe,
20 a new microcentrifuge tube was placed on the bottom of the column and the column was again centrifuged as before. This process was repeated until six fractions had been collected. About 10% of each fraction was electrophoresed on a 1% agarose gel to
25 determine the size distribution of the cDNA in each fraction. The remainder of each fraction was

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extracted with an equal volume of phenol:chloroform and then chloroform as described above and precipitated by the addition of 2 volumes of 100% ethanol. After overnight incubation at -20°C the

5 cDNA was recovered by centrifugation in a microcentrifuge at 14,000 rpm for 60 minutes at 4°C. Each cDNA fraction was washed with 200 NL of 80% ethanol and dried as described above. cDNA fraction 1 was resuspended in 3 μ L of sterile water, and cDNA

10 fraction 2 was resuspended in 10.5 μ L of sterile water. One-half μ L of each of the two fractions was used to determine the quantity of DNA using the ethidium bromide plate detection method. Fractions 1 and 2, containing the largest cDNA molecules, were

15 combined. The 12.5 mL combined fractions contained approximately 100 ng of cDNA. This fraction was reduced to 2.5 μ L in a Speed-Vac and stored on ice. cDNA fraction 3 was resuspended in 10.5 μ L of sterile water, and saved at -20°C for later use.

20 One-hundred ng of cDNA from fraction 1 and 2 were ligated into 1 μ g of Uni-ZAP™ (Stratagene, La Jolla, CA), a lambda ZAP vector that had been digested with *Eco*RI and *Xho*I. Fraction 1 and 2 cDNA (2.5 μ L) were added to 0.5 μ L of 10 X ligation

25 buffer, 0.5 μ L 10 mM ATP, 1 μ L of 1 μ g/ μ L Uni-Zap XR vector and 0.5 μ L of 4 Weiss U/ μ L T4 DNA ligase.

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The reaction was incubated at 8°C for about 44 hours. A 1 μ L aliquot of the ligation reaction was added to one aliquot of the 'Freeze-Thaw' extract from the Gigapack II Gold bacteriophage λ packaging kit (Stratagene, La Jolla, CA). Fifteen microliters of Sonic extract were added and the contents were gently mixed. The packaging was carried out at room temperature. After 2 hours, 500 μ L of SM buffer and 20 μ L of chloroform were added to each packaging reaction and the debris was removed by a short centrifugation in a microcentrifuge. The packaged phages were moved to a new microcentrifuge tube. Ten μ L of chloroform were added and the packages phages were stored at 4°C until used. A titer of this primary library indicated the presence of 0.7×10^6 recombinant plaques.

c) **Amplification of primary library.**

Six-hundred μ L of *E. coli* XL1-Blue MRF' (Stratagene, La Jolla, CA), grown to a density of 0.5 at O.D.₆₀₀, and 32.5 μ L of primary library stock were added to each of 16 tubes. After incubation at 37°C for 15 min, 6.0 mL of 48°C top agar (5 g/L NaCl, 2 g/L MgSO₄·7H₂O, 5 g/L yeast extract, 10 g/L NZ amine [pH 7.5], and 0.7% agarose) were added to each tube and the contents were plated on 150 X 15 mm NZY plates (5 g/L NaCl, 2 g/L MgSO₄·7H₂O, 5 g/L

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yeast extract, 10 g/L NZ amine [pH 7.5], and 15 g/L Difco agar). The plates were incubated overnight at 37°C and then overlayed with 10 mL of SM buffer and incubated for a further 8 hours at 4°C with gentle shaking. The SM buffer was collected with a sterile pipette and stored in a sterile 250 mL centrifuge bottle. Each plate was rinsed with an additional 10 mL of SM buffer which were collected and added to the previous SM buffer. Chloroform, to a final concentration of 5%, was added and the phage solution was incubated at room temperature for 15 minutes and then centrifuged at 2,000 X g for 10 minutes to remove cell debris. The supernatant was recovered to a sterile polypropylene bottle and chloroform was added to a final concentration of 0.3%. The amplified library was stored at 4°C.

d) **Plating of amplified library for screening for specific genes.**

The amplified library was titered as described above. Approximately 50,000 recombinant plaques were added to 600 µL of *E. coli* XL1-Blue MRF' that were grown as described above. After 15 min at 37°C, 6.5 mL of 48°C top agar were added and the cells were plated on 150 X 15 mm NZY plates. Four plates containing a total of 200,000 recombinant plaques were prepared and incubated at 37°C overnight. The plates were then chilled for 4 hours

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at 4°C, then used for preparing plaque lifts as described below.

5 e) Identification and Construction of
Oligonucleotides Homologous to Coffee ACC
Synthase Genes

In previous studies, described in United States patent application serial number 08/485,107 the
10 specification of which is incorporated herein by reference, we identified base sequences common to ACC synthase occurring in a variety of plants, referred to herein as consensus sequences. Based on these studies, we developed a set of three (3) fully
15 degenerate primers for PCR amplification of regions of coffee first strand cDNA corresponding to consensus sequences. The sequence of the primers used is:

ACS167: 5' -GCCAAGCTTC CRTGRTARTCYTGRAA-3'

20 (SEQ. ID NO. 3)

ACS289: 5' -TTYCARGAYTAYCAYGGHYT-3'

(SEQ. ID NO. 4)

ACS885: 5' -CCHGGDARNCCYAWRTCTTT-3'

(SEQ. ID NO. 5)

25 f) Reverse Transcriptase reaction to obtain first-strand coffee cDNA.

The reverse transcriptase reaction to obtain first-strand cDNA was performed in a final volume of
30 20 µL using the GeneAmp RNA PCR Core Kit (Perkin

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Elmer, Foster City, CA). First, 0.9 μ g of coffee fruit mRNA in 3 μ L water was mixed with 1 μ L of 50 μ M random hexamer and 6 μ L of sterile water in a microcentrifuge tube and incubated at 65°C for 5 minutes. The mixture was left at room temperature for 2 minutes and the liquid was recovered to the bottom of the tube by a brief centrifugation. To this mixture 2 μ L PCR buffer II (from the above mentioned kit), 4 μ L 25 mM MgCl₂, 2 μ L 10 mM dNTP's, 1 μ L RNAsin (20 u/ μ L), and 1 μ L reverse transcriptase (50 u/ μ L) were added. The reaction was incubated at 42°C for 1 hour after which the reverse transcriptase was heat inactivated in a 95°C water bath for 5 minutes.

15 g) **Polymerase chain reaction to amplify coffee ACC-synthase gene.**

A polymerase chain reaction (PCR) (Saiki et al., 1988) was performed using the GeneAmp Kit described above in a 50 μ L reaction containing 10 μ L first-strand cDNA mix, 4 μ L PCR buffer II, 1 μ L 25 mM MgCl₂, 2.5 μ L of 20 μ M AC5167 primer (SEQ. ID NO. 3), 2.5 μ L 20 μ M AC5885 primer (SEQ. ID. NO. 5), 29.5 μ L sterile H₂O, and 0.5 μ L Tag DNA polymerase (5 u/ μ L). PCR conditions were 35 cycles of 94°C for 1 minute, 44°C for 1 minute, and 72°C for 2 minutes. The product of the PCR reaction was analyzed by agarose gel electrophoresis using 1.5% SeaPlaque

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agarose (FMC BioProducts, Rockland, ME) and Hae III-digested ϕ X174 DNA (Promega Corporation, Madison, WI) as size markers. A single PCR product of approximately 650 bp was obtained.

5 h) **Amplification of PCR product with different primers.**

The 650 bp fragment obtained above was excised from the gel and placed in a 1.5 mL microcentrifuge tube. After the addition of 200 μ L of sterile water, the 650 bp fragment was heated to 90°C for 5 minutes, cooled to room temperature and centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge. The supernatant containing the amplified DNA was removed and placed in a new sterile 1.5 mL microcentrifuge tube. A 25 μ L PCR reaction was carried out using 0.4 μ L of the previously amplified DNA as template, 2.5 μ L 10 X PCR buffer (10 mM Tris-HCl pH 9.0, 0.1% triton X-100), 2 μ L 25 mM $MgCl_2$, 5 μ L of 1 mM dNTPs, 1 μ L of 20 μ M ACS289 primer (SEQ. ID. NO. 5), 1 μ L of 20 μ M ACS885 primer (table 2), 12.8 μ L H_2O , and 0.3 μ L Tag DNA polymerase (5 u/ μ L) (Promega Corporation, Madison, WI). The PCR was performed using 35 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes. Five μ L of this reaction was electrophoresed in a 1.5% agarose gel as described above. A single product of approximately 603 bp was observed. Eighty μ L of

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- sterile water, 10 μ L of 3 M sodium acetate (pH 5.2), and 220 μ L of 100% ethanol was added to the remainder of the reaction. After incubation at 20°C overnight, the DNA was recovered by
- 5 centrifugation at 4°C for 30 minutes at 14,000 rpm. The DNA was washed with 400 μ L of ice-cold 75% ethanol and resuspended in 25 μ L of sterile water. The DNA concentration was determined to be 10 ng/ μ L using the ethidium bromide plate assay.
- 10 i) **Labeling Coffee Fruit-Specific ACC Synthase DNA**
- A random primed probe was produced using the PCR-generated ACC synthase DNA and the Prime-a-Gene Kit (Promega Corporation, Madison, WI). Two and one-half μ L of the DNA (25 ng) was added to 27.5 μ L
- 15 of sterile water and the DNA was denatured by boiling for 5 min. Ten μ L of 5 X labeling buffer, 2 μ L of unlabeled dNTP's [20 μ M each; dCTP, dGTP, dTTP], 2 μ L 1 mg/mL acetylated BSA, 1 μ L 5u/ μ L *E. coli* DNA polymerase I Klenow fragment and 5 μ L (50
- 20 μ Ci) of [α -³²P]dATP (3,000 Ci/mmol) (Dupont-NEN) were added to give a final volume of 50 μ L. After 1 hr at room temperature, the reaction was terminated by the addition of 2 μ L of 0.5 M Na₂EDTA and boiling for 2 min.
- 25 j) **Screening of amplified library with the ACC synthase-specific probe.**

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Plaque lifts of the four 150x15 mm NZY plates containing 50,000 recombinant clones each were prepared. Four 132 mm Magna nylon transfer membranes (Micron Separations, Incorporated, Westborough, MA) were wetted by placing them on chromatography paper saturated with 5 X SSC buffer for approximately 10 sec. The membranes were placed on the plates containing the recombinant plaques for 5 min, removed and incubated, phage containing side up, for 2 min on chromatography paper saturated with 0.5 M NaOH and 1.5 M NaCl. The membranes were then neutralized by transferring onto chromatography paper saturated with 0.5 M tris-HCl (pH 8.0) and 1.5 M NaCl, for 5 min. After a brief 20 sec treatment on chromatography sheets saturated with 2 X SCC containing 0.2 M tris-hcl (pH 7.5), the filters were blotted dry. After 1 hour of air drying, DNA was cross-linked to the membranes by treatment with 12,000 μ Joules of a 260 nm UV light in a UV Stratalinker 1800 (Stratagene, La Jolla, CA).

The four membranes were prehybridized at 65°C for 2 hours in 100 mL 6 X SSPE (52.2 g/L NaCl, 8.3 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2.2 g/L Na_2EDTA , [pH 7.4]), 5 X Denhardt's solution (1 g/L Ficoll, 1 g/L polyvinylpyrrolidone, 1 g/L BSA [pentax fraction V]), 0.5% SDS and 100 μ g/mL denatured herring sperm

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DNA in a Hybaid Mark II hybridization oven (National Labnet Company, Woodbridge, NJ) using HB-OV-BL bottles.

Hybridization was carried out at 65°C for 12
5 hours in 10 mL of 6 X SSPE containing 0.5% SDS, 100
μg/mL denatured herring sperm DNA, and 52 μL of the
random primed probe described above. At the end of
the hybridization period the hybridization solution
was removed and the membranes were briefly washed
10 with 100 mL of 2 X SSC containing 0.5% SDS at 65°C.
They were then washed for an additional 30 min with
the same amount of fresh buffer again at 65°C. The
membranes were washed twice more for 30 min at 65°C
with 100 mL of 0.2 X SSC containing 0.5% SDS,
15 wrapped in a cellophane envelope and exposed to pre-
flashed Fuji RX_{GCU} X-ray film at -70°C for 24 hours.
Ten positive clones were obtained. The region of
the original plates corresponding to the identified
plaques were removed and placed in 1 mL of SM buffer
20 containing 20 μL chloroform. Of these ten, 5 were
re-plated at lower densities and rescreened as above
to obtain individual plaques.

**k) Characterization of Coffee-Fruit ACC synthase
cDNA clones.**

25

The size of the putative coffee ACC synthase
cDNA clones was determined by polymerase chain
reaction using primers homologous to a portion of

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the T3 and T7 promoters present in the cloning vector and flanking the cDNA insertion site. The sequence of the primers are:

T3: 5'-TAATACGACTCACTATAGGG-3' (SEQ. ID NO. 6)

5 T7: 5'-AATTAACCCTCACTAAAGGG-3' (SEQ. ID NO. 7)

The conditions for PCR were as described above except that the temperature cycle was 95°C for 1 min., 50°C for 1 min. and 72°C for 2 min. Analysis was by agarose gel electrophoresis as before.

10 The three largest clones were recovered as phagemids by *in vivo* excision. Two hundred μ L of phage stock from a single plaque was mixed with 200 μ L of *E. coli* XL1-Blue MRF' grown to a density at O.D.₆₀₀ of 1.0. One μ L of ExAssist (Stratagene, La
15 Jolla, CA) helper phage ($>1 \times 10^6$ pfu/ μ L) was added and the tubes were incubated at 37°C for 15 min. Three mL of sterile LB broth were added and they were incubated for 3 hours at 37°C with shaking. After heating at 70°C for 20 min and centrifugation
20 at 1,000 X g for 15 min, 1 mL of the supernatant, containing the excised pBluescript phagemid packaged as filamentous phage particles, was transferred to a sterile 1.5 mL microcentrifuge tube and stored at 4°C. Phagemids were recovered by adding 25 μ L of
25 the stock solution to 200 μ L of *E. coli* Solar cells (Stratagene, La Jolla, CA) grown to a density of 1

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when measured at O.D.₆₀₀. After incubation at 37°C for 15 min, 200 µL of the cell mixture was plated on 100 X 15 mm NZY agar plates containing 50 µg/mL ampicillin. The plates were incubated overnight at 37°C. Individual colonies were picked into 10 mL of LB broth containing 50 µg/mL ampicillin and grown overnight in a 37°C shaking incubator. The cells were concentrated in a 1.5 mL sterile microcentrifuge tube by repeated centrifugation and the phasmid DNA was purified using the plasmid mini kit from QIAGEN. The bacterial pellets were washed with water and resuspended in 0.3 mL of buffer P1. Next, 0.3 mL of alkaline lysis buffer P2 was added, mixed gently, and incubated for less than 5 min at room temperature. Following the addition of 0.3 mL of chilled buffer P3 and mixing by inverting the tubes 6 times, the extracts were incubated on ice for 10 min and centrifuged at 14,000 rpm for 15 min in a microcentrifuge. The supernatants were removed and applied to QIAGEN-tip 20 columns that had been previously equilibrated with 1 mL of QDT buffer. The extracts were allowed to enter the resin of the columns by gravity flow. Once the flow had stopped, the columns were washed 4 times with 1 mL buffer QC. The DNAs were eluted by washing the QIAGEN-tip 20 columns with 0.8 mL buffer QF which was collected

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into 1.5 mL microcentrifuge tubes. The DNA was precipitated by the addition of 0.7 volumes (560 μ L) of isopropanol. The tubes were immediately centrifuged at 14,000 rpm for 30 min and the supernatant carefully removed. The pellets, containing the DNA, were washed 20 with 1 mL of ice-cold 70% ethanol, centrifuged as above, and air dried for 5 min. The DNA was resuspended in 50 μ L sterile H₂O. The concentration of DNA from one plasmid isolation was 0.1 μ g/ μ L by fluorometric analysis.

Sequencing reactions were performed by mixing 8 μ L of phagmid DNA (0.8 μ g) with 4 μ L of either T3 or T7 sequencing primers (0.8 pmol/ μ L). Automated DNA sequencing was carried out on these samples at the University of Hawaii Biotechnology Service Center. About 350 bp of sequence from both the 5' and the 3' end of the cDNA was obtained. New sequencing primers were synthesized based on sequences near the end of the previous sequences and used in the same manner to complete the sequence of both strands of the cDNA. The complete sequence of the coffee fruit-expressed ACC synthase cDNA is given in Figure 1. The deduced amino acid sequence of the coffee fruit-expressed ACC synthase is given in Figure 2.

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The sequence of the coffee ACC synthase cDNA clone and deduced protein was compared with other ACC synthase genes present in GenBank. The cDNA isolated from coffee fruit shows from 68.3% to 58.1% identity to other ACC synthases present in GenBank. And, the protein sequence deduced from this cDNA shows from 67.9% to 50.5% identity to other ACC synthases. However, this cDNA is unique in that no other sequence greater than 1500 bp showed greater than 68.3% identity to it.

EXAMPLE 2***Isolation of Coffee Fruit-Specific ACC Oxidase***

- a) **Synthesis of ACC Oxidase specific oligonucleotide primers.**

The isolation of total RNA, mRNA, and the synthesis of coffee fruit-specific cDNA was as described above.

Twelve ACC oxidase sequences, obtained from GenBank, were aligned using the Pileup program of GCG (Genetics Computer Group, Madison, WI). A region approximately 1000 bp from the translation start codon was found to be conserved and a degenerate oligonucleotide primer

5'-TCATIGCKKCRKIGGTTC-3' (SEQ. ID NO. 8) corresponding to this region was synthesized. Inosine (I) was placed at positions showing no sequence conservation, since position could be any

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of A, T, G or C. Positions showing two-fold ambiguity were prepared with mixed residues (T/G or A/G). We also prepared a second primer homologous to a region of the papaya fruit-expressed ACC oxidase cDNA that had been previously cloned in our laboratory and situated approximately 372 bp from the translational start codon:

5'-GACACTGTGGAGAGGCTGAC-3' (SEQ. ID NO. 9)

The two primers were used in a PCR reaction to amplify a portion of the coffee fruit-expressed ACC oxidase. The PCR contained 0.2 μ L (10 ng) cDNA fraction 3 (described in Example 1), 5 μ L 10 X PCR buffer, 3 μ L 25 mM $MgCl_2$, 1 μ L of each of the four 10 mM dNTPs, 1 μ L of a 20 μ M solution of each primer, 0.3 μ L Taq DNA polymerase (promega Corporation, Madison, WI) and 38.5 μ L water. PCR conditions were 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. A 5 min incubation at 72°C was carried out after the last cycle. A 20 μ L aliquot of the product was electrophoresed in a 1.5% agarose gel as described previously and revealed an approximately 800 bp product. The DNA was excised from the gel and mixed with 200 μ L of sterile water in a 1.5 mL microcentrifuge tube. After boiling for 5 min, 2 μ L was used as a template in a 50 μ L PCR reaction as above using the same primers. Gel

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electrophoresis performed as described above using 20 μ L of the PCR reaction indicated the presence of a single 800 bp product. To the remaining 30 μ L of the PCR reaction 20 μ L chloroform and 100 μ L water was added. The contents were mixed and centrifuged for 2 minutes at 14,000 rpm in a microcentrifuge. The upper aqueous phase containing the DNA was removed to a clean microcentrifuge tube. A portion of this DNA was radioactively labeled by random primed synthesis as described above.

b) Screening of amplified library with random primed probe.

The amplified coffee-fruit cDNA described in Example 1 was used to prepare four 150 X 10 mm NZY plates as previously described. Prehybridization, hybridization and recovery of clones was as previously described except that the ACC oxidase sequence obtained by PCR was used as the probe.

20 c) Characterization of Coffee-Fruit ACC-oxidase cDNA clones.

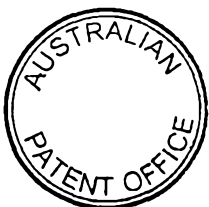
The size of the coffee ACC-oxidase cDNA clones was determined by polymerase chain reaction using primers homologous to the T3 and T7 promoters as described in Example 1.

The sequence of the largest coffee ACC oxidase cDNA clone was obtained as described in Example 1 and compared with ACC oxidase genes present in

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GenBank. Figure 3 gives the sequence of the coffee fruit-specific ACC oxidase. Figure 4 gives the deduced amino acid sequence of this protein. The cDNA was determined to encode ACC oxidase because it is from 50.4% to 82.5% identical to other ACC synthases nucleic acid sequences present in GenBank. Also, the deduced protein sequence is from 32.5% to 86.5% identical to other ACC oxidases.

10



EXAMPLE 3 GENERATION OF PLANTLETS

a) Construction of vectors for the expression of anti-sense ACC synthase and ACC oxidase transcripts.

The ACC synthase and ACC oxidase cDNAs can be used to modify ethylene content in coffee by, for example, antisense expression or co-suppression. An example of its use with the vector pKR1 is described. This is only an example and many other plant transformation vectors could be used in conjunction with the ACC synthase and ACC oxidase cDNAs. pKR1 was created by modification of pBI-121 (Clontech Laboratories) as follows.

Two 38-base pair synthetic sequences containing *lox* recognition sites for the *cre* site-specific recombinase were inserted surrounding the neomycin phosphotransferase II (NPT II) selectable marker gene of pBI-121. These *lox* sites allow for the removal of the NPT II gene from the construct after it is integrated into the plant genome [Dale and Ow, *Proc. Natl. Acad. Sci. USA* 88:10558 (1991)], but are not pertinent to the function of the ACC synthase and ACC oxidase cDNAs in antisense.

Three synthetic oligonucleotides were synthesized based on the *loxP* sequences defined by Dale and Ow (*supra*). The sequence of these oligonucleotides are:

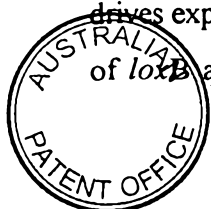
loxA: 5'-AGCTATAACTTCGTATAGCATACATTATACGAAGTTAT-3'

loxB: 5'-AGCTATAACTTCGTATAATGTATGCTATACGAAGTTAT-3', and

loxC: 5'-ATAACTTCGTATAGCATACATTATACGAAGTTATAGCT-3'.

loxB is the complementary strand to both *loxA* and *loxC*. When *loxA* and *loxB* were annealed they formed a double-stranded molecule with 4-base overhangs complementary to *Hind*III overhangs which allows insertion of the double-stranded sequence into a *Hind*III site such as that found after the NOS transcription termination sequence adjacent to the NPT II gene in pBI-121. Annealing of *loxC* with *loxB* produces a blunt-ended double-stranded DNA containing a *lox* recognition site.

Synthetic *lox* sites were inserted surrounding the NPT II gene of pBI-121 as follows. pBI-121 was digested with *Pme*I (New England Biolabs, Beverly, MA) at 37°C for 2 hours in reaction buffer supplied by the manufacturer. pBI-121 has a single *Pme*I site just proximal to the NOS promoter that drives expression of the NPT II gene. A synthetic *lox* site was generated by annealing equimolar amounts of *loxB* and *loxC* by heating at 95°C, slowly cooling to room temperature and ligating into the *Pme*I-



digested pBI-121. The 30 μ L ligation reaction contained ligation buffer (New England Biolabs, Beverly, MA), 60 nmoles *PmeI*-digested pBI-121, 3 μ L of a 1 μ M stock solution of annealed *loxB* / *loxC*, 4 units *PmeI* and 4,000 units of high concentration T4 DNA ligase (New England Biolabs, Beverly, MA). Ligation was at 16°C overnight. One to 4 μ L of the ligation reaction were electroporated into *E. coli* XL1-Blue cells (Stratagene) and plated onto LB plates containing 50 μ g/mL kanamycin, 50 μ L of 20 mg/mL X-gal and 10 μ L of 100 mM IPTG. White colonies were picked to fresh LB-kanamycin master plates.

Colonies containing the *lox* site were identified by colony hybridization. The master plates were grown for 4 hours at 37°C and blotted to nylon membranes (MSI). The membranes were placed on fresh LB-kanamycin plates and grown at 37°C overnight. The membranes were floated on 0.5 M NaOH for 10 minutes, neutralized by floating on 0.5 M Tris-HCl (pH 8.0) containing 0.5 M NaCl for 2 minutes and rinsed in 2 X SSC.

The membranes were pre-hybridized in 20 mL of 6 X SSPE, 5 X Denhardt's solution, 0.5% SDS and 100 μ g/mL fragmented herring sperm DNA at 55°C for 3 hours. The pre-hybridization solution was replaced with 10 mL of fresh solution containing 8.4×10^6 cpm of *loxC* labeled at the 5' end with [32 P] using T4 polynucleotide kinase. The 50 μ L labeling reaction contained 50 pmoles of *loxC*, polynucleotide kinase reaction buffer (Promega), 15 μ L of 3,000 Ci/mmol [32 P] ATP (DuPont-NEN) and 20 units T4 polynucleotide kinase (Promega). The reaction was incubated at 37°C for 10 minutes and the product was separated from the unincorporated ATP using a Sephadex G-25 spin column. Hybridization was at 55°C overnight. The filters were washed at 55°C twice in 100 mL of 2 X SSC containing 0.5% SDS, once with 100 mL of 1 X SSC containing 0.5% SDS and autoradiographed as described previously. Several colonies were found to hybridize intensely and were selected for further characterization. Plasmid DNA was extracted using the Magic Minipreps DNA Purification System[®] (Promega) and digested with *PmeI* as described above. Plasmids containing the *lox* site will no longer have a *PmeI* site. Plasmids that were resistant to digestion by *PmeI* were further analyzed by automated DNA sequencing at the University of Hawaii Biotechnology Service Center, to confirm insertion of a *lox* site.

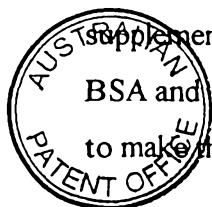
A plasmid containing the *lox* site in the desired orientation was digested with *HindIII* mixed with *loxA* / *loxB* heteroduplex that contains *HindIII* sticky-ends but not a complete *HindIII* restriction site, annealed as described above and ligated. The ligation reaction contained 2.5 μ g of *HindIII*-digested



plasmid, 1.25 pmoles of *loxA* / *loxB*, ligation buffer (Promega), 6 units of T4 DNA ligase (Promega), 1.25 units of *Hind*III (Promega) in a final volume of 30 μ L. The reaction was incubated at room temperature for 1 hour, heated at 80°C for 10 min and introduced into *E. coli* XL1-Blue cells by electroporation (Stratagene). Random plasmids were screened for loss of the *Hind*III site by digestion with *Hind*III as above. Final confirmation of this plasmid structure, designated pKR1, was obtained by DNA sequencing as described above.

pKR1 was digested with *Sac*I. The 173 μ L reaction contained 10 μ g pKR1, multicore buffer (Promega), and 20 units of *Sac*I (Promega). After 1 hour at 37°C, 0.7 μ L of 25 mM stocks of dATP, dCTP, dGTP and dTTP and 10 units of T4 DNA polymerase (Promega) were added. This reaction, which will make the *Sac*I digestion products blunt-ended, was incubated at 15°C for 30 minutes. After inactivation of the T4 DNA polymerase by incubation at 75°C for 15 minutes, 24 units of *Sma*I were added and the reaction was incubated at room temperature for two hours. The reaction was stopped by heating at 80°C for 15 minutes. The DNA was precipitated by the addition of 17 μ L of 3 M sodium acetate and 375 μ L of 100% ethanol. After 1 hour at -70°C the DNA was recovered by centrifugation in a microcentrifuge at full-speed for 20 min at 4°C. The DNA was washed with 70% ethanol, dried under vacuum and dissolved in 88 μ L water. Ten μ L of 10 X calf intestinal alkaline phosphatase buffer (Promega) and 20 units of calf intestinal alkaline phosphatase were added and the reaction was incubated at 37°C for 2 hours. The reaction was stopped by the addition of 4 μ L of 0.5 M EDTA and heating at 75°C for 10 minutes. The sample was extracted with an equal volume of water saturated phenol, then with an equal volume of phenol:chloroform (1:1) and finally with chloroform. The DNA was recovered by precipitation after adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol.

The coffee ACC synthase and ACC oxidase cDNA inserts were released from original plasmids, pACS and pACO, using restriction enzymes as follows. Ten μ g of pACS plasmid was digested in 100 μ L containing 10 μ L of 10 X Multicore buffer (Promega), 40 units *Sma*I. After incubation at 25°C overnight, 40 units of *Bsr*SI were added and the reaction was continued at 67°C. After 2 hours at 67°C, the reaction tube was cooled in ice for 10 min followed by incubation at 37°C. The reaction mix was supplemented with 1 μ L of 10 mM dNTP (dATP, dCTP, dGTP and dTTP), 1 μ L of 1 mg/mL acetylated BSA and 15 units of T4 DNA polymerase (Promega). The reaction was incubated at 37°C for 5 minutes to make the DNA blunt-ended. After inactivation of the T4 DNA polymerase by incubation at 75°C for



30 minutes, the volume was reduced to 50 μ L in a Speed-Vac[®]. The digestion products were separated by electrophoresis on a 1% SeaPlaque agarose gel. The 1.6 kb coffee ACC synthase cDNA was excised from the agarose gel and the cDNA insert recovered by digestion of the agarose with 1.12 units of *Agar ace* (Promega). After incubation at 45°C for 30 min, the cDNA was precipitated by adding 24 μ L of 3 M sodium acetate (pH 5.2) and 600 μ L of 95% ethanol. The ethanol-precipitated cDNA was centrifuged for 30 min at room temperature, the supernatant discarded, and the pellet was washed with ice-cold 70% ethanol and centrifuged as above. The pellet was dissolved in 100 μ L water and subsequently used in the blunt-end ligation into the pKR1 vector.

The ACC oxidase cDNA insert was prepared in a similar manner described for the pACS cDNAs above. Ten μ g of pACO plasmid was digested in 100 μ L containing 10 μ L of 10 X buffer C (Promega), 1 μ L of 1 mg/mL acetylated BSA, 30 units of *Bali* and 30 units of *bamboo*. After incubation at 37°C for 2 hours, the reaction mix was supplemented with 1 μ L of 10 mM dNTP (dATP, dCTP, dGTP and dTTP) and 15 units of T4 DNA polymerase (Promega). The reaction was incubated at 37°C for 5 minutes to generate blunt-ends. After inactivation of the T4 DNA polymerase by incubation at 75°C for 30 minutes, the volume was reduced to 50 μ L in a Speed-Vac[®]. The digestion products were separated by electrophoresis on a 1% SeaPlaque agarose gel. The 1 kb coffee ACC oxidase cDNA was excised from the agarose gel and the cDNA insert recovered by digestion of the agarose with 1.12 units of *Agar ace* (Promega). The purification of the cDNA insert from agarose was as described for the ACC synthase insert. The pellet was dissolved in 50 μ L water and subsequently used in the blunt-end ligation into the pKR1 vector.

The purified ACC synthase and ACC oxidase inserts were ligated in pKR1 vector by mixing 500 ng of pKR1 vector (*SmaI/SacI* fragment blunt ended and phosphatased) and 150 ng of either ACC synthase or ACC oxidase insert in separate tubes. The volume of each vector/insert mixture was reduced to 8 μ L using a Speed-Vac[®]. To each of the vector/insert tubes, 1 μ L of 10 X ligase buffer (Promega) and 1 μ L of T4 DNA ligase (10 units) were added. The contents were incubated at 8°C for 48 hours.

One μ L of each ligation product was mixed with 40 μ L of XL-1 Blue cells (previously prepared for electroporation and stored at -70°C) and electroporated using "Electro Cell Manipulator 600" (ECM2000, BTX Inc., CA) at 2.35 KV for 5 msec. Immediately after electroporation, 1 mL of LB buffer was added to each tube and incubated in a rotary shaker for 1 hour at 250 RPM. After the incubation, the

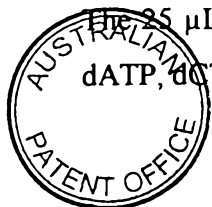


bacteria were precipitated by centrifugation at 1400 RPM for 2 min and the volume was reduced to 100 μ L. Fifty μ L of the mixture was plated into LB plates containing 40 μ g/mL kanamycin and incubated overnight at 37°C. Colonies growing in kanamycin plates were further screened to identify clones that included the ACC synthase or ACC oxidase inserts. Individual clones were harvested using sterile tooth picks and laid on fresh LB-kanamycin plates in a grid pattern. There were three plates for each cDNA insert (ACC synthase and ACC oxidase). After overnight growth in the grid, the bacteria were replica blotted in Magna nylon membranes (MSI, MA). The membranes were sequentially treated in 10% SDS for 5 min, 0.5 M NaOH and 1.5 M NaCl for 15 min, 0.5 M Tris-HCl and 1.5 M NaCl for 15 min, and 2 X SSC and 0.2 M Tris-HCl for 5 min. The membranes were allowed to air dry and baked at 80°C for 20 min and cross-linked by 120,000 μ Joules of UV illumination (Strata Linker UV crosslinker 1800, Stratagene, CA).

The membranes were pre-hybridized for 2 hours in 6 X SSPE, 5X Denhardt's solution, 0.5% SDS and 100 μ g/mL herring sperm DNA at 65°C. Probes for ACC synthase and ACC oxidase were synthesized using Ready-to-Go DNA labeling beads (Pharmacia). Fifty ng of each cDNA insert was denatured by boiling in 45 μ L water and quenched in ice. The 45 μ L denatured DNA was mixed with Ready-to-Go DNA labeling beads and 5 μ L of [32 -P] dCTP (3000 Ci/mmol) and incubated at 37°C for 30 min. After the probe synthesis, the tubes were boiled in water for 4 min and quenched in ice. The pre-hybridization buffer was discarded and 10 mL of pre-heated hybridization buffer (6 X SSPE, 0.5% SDS and 100 μ g/mL herring sperm DNA) was put into each hybridization bottle followed by the addition of the denatured probe. Hybridization was performed overnight at 65°C.

The membranes were washed briefly with 2 X SSC and 0.5% SDS. A second wash with the same buffer was performed for 30 min. The membranes were washed 2 X with 0.2 X SSC and 0.5 X SDS. Each wash was for 30 min. The membranes were then autoradiographed. Five pKR1 clones with ACC synthase and 24 clones with ACC oxidase were identified upon developing the autoradiogram. The gene orientation in each clone was identified employing PCR, restriction digestion and sequencing the vector/insert junction. A pinch of bacterial colony from each of the 29 clones (5 ACC synthase and 24 ACC oxidase) were picked using toothpicks and suspended in 20 μ L sterile Milli-Q water (cell dilution).

The 25 μ L PCR reactions contained 2 μ L of the above cell dilutions, 0.5 μ L of a 10 mM stock of each dATP, dGTP, dGTP and dTTP, 1.5 μ L 25 mM $MgCl_2$, 2.5 μ L 10 X buffer (Promega), 1 μ L each of three



20 μ M primers given below, 0.3 μ L 5 units/ μ L *Taq* DNA polymerase (Promega) and 16.2 μ L sterile Milli-Q water. The primers used for ACC synthase clones were: 35S primer (5'-CCA CTA TCC TTC GCA AGA CC-3'); ACSR₇ (5'-TTG CCA TCT TCG ACA AGA CT-3'); and ACSL₄ (5'-CTG TTG TCA GCT GTG CTA-3'). Likewise the primers used for ACC oxidase clones were: 35S primer; ACOR₄ (5'-GGA CTT CTG AGA TGT TGG AA-3'); and ACOL₁ (5'-TGG TGG AGA GCA AGG AAT TG-3'). The thermal cycling conditions were 10 minutes at 94°C; 35 cycles of 1 minute at 94°C, 1 minute at 50°C, 1 minute at 72°C, and 5 minutes at 72°C. The expected sizes of the PCR products for pKR1/ACC synthase construct were 320 bp (35S and ACSR₇) for the sense orientation and 850 bp (35S and ACSL₄) for the antisense orientation. Similarly, the expected products for pKR1/ACC oxidase construct were 400 bp (35S and ACOR₄) for the sense orientation and 800 bp (35S and ACOL₁) for the antisense orientation. For both ACC synthase and ACC oxidase, one plasmid each with the cDNA in the sense and in the antisense direction were further analyzed by DNA sequencing of the junctions between the plasmid and cDNA to confirm the orientation. DNA sequencing was carried out as previously described. The binary vectors with ACC synthase gene in antisense orientation (designated pKRCACS-A), ACC synthase in sense orientation (pKRCACS-S), ACC oxidase in antisense orientation (pKRCACO-A) and ACC oxidase in sense orientation (pKRCACO-S) are shown in Figures 1-4, respectively, here attached..

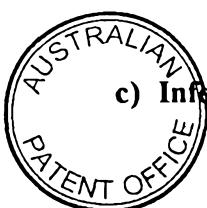


b) Electroporation of *Agrobacterium* with pKR1 plasmid containing the ACC synthase (pKRCACS-S and pKRCACS-A) and ACC oxidase (pKRCACO-S and pKRCACO-A) cDNAs gene in sense and antisense orientation.

Agrobacterium strain LBA 4404 was grown from a single colony to OD₆₀₀ in 100 mL YM liquid media (0.4 g/L Yeast extract, 10g/L mannitol, 0.1 g/L NaCl, 0.2 g/L MgSO₄·7H₂O, 0.5 g/L K₂HPO₄, pH 7.5) in a rotary shaker at 29°C for 48 hours. The cells were concentrated by centrifugation at 4000 X g to 6 X 10¹¹ cells/mL. Electroporation was performed using the "Electro Cell Manipulator 600" (ECM 600, BTX Inc., CA). A 3.5 µL aliquot of either pKRCACS-A, pKRCACO-A, pKRCACS-S or pKRCACO-S plasmid solution containing 200 ng of DNA was mixed with 50 µL of concentrated *Agrobacterium* cells and transferred to a pre-cooled 2 mm gap cuvette (BTX Inc., CA) and a 5 ms pulse at 2.35 kV was applied. One mL of YM liquid media containing 50 µg/mL kanamycin was added to the electroporated cells and they were allowed to recover for 1 hour in a 29°C shaker at 250 RPM. The cells were centrifuged at 4000 X g and resuspended in 100 µL fresh YM liquid media. Transformed bacteria were selected on YM-agar plates (YM liquid media containing 15 g/L Bacto-agar) supplemented with 50 µg/mL kanamycin.

Confirmation of *Agrobacterium* transformation after incubation at 29°C for 48 hours was obtained by picking 10 colonies from each transformation to fresh YM-agar/kanamycin plates on a marked grid. The presence and orientation of the ACC synthase and ACC oxidase gene insert was determined by PCR using the 35S primer in conjunction with one of the internal gene-specific primers. A small amount of each colony was transferred to 20 µL sterile Milli-Q water using a sterile toothpick. PCR reactions were performed as described above using 2 µL of these cell suspensions as template DNA. For the ACC synthase sense orientation the 35S and ACSR₇ primers were used and produced a product of the expected size, 320 bp. The ACC synthase antisense primers 35S and ACSL₄ gave the expected product of 850 bp. Similarly, for the ACC oxidase sense orientation the 35S and ACOR₄ primers were used and produced a product of the expected size, 400 bp. The ACC oxidase antisense primers 35S and ACOL₁ gave the expected product of 800 bp. We selected one colony of each orientation for transformation of coffee leaf tissue.

c) Infection of coffee leaf tissue with *Agrobacterium* containing the



pKRCACS-S, pKRCACO-S, pKRCACS-A and pKRCACO-A plasmids.

Mature young coffee leaves from plagiotrophic shoots were sterilized in 30% Clorox for 30 minutes and rinsed three times in sterilized distilled water. Approximately 7 mm² pieces from the lamina between the midrib and the margin were excised and placed in MS liquid media (Murashige and Skoog, 1962) with an *Agrobacterium* slurry of 10⁹ cells/mL and co-cultivated for three hours. The leaf tissue was blotted dry with sterilized paper towels and co-cultivated with the remaining *Agrobacterium* for three days on MS media solidified by the addition of 2.0 g/L Phytigel.

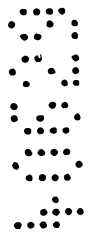
The leaf tissue was again blotted with sterile paper towels to remove remaining *Agrobacteria* and then transferred into callus induction medium (MS medium containing 2,4-D and kinetin; Sondahl and Sharp, 1977), containing 500 µg/mL carbenicillin, and either 100 to 300 µg/mL kanamycin monosulfate or 10 to 20 µg/mL geneticin (G418). After 13 days of culture at 25°C in the dark, primary callus started to appear.

d) Subculturing of coffee leaf callus tissue containing the pKRCACS-S, pKRCACO-S, pKRCACS-A or pKRCACO-A plasmids.

The antibiotic resistant calli were subcultured monthly using embryo induction media M II (basal salts, half-strength MS salts, 10mg thiamine HCl, 40 mg cysteine HCl, 100mg myo-inositol, 40 g sucrose, 2 mg BA, 1 mg pyridoxine, 1 mg nicotinic acid, 2.0 g phytigel, pH 5.65; Yasuda and Fujii, 1985) containing 300 µg/mL carbenicillin, and 150-200 µg/mL kanamycin for three months. These calli were then subcultured for thirty days into M II medium containing 100 µg/mL kanamycin, and for a further thirty days into M II medium containing 50 µg/mL kanamycin. Somatic embryos formed in this last medium. The somatic embryos developed into plantlets on germination media M III lacking growth regulators (basal salts, full-strength MS salts, 10mg thiamine HCl, 40 mg cysteine HCl, 100 mg myo-inositol, 40 g sucrose, 2 g phytigel, pH 5.65; Sondahl and Sharp, 1977) under fluorescent light.



The foregoing examples are for illustrative purposes only, and should not be viewed as limiting the scope of applicants' invention, which is set forth in the claims appended hereto.



SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: STILES, JOHN I.
MOISYADI, ISTEFO
NEUPANE, KABI R.
- (ii) TITLE OF INVENTION: PURIFIED PROTEINS, RECOMBINANT
DNA SEQUENCES AND PROCESSES FOR CONTROLLING THE
RIPENING OF COFFEE
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: JONES, DAY, REAVIS & POGUE
 - (B) STREET: NORTH POINT, 901 LAKESIDE AVENUE
 - (C) CITY: CLEVELAND
 - (D) STATE: OHIO
 - (E) COUNTRY: USA
 - (F) ZIP: 44114
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb
storage
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: MS-DOS v. 5.1
 - (D) SOFTWARE: WordPerfect v. 6.1
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/695,412
 - (B) FILING DATE: 12-AUG-1996
 - (C) CLASSIFICATION: 435
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US08/485,107
 - (B) FILING DATE: 07-JUN-1995
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: GRIFFITH, CALVIN P.
 - (B) REGISTRATION NUMBER: 34,831
 - (C) REFERENCE/DOCKET NUMBER: 265036600002
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (216) 586-7050
 - (B) TELEFAX: (216) 579-0212

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 amino acid residues
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: N/A
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Fragment A

(B) LOCATION: 17..1480

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile Asn Tyr Ala Ser Gly Ala Ser Gly Ile Leu Asp Gln Xaa Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile Asn Tyr Ala Ser Gly Ala Ser Gly Ile Leu Asp Gln Thr
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID

(A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: Internal

(ix) FEATURE:

(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATNAAYTAYG CNAGYGGNGC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: OTHER NUCLEAR ACID
 - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: INTERNAL
- (ix) FEATURE
 - (A) OTHER INFORMATION: N IS INOSINE
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
ATNAAYTAYG CNAGYGGNGC 20

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: OTHER NUCLEIC ACID
 - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: INTERNAL
- (ix) FEATURE
 - (A) OTHER INFORMATION: N IS INOSINE
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
CGNCCAGNCG NYTAYTTNAT 20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
 - (A) DESCRIPTION: PRIMER
- (v) FRAGMENT TYPE: INTERNAL
- (ix) FEATURE
 - (A) OTHER INFORMATION: N IS INOSINE
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
CGNCCYCTYG CYTAYTTNAT 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: INTERNAL

(ix) FEATURE

- (D) OTHER INFORMATION: Xaa is either Thr or Asp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gln Tyr Val Pro Cys Tyr Phe Xaa Phe Ile Asp Asp Gln Asp
1 5 10 14

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID

- (A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: Internal

(ix) FEATURE

- (A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAWTATGTNC CNTGTTATTT 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: OTHER NUCLEIC ACID

- (A) DESCRIPTION: PRIMER

(v) FRAGMENT TYPE: Internal

(ix) FEATURE

(A) OTHER INFORMATION: N IS INOSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAWTAWCAHG GNACWTATTG 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 488 amino acid residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 178..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Gly	Trp	Lys	Ala	Tyr	Asp	Ser	Asp	Pro	Tyr	His	Pro	Thr	Arg	Asn	35	40	45	
Pro	Asn	Gly	Val	Ile	Gln	Met	Gly	Leu	Ala	Glu	Asn	Gln	Leu	Cys	50	55	60	
Phe	Asp	Leu	Ile	Glu	Glu	Trp	Val	Leu	Asn	Asn	Pro	Glu	Ala	Ser	65	70	75	
Ile	Cys	Thr	Ala	Glu	Gly	Ala	Asn	Lys	Phe	Met	Glu	Val	Ala	Ile	80	85	90	
Tyr	Gln	Asp	Tyr	His	Gly	Leu	Pro	Glu	Phe	Arg	Asn	Ala	Val	Ala	95	100	105	
Arg	Phe	Met	Glu	Lys	Val	Arg	Gly	Asp	Arg	Val	Lys	Phe	Asp	Pro	110	115	120	
Asn	Arg	Ile	Val	Met	Ser	Gly	Gly	Ala	Thr	Gly	Ala	His	Glu	Thr	125	130	135	

Leu	Ala	Phe	Cys	Leu	Ala	Asp	Pro	Glu	Asp	Ala	Phe	Leu	Val	Pro	140	145	150
Thr	Pro	Tyr	Tyr	Pro	Gly	Phe	Asp	Arg	Asp	Leu	Arg	Trp	Arg	Thr	155	160	165
Gly	Met	Gln	Leu	Leu	Pro	Ile	Val	Cys	Arg	Ser	Ser	Asn	Asp	Phe	170	175	180
Lys	Val	Thr	Lys	Glu	Ser	Met	Glu	Ala	Ala	Tyr	Gln	Lys	Ala	Gln	185	190	195
Glu	Ala	Asn	Ile	Arg	Val	Lys	Gly	Phe	Leu	Leu	Asn	Asn	Pro	Ser	200	205	210
Asn	Pro	Leu	Gly	Thr	Val	Leu	Asp	Arg	Glu	Thr	Leu	Ile	Asp	Ile	215	220	225
Val	Thr	Phe	Ile	Asn	Asp	Lys	Asn	Ile	His	Leu	Ile	Cys	Asp	Glu	230	235	240
Ile	Tyr	Ser	Ala	Thr	Val	Phe	Ser	Gln	Pro	Glu	Phe	Ile	Ser	Ile	245	250	255
Ser	Glu	Ile	Ile	Glu	His	Asp	Val	Gln	Cys	Asn	Arg	Asp	Leu	Ile	260	265	270
His	Leu	Val	Tyr	Ser	Leu	Ser	Lys	Asp	Leu	Gly	Phe	Pro	Gly	Phe	275	280	285
Arg	Val	Gly	Ile	Leu	Tyr	Ser	Tyr	Asn	Asp	Ala	Val	Val	Ser	Cys	290	295	300
Ala	Arg	Lys	Met	Ser	Ser	Phe	Gly	Leu	Val	Ser	Thr	Gln	Thr	Gln	305	310	315
His	Leu	Ile	Ala	Ser	Met	Leu	Ser	Asp	Glu	Ala	Phe	Met	Asp	Lys	320	325	330
Ile	Ile	Ser	Thr	Ser	Ser	Glu	Arg	Leu	Ala	Ala	Arg	His	Gly	Leu	335	340	345
Phe	Thr	Arg	Gly	Leu	Ala	Gln	Val	Gly	Ile	Gly	Thr	Leu	Lys	Ser	350	355	360
Ser	Ala	Gly	Leu	Tyr	Phe	Trp	Met	Asp	Leu	Arg	Arg	Leu	Leu	Arg	365	370	375
Glu	Ser	Thr	Phe	Glu	Ala	Glu	Met	Glu	Leu	Trp	Arg	Ile	Ile	Ile	380	385	390
His	Glu	Val	Lys	Leu	Asn	Val	Ser	Pro	Gly	Leu	Ser	Phe	His	Cys	395	400	405

Ser	Glu	Pro	Gly	Trp	Phe	Arg	Val	Cys	Phe	Ala	Asn	Met	Asp	Asp
				410					415					420
Glu	Ser	Val	Arg	Val	Ala	Leu	Arg	Arg	Ile	His	Lys	Phe	Val	Leu
				425					430					435
Val	Gln	Gly	Lys	Ala	Thr	Glu	Pro	Thr	Thr	Pro	Lys	Ser	Arg	Cys
				440					445					450
Gly	Ser	Ser	Lys	Leu	Gln	Leu	Ser	Leu	Ser	Phe	Arg	Arg	Leu	Asp
				455					460					465
Glu	Arg	Val	Met	Gly	Ser	His	Met	Met	Ser	Pro	His	Ser	Pro	Met
				470					475					480
Ala	Ser	Pro	Leu	Val	Arg	Ala	Thr							
				485										

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2040 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix)Feature:

(A)NAME/KEY:CDS

(B)LOCATION:178..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAATCTCTT	CTAAAATCAA	CCATTCTCTT	CATTCTTCAC	TTGACAAGGC	50
CACTGCATTC	TTCATTCTTT	CTTGATATAT	AGCCATTTTT	TTCATTCTTT	100
CTTGATATAT	AGCCATTTTT	TTCATTCTTT	CTTCATTCAT	TGTCTGGAGA	150
AGTTGGTTGA	GTTTTCTTGA	AAATTCAAGC	AAAACA	ATG GAG TTC AGT	198
				Met Glu Phe Ser	
				1	
TTG AAA AAC GAA CAA CAA CTC TTG TCG AAG ATG GCA ACC	240				
Leu Lys Asn Glu Gln Gln Gln Leu Leu Ser Lys Met Ala Thr					
5		10		15	

AAC	GAT	GGA	CAT	GGC	GAA	AAC	TCG	CCT	TAT	TTT	GAT	GGT	TGG	282
Asn	Asp	Gly	His	Gly	Glu	Asn	Ser	Pro	Tyr	Phe	Asp	Gly	Trp	
	20					25					30			
AAG	GCA	TAT	GAT	AGT	GAT	CCT	TAC	CAT	CCC	ACC	AGA	AAT	CCT	324
Lys	Ala	Tyr	Asp	Ser	Asp	Pro	Tyr	His	Pro	Thr	Arg	Asn	Pro	
		35					40					45		
AAT	GGT	GTT	ATA	CAG	ATG	GGA	CTC	GCA	GAA	AAT	CAG	TTA	TGC	366
Asn	Gly	Val	Ile	Gln	Met	Gly	Leu	Ala	Glu	Asn	Gln	Leu	Cys	
			50					55					60	
TTT	GAT	TTG	ATC	GAG	GAA	TGG	GTT	CTG	AAC	AAT	CCA	GAG	GCT	408
Phe	Asp	Leu	Ile	Glu	Glu	Trp	Val	Leu	Asn	Asn	Pro	Glu	Ala	
				65					70					
TCC	ATT	TGC	ACA	GCA	GAA	GGA	GCG	AAC	AAA	TTC	ATG	GAA	GTT	450
Ser	Ile	Cys	Thr	Ala	Glu	Gly	Ala	Asn	Lys	Phe	Met	Glu	Val	
	75				80					85				
GCT	ATC	TAT	CAA	GAT	TAT	CAT	GGC	TTG	CCA	GAG	TTC	AGA	AAT	492
Ala	Ile	Tyr	Gln	Asp	Tyr	His	Gly	Leu	Pro	Glu	Phe	Arg	Asn	
	90					95					100			
GCT	GTA	GCA	AGG	TTC	ATG	GAG	AAG	GTG	AGA	GGT	GAC	AGA	GTC	534
Ala	Val	Ala	Arg	Phe	Met	Glu	Lys	Val	Arg	Gly	Asp	Arg	Val	
		105					110					115		
AAG	TTC	GAT	CCC	AAC	CGC	ATT	GTG	ATG	AGT	GGT	GGG	GCA	ACC	576
Lys	Phe	Asp	Pro	Asn	Arg	Ile	Val	Met	Ser	Gly	Gly	Ala	Thr	
			120					125					130	
GGA	GCT	CAT	GAA	ACT	CTG	GCC	TTC	TGT	TTA	GCT	GAC	CCT	GAA	618
Gly	Ala	His	Glu	Thr	Leu	Ala	Phe	Cys	Leu	Ala	Asp	Pro	Glu	
				135					140					
GAT	GCG	TTT	TTG	GTA	CCC	ACA	CCA	TAT	TAT	CCA	GGA	TTT	GAT	660
Asp	Ala	Phe	Leu	Val	Pro	Thr	Pro	Tyr	Tyr	Pro	Gly	Phe	Asp	
	145				150					155				
CGG	GAT	TTG	AGG	TGG	CGA	ACA	GGG	ATG	CAA	CTT	CTT	CCA	ATT	702
Arg	Asp	Leu	Arg	Trp	Arg	Thr	Gly	Met	Gln	Leu	Leu	Pro	Ile	
	160					165					170			

GTT	TGT	CGC	AGC	TCC	AAT	GAT	TTT	AAG	GTC	ACT	AAA	GAA	TCC	744
Val	Cys	Arg	Ser	Ser	Asn	Asp	Phe	Lys	Val	Thr	Lys	Glu	Ser	
		175					180					185		
ATG	GAA	GCT	GCT	TAT	CAG	AAA	GCT	CAA	GAA	GCC	AAC	ATC	AGA	786
Met	Glu	Ala	Ala	Tyr	Gln	Lys	Ala	Gln	Glu	Ala	Asn	Ile	Arg	
			190					195					200	
GTA	AAG	GGG	TTC	CTC	TTA	AAT	AAT	CCA	TCA	AAT	CCA	TTG	GGA	828
Val	Lys	Gly	Phe	Leu	Leu	Asn	Asn	Pro	Ser	Asn	Pro	Leu	Gly	
				205					210					
ACT	GTT	CTT	GAC	AGG	GAA	ACT	TTG	ATT	GAT	ATA	GTC	ACA	TTC	870
Thr	Val	Leu	Asp	Arg	Glu	Thr	Leu	Ile	Asp	Ile	Val	Thr	Phe	
215					220					225				
ATC	AAT	GAC	AAA	AAT	ATC	CAC	TTG	ATT	TGT	GAT	GAG	ATA	TAT	912
Ile	Asn	Asp	Lys	Asn	Ile	His	Leu	Ile	Cys	Asp	Glu	Ile	Tyr	
	230					235					240			
TCT	GCC	ACC	GTC	TTC	AGC	CAG	CCC	GAA	TTC	ATC	AGC	ATC	TCT	954
Ser	Ala	Thr	Val	Phe	Ser	Gln	Pro	Glu	Phe	Ile	Ser	Ile	Ser	
		245					250					255		
GAA	ATA	ATT	GAG	CAT	GAT	GTT	CAA	TGC	AAC	CGT	GAT	CTC	ATA	996
Glu	Ile	Ile	Glu	His	Asp	Val	Gln	Cys	Asn	Arg	Asp	Leu	Ile	
			260					265					270	
CAT	CTT	GTG	TAT	AGC	CTG	TCC	AAG	GAC	TTG	GGC	TTC	CCT	GGA	1038
His	Leu	Val	Tyr	Ser	Leu	Ser	Lys	Asp	Leu	Gly	Phe	Pro	Gly	
				275					280					
TTC	AGA	GTT	GGC	ATT	TTG	TAT	TCA	TAT	AAT	GAC	GCT	GTT	GTC	1080
Phe	Arg	Val	Gly	Ile	Leu	Tyr	Ser	Tyr	Asn	Asp	Ala	Val	Val	
285					290					295				
AGC	TGT	GCT	AGA	AAA	ATG	TCG	AGT	TTC	GGC	CTT	GTT	TCA	ACA	1122
Ser	Cys	Ala	Arg	Lys	Met	Ser	Ser	Phe	Gly	Leu	Val	Ser	Thr	
	300					305					310			
CAA	ACT	CAG	CAT	CTG	ATT	GCA	TCA	ATG	TTA	TCG	GAC	GAA	GCA	1164
Gln	Thr	Gln	His	Leu	Ile	Ala	Ser	Met	Leu	Ser	Asp	Glu	Ala	
		315					320					325		

TTT	ATG	GAC	AAA	ATC	ATT	TCC	ACG	AGC	TCA	GAG	AGA	TTA	GCT	1206
Phe	Met	Asp	Lys	Ile	Ile	Ser	Thr	Ser	Ser	Glu	Arg	Leu	Ala	
			330					335					340	
GCA	AGG	CAT	GGT	CTT	TTC	ACA	AGA	GGA	CTT	GCT	CAA	GTA	GGC	1248
Ala	Arg	His	Gly	Leu	Phe	Thr	Arg	Gly	Leu	Ala	Gln	Val	Gly	
			345					350						
ATT	GGC	ACC	TTA	AAA	AGC	AGT	GCG	GGC	CTT	TAT	TTC	TGG	ATG	1290
Ile	Gly	Thr	Leu	Lys	Ser	Ser	Ala	Gly	Leu	Tyr	Phe	Trp	Met	
355					360					365				
GAC	TTA	AGG	AGA	CTC	CTC	AGG	GAG	TCC	ACA	TTT	GAG	GCA	GAA	1332
Asp	Leu	Arg	Arg	Leu	Leu	Arg	Glu	Ser	Thr	Phe	Glu	Ala	Glu	
	370					375					380			
ATG	GAA	CTT	TGG	AGG	ATC	ATA	ATA	CAT	GAA	GTC	AAG	CTC	AAT	1374
Met	Glu	Leu	Trp	Arg	Ile	Ile	Ile	His	Glu	Val	Lys	Leu	Asn	
		385					390					395		
GTT	TCA	CCA	GGC	TTA	TCT	TTC	CAT	TGC	TCA	GAA	CCA	GGA	TGG	1416
Val	Ser	Pro	Gly	Leu	Ser	Phe	His	Cys	Ser	Glu	Pro	Gly	Trp	
			400					405					410	
TTC	AGA	GTT	TGC	TTT	GCC	AAC	ATG	GAC	GAC	GAA	AGT	GTG	AGA	1458
Phe	Arg	Val	Cys	Phe	Ala	Asn	Met	Asp	Asp	Glu	Ser	Val	Arg	
				415					420					
GTT	GCT	CTC	AGA	AGA	ATC	CAC	AAA	TTT	GTG	CTT	GTT	CAG	GGC	1500
Val	Ala	Leu	Arg	Arg	Ile	His	Lys	Phe	Val	Leu	Val	Gln	Gly	
425					430					435				
AAG	GCA	ACA	GAG	CCA	ACA	ACT	CCA	AAG	AGT	CGC	TGC	GGA	AGC	1542
Lys	Ala	Thr	Glu	Pro	Thr	Thr	Pro	Lys	Ser	Arg	Cys	Gly	Ser	
	440					445					450			
AGC	AAA	CTT	CAA	CTC	AGC	TTA	TCT	TTC	CGC	AGA	TTG	GAC	GAA	1584
Ser	Lys	Leu	Gln	Leu	Ser	Leu	Ser	Phe	Arg	Arg	Leu	Asp	Glu	
		455					460					465		
AGG	GTG	ATG	GGA	TCG	CAT	ATG	ATG	TCC	CCT	CAC	TCC	CCG	ATG	1626
Arg	Val	Met	Gly	Ser	His	Met	Met	Ser	Pro	His	Ser	Pro	Met	
			470					475					480	

GCT TCA CCT TTG GTT CGG GCT ACA TAAATCATT CTTGATCAGA 1670
 Ala Ser Pro Leu Val Arg Ala Thr
 485

TCATATAGCA AAGATTCCTG AGTAAATACT CGAAACCCTT TCTGGATAAC 1720
 TGAAAAGAGA GTTGTGATT CTTTGCTGTA TCATACAAAC ACGTTACAGG 1770
 CATTTTTTGG CCATCTGATG CGTGCAAATT GCATCAAATG CTTTTATTAT 1820
 TGTCATATTC ATTTGTGTAC CTTGGTTTTC CTTGCCCTTC AGTCCTCCTT 1870
 GTTTTTTGTT TCTTTGTTAT TATTTTCTTC CAGTTGATCA GTTAAACGAA 1920
 GGAAGCTCAA TTGTTTCAAG CTATTAGTAA CAGATCATTT TGTAATAGCA 1970
 ATAGTTTCAG GATTCTGAAA TGAAAGTTTA TCATTTTCC ATCATTTTAA 2020
 AAAAAAAAAA AAAAAAAAAA 2040

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 318 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 46..1003

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

Met	Ala	Thr	Phe	Pro	Leu	Ile	Asp	Met	Glu	Lys	Leu	Asp	Gly	Glu
1				5					10					15
Glu	Arg	Ala	Ala	Thr	Met	Gly	Val	Ile	Lys	Asp	Ala	Cys	Glu	Ser
				20					25					30
Trp	Gly	Phe	Phe	Glu	Val	Leu	Asn	His	Gly	Ile	Ser	Asn	Glu	Leu
				35					40					45
Met	Asp	Thr	Val	Glu	Arg	Leu	Thr	Lys	Glu	His	Tyr	Lys	Lys	Cys
				50					55					60

Met	Glu	Leu	Lys	Phe	Lys	Glu	Met	Val	Glu	Ser	Lys	Glu	Leu	Glu		65	70	75
Ala	Val	Gln	Thr	Glu	Ile	Asn	Asp	Leu	Asp	Trp	Glu	Ser	Thr	Phe		80	85	90
Phe	Leu	Arg	His	Leu	Pro	Val	Ser	Asn	Ile	Ser	Glu	Val	Pro	Asp		95	100	105
Leu	Asp	Asp	Glu	Tyr	Arg	Lys	Val	Met	Lys	Glu	Phe	Ala	Leu	Gln		110	115	120
Leu	Glu	Lys	Leu	Ala	Glu	Leu	Leu	Leu	Asp	Leu	Leu	Cys	Glu	Asn		125	130	135
Leu	Gly	Leu	Glu	Lys	Gly	Tyr	Leu	Lys	Lys	Ala	Phe	Tyr	Gly	Thr		140	145	150
Lys	Gly	Pro	Thr	Phe	Gly	Thr	Lys	Val	Ser	Asn	Tyr	Pro	Pro	Cys		155	160	165
Pro	Arg	Pro	Glu	Leu	Ile	Lys	Gly	Leu	Arg	Ala	His	Thr	Asp	Ala		170	175	180
Gly	Gly	Ile	Ile	Leu	Leu	Phe	Gln	Asp	Asp	Lys	Val	Ser	Gly	Leu		185	190	195
Gln	Leu	Leu	Lys	Asp	Gly	Glu	Trp	Val	Asp	Val	Pro	Pro	Met	Arg		200	205	210
His	Ser	Ile	Val	Ile	Asn	Ile	Gly	Asp	Gln	Leu	Glu	Val	Ile	Thr		215	220	225
Asn	Gly	Lys	Tyr	Lys	Ser	Val	Met	His	Arg	Val	Ile	Ala	Gln	Pro		230	235	240
Asp	Gly	Asn	Arg	Met	Ser	Leu	Ala	Ser	Phe	Tyr	Asn	Pro	Gly	Ser		245	250	255
Asp	Ala	Val	Ile	Tyr	Pro	Ala	Pro	Ala	Leu	Val	Glu	Lys	Glu	Ala		260	265	270
Glu	Asp	Lys	Gln	Ile	Tyr	Pro	Lys	Phe	Val	Phe	Glu	Asp	Tyr	Met		275	280	285
Lys	Leu	Tyr	Ala	Gly	Leu	Lys	Phe	Gln	Ala	Lys	Glu	Pro	Arg	Phe		290	295	300
Glu	Ala	Met	Lys	Ala	Val	Glu	Ser	Thr	Val	Asn	Leu	Gly	Pro	Ile		305	310	315
Ala	Thr	Val														318		

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1320 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix)Feature:

(A)NAME/KEY:CDS

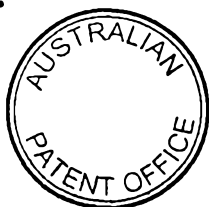
(B)LOCATION:46..1003

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGTAAACGAA GCATAAGCAC AAGCAAACAC AAAC TAGAAA GAGAG ATG	48
Met	
1	
GCT ACA TTC CCC CTA ATC GAC ATG GAG AAG CTT GAC GGT GAA	90
Ala Thr Phe Pro Leu Ile Asp Met Glu Lys Leu Asp Gly Glu	
5 10 15	
GAG AGG GCT GCC ACT ATG GGA GTC ATA AAA GAT GCT TGT GAA	132
Glu Arg Ala Ala Thr Met Gly Val Ile Lys Asp Ala Cys Glu	
20 25	
AGC TGG GGC TTC TTT GAG GTG TTG AAT CAT GGG ATA TCT AAT	174
Ser Trp Gly Phe Phe Glu Val Leu Asn His Gly Ile Ser Asn	
30 35 40	
GAG CTC ATG GAC ACA GTG GAG AGG CTA ACA AAG GAG CAT TAC	216
Glu Leu Met Asp Thr Val Glu Arg Leu Thr Lys Glu His Tyr	
45 50 55	
AAG AAA TGT ATG GAA CTA AAG TTC AAG GAA ATG GTG GAG AGC	258
Lys Lys Cys Met Glu Leu Lys Phe Lys Glu Met Val Glu Ser	
60 65 70	
AAG GAA TTG GAA GCT GTT CAG ACT GAG ATC AAT GAT TTG GAC	300
Lys Glu Leu Glu Ala Val Gln Thr Glu Ile Asn Asp Leu Asp	
75 80 85	

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A substantially pure ACC synthase from a coffee plant comprising the amino acid sequence: (SEQ ID NO:10).
2. The ACC synthase of claim 1, wherein the coffee plant is *Coffea arabica*.
3. A substantially pure nucleic acid sequence that codes on expression for an ACC synthase produced by a coffee plant and comprising the nucleotide sequence set forth in (SEQ ID NO:11).
4. A substantially pure nucleic acid sequence that codes on expression for an ACC synthase produced by a coffee plant, wherein the ACC synthase has the amino acid sequence: (SEQ ID NO:10).
5. A substantially pure ACC oxidase from a coffee plant comprising the amino acid sequence: (SEQ ID NO:12).
6. The ACC oxidase of claim 5, wherein the coffee plant is *Coffea arabica*.
7. A substantially pure nucleic acid sequence that codes on expression for an ACC oxidase produced by a coffee plant and comprising the nucleotide sequence set forth in (SEQ ID NO:13).
8. A substantially pure nucleic acid sequence that codes on expression for an ACC oxidase produced by a coffee plant, wherein the ACC oxidase has the amino acid sequence: (SEQ ID NO:12).
9. The nucleic acid sequence of any of claims 3, 4, 7 or 8, wherein the coffee plant is *Coffea arabica*.
10. A coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10), wherein the RNA has a length sufficient to interfere with the expression of the ACC synthase.
11. A coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12), wherein the RNA has a length sufficient to interfere with the expression of the ACC oxidase.



12. A coffee plant transformed with (I) the nucleic acid sequence according to claim 7, and (ii) the nucleic acid sequence according to claim 8.
13. A coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10), wherein the RNA has a length sufficient to interfere with the expression of the ACC synthase.
14. A coffee plant transformed with a nucleic acid sequence isolated from a coffee plant, wherein the nucleic acid sequence codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12), wherein the RNA has a length sufficient to interfere with the expression of the ACC oxidase.
15. A coffee plant transformed with (I) the nucleic acid sequence according to claim 13, and (ii) the nucleic acid sequence according to claim 14.
16. A coffee plant transformed with an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10).
17. The coffee plant of claim 16, wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation.
18. The coffee plant of claim 16, wherein the nucleic acid sequence is operably linked to a transcription promoter in a sense orientation.
19. A coffee plant transformed with an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).
20. The coffee plant of claim 19, wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation.
21. The coffee plant of claim 19, wherein the nucleic acid sequence is operably linked to a transcription promoter in a sense orientation.
22. A coffee plant transformed with a first isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10), and a second isolated nucleic acid comprising the nucleotide sequence that codes on expression for the amino acid sequence (SEQ ID NO:12).
23. The coffee plant of claim 22, wherein at least one of the first or second nucleic acid sequences is operably linked to a transcription promoter in an antisense orientation.



24. A transformed coffee plant produced by the process of inserting into the plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10).
25. A transformed coffee plant produced by the process of inserting into the plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12).
26. A transformed coffee plant produced by the process of inserting into the plant genome (i) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10), and (ii) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is antisense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12).
27. A transformed coffee plant produced by the process of inserting into the plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10).
28. A transformed coffee plant produced by the process of inserting into the plant genome a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12).
29. A transformed coffee plant produced by the process of inserting into the plant genome (i) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC synthase having the amino acid sequence: (SEQ ID NO:10), and (ii) a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that is sense to the mRNA that codes on expression for an ACC oxidase having the amino acid sequence: (SEQ ID NO:12).
30. A coffee bean from the coffee plant according to any one of claims 19 through 29.
31. A transforming vector comprising a transcription promoter operably linked to:
 - (a) the nucleic acid sequence: (SEQ ID NO:11); or
 - (b) a nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10); or
 - (c) the nucleic acid sequence: (SEQ ID NO:13); or



- (d) a nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).
32. The transforming vector of claim 31, wherein the nucleic acid sequence is operably linked to the transcription promoter in a sense orientation.
33. The transforming vector of claim 31, wherein the nucleic acid sequence is operably linked to the transcription promoter in an antisense orientation.
34. A coffee plant cell transformed with an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10).
35. The coffee plant cell of claim 34, wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation.
36. The coffee plant cell of claim 34, wherein the nucleic acid sequence is operably linked to a transcription promoter in a sense orientation.
37. A coffee plant cell transformed with an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).
38. The coffee plant cell of claim 37, wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation.
39. The coffee plant cell of claim 37, wherein the nucleic acid sequence is operably linked to a transcription promoter in a sense orientation.
40. A coffee plant cell transformed with (i) an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10), and (ii) an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).
41. The coffee plant cell of claim 40, wherein at least one of the nucleic acid sequences is operably linked to a transcription promoter in an antisense orientation.
42. The coffee plant cell of claim 40, wherein at least one of the nucleic acid sequences is operably linked to a transcription promoter in a sense orientation.
43. A transformed coffee plant cell produced by the process of inserting a transforming vector into a coffee plant cell, wherein the transforming vector comprises a transcription promoter operably linked to (i) an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10), or (ii) an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).



44. The transformed coffee plant cell of claim 43, wherein the nucleic acid sequence is operably linked to the transcription promoter in a sense orientation.
45. The transformed coffee plant cell of claim 43, wherein the nucleic acid sequence is operably linked to the transcription promoter in an antisense orientation.
46. A transformed coffee plant cell produced by the process of inserting a first transforming vector and a second transforming vector into a coffee plant cell, wherein the first transforming vector comprises a transcription promoter operably linked to an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:10), and the second transforming vector comprises a transcription promoter operably linked to an isolated nucleic acid sequence that codes on expression for the amino acid sequence: (SEQ ID NO:12).
47. The transformed coffee plant cell of claim 46, wherein at least one of the nucleic acid sequences is operably linked to its transcription promoter in an antisense orientation.
48. A coffee plant regenerated from the transformed coffee plant cell according to any of claims 34 through 47.
49. A coffee bean from any of the coffee plants of claim 48.
50. A method for transforming a coffee plant cell with a nucleic acid sequence that codes on transcription for an RNA that is antisense to the mRNA that codes for an ACC synthase from a coffee plant, comprising the steps of:

providing a transforming vector comprising a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC synthase having the amino acid sequence: (SEQ ID NO:10), wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation; and

inserting the transforming vector into a coffee plant cell, wherein the nucleic acid sequence thereafter becomes inserted into the genome of the coffee plant cell.

51. A method for transforming a coffee plant cell with a nucleic acid sequence that codes on transcription for an RNA that is antisense to the mRNA that codes for an ACC oxidase from a coffee plant, comprising the steps of:

providing a transforming vector comprising a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC oxidase having the amino acid sequence: (SEQ ID NO:12),



wherein the nucleic acid sequence is operably linked to a transcription promoter in an antisense orientation; and

inserting the transforming vector into a coffee plant cell, wherein the nucleic acid sequence thereafter becomes inserted into the genome of the coffee plant cell.

52. A method for transforming a coffee plant cell with (i) a nucleic acid sequence that codes on transcription for an RNA that is antisense to the mRNA that codes for an ACC synthase from a coffee plant, and (ii) a nucleic acid sequence that codes on transcription for an RNA that is antisense to the mRNA that codes for an ACC oxidase from a coffee plant, comprising the steps of:

providing a first transforming vector comprising a first transcription promoter operably linked to a first nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC synthase having the amino acid sequence: (SEQ ID NO:10),

providing a second transforming vector comprising a second transcription promoter operably linked to a second nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC oxidase having the amino acid sequence: (SEQ ID NO:12),

wherein at least one of the first or second nucleic acid sequences is operably linked to its transcription promoter in an antisense orientation; and

inserting each of the transforming vectors into a coffee plant cell, wherein each of the nucleic acid sequences thereafter becomes inserted into the genome of the coffee plant cell.

53. A method for transforming a coffee plant cell with a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC synthase from a coffee plant, comprising the steps of:

providing a transforming vector comprising a transcription promoter operably linked to a nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC synthase having the amino acid sequence: (SEQ ID NO:10), wherein the nucleic acid sequence is operably linked to the promoter in a sense orientation; and

inserting the transforming vector into a coffee plant cell, wherein the nucleic acid sequence thereafter become inserted into the genome of the coffee plant cell.



54. A method for transforming a coffee plant cell with a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC oxidase from a coffee plant, comprising the steps of:

providing a transforming vector comprising a transcription promoter operably linked to a nucleic acid sequence from a coffee plant that codes on for an RNA that has a length sufficient to interfere with the expression of an ACC oxidase having the amino acid sequence: (SEQ ID NO:12), wherein the nucleic acid sequence is operably linked to the transcription promoter in a sense orientation; and

inserting the transforming vector into a coffee plant cell, wherein the nucleic acid sequence thereafter become inserted into the genome of the coffee plant cell.

55. A method for transforming a coffee plant cell with (i) a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC synthase from a coffee plant, and (ii) a nucleic acid sequence that codes on transcription for an RNA that is sense in orientation to the mRNA that codes for an ACC oxidase from a coffee plant, comprising the steps of:

providing a first transforming vector comprising a first transcription promoter operably linked to a first nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC synthase having the amino acid sequence: (SEQ ID NO:10),

providing a second transforming vector comprising a second transcription promoter operably linked to a second nucleic acid sequence isolated from a coffee plant that codes on transcription for an RNA that has a length sufficient to interfere with the expression of an ACC oxidase having the amino acid sequence: (SEQ ID NO:12),

wherein at least one of the nucleic acid sequences is operably linked to its transcription promoter in a sense orientation; and

inserting each of the transforming vectors into a coffee plant cell, wherein each of the nucleic acid sequences thereafter becomes inserted into the genome of the coffee plant cell.

DATED: 28 January, 1999

PHILLIPS ORMONDE & FITZPATRICK

Attorneys for:

UNIVERSITY OF HAWAII



Fig 1: DEDUCED AMINO ACID SEQUENCE OF ACC SYNTHASE FROM *COFFEA ARABICA* (SEQ. ID. NO. 10)

	Met	Glu	Phe	Ser	Leu	Lys	Asn	Glu	Gln	Gln	Gln	Leu	Leu	Ser	Lys		
	1					5						10			15		
5																	
	Met	Ala	Thr	Asn	Asp	Gly	His	Gly	Glu	Asn	Ser	Pro	Tyr	Phe	Asp	Gly	
						20					25				30		
	Trp	Lys	Ala	Tyr	Asp	Ser	Asp	Pro	Tyr	His	Pro	Thr	Arg	Asn	Pro	Asn	
10						35					40				45		
	Gly	Val	Ile	Gln	Met	Gly	Leu	Ala	Glu	Asn	Gln	Leu	Cys	Phe	Asp	Leu	
						50					55				60		
15	Ile	Glu	Glu	Trp	Val	Leu	Asn	Asn	Pro	Glu	Ala	Ser	Ile	Cys	Thr	Ala	
						65					70				75		
	Glu	Gly	Ala	Asn	Lys	Phe	Met	Glu	Val	Ala	Ile	Tyr	Gln	Asp	Tyr	His	
						80					85				90	95	
20																	
	Gly	Leu	Pro	Glu	Phe	Arg	Asn	Ala	Val	Ala	Arg	Phe	Met	Glu	Lys	Val	
											100				105	110	
	Arg	Gly	Asp	Arg	Val	Lys	Phe	Asp	Pro	Asn	Arg	Ile	Val	Met	Ser	Gly	
25																	
						115					120				125		

Gly Ala Thr Gly Ala His Glu Thr Leu Ala Phe Cys Leu Ala Asp Pro
 130 135 140

5 Glu Asp Ala Phe Leu Val Pro Thr Pro Tyr Tyr Pro Gly Phe Asp Arg
 145 150 155

Asp Leu Arg Trp Arg Thr Gly Met Gln Leu Leu Pro Ile Val Cys Arg
 160 165 170 175

10 Ser Ser Asn Asp Phe Lys Val Thr Lys Glu Ser Met Glu Ala Ala Tyr
 180 185 190

15 Gln Lys Ala Gln Glu Ala Asn Ile Arg Val Lys Gly Phe Leu Leu Asn
 195 200 205

Asn Pro Ser Asn Pro Leu Gly Thr Val Leu Asp Arg Glu Thr Leu Ile
 210 215 220

20 Asp Ile Val Thr Phe Ile Asn Asp Lys Asn Ile His Leu Ile Cys Asp
 225 230 235

Glu Ile Tyr Ser Ala Thr Val Phe Ser Gln Pro Glu Phe Ile Ser Ile
 240 245 250 255

25 Ser Glu Ile Ile Glu His Asp Val Gln Cys Asn Arg Asp Leu Ile His
 260 265 270

Leu Val Tyr Ser Leu Ser Lys Asp Leu Gly Phe Pro Gly Phe Arg Val
 275 280 285

5 Gly Ile Leu Tyr Ser Tyr Asn Asp Ala Val Val Ser Cys Ala Arg Lys
 290 295 300

Met Ser Ser Phe Gly Leu Val Ser Thr Gln Thr Gln His Leu Ile Ala
 305 310 315

10 Ser Met Leu Ser Asp Glu Ala Phe Met Asp Lys Ile Ile Ser Thr Ser
 320 325 330 335

Ser Glu Arg Leu Ala Ala Arg His Gly Leu Phe Thr Arg Gly Leu Ala
 340 345 350

15 Gln Val Gly Ile Gly Thr Leu Lys Ser Ser Ala Gly Leu Tyr Phe Trp
 355 360 365

Met Asp Leu Arg Arg Leu Leu Arg Glu Ser Thr Phe Glu Ala Glu Met
 370 375 380

20 Glu Leu Trp Arg Ile Ile Ile His Glu Val Lys Leu Asn Val Ser Pro
 385 390 395

25 Gly Leu Ser Phe His Cys Ser Glu Pro Gly Trp Phe Arg Val Cys Phe
 400 405 410 415

Ala Asn Met Asp Asp Glu Ser Val Arg Val Ala Leu Arg Arg Ile His
420 425 430

Lys Phe Val Leu Val Gln Gly Lys Ala Thr Glu Pro Thr Thr Pro Lys
5 435 440 445

Ser Arg Cys Gly Ser Ser Lys Leu Gln Leu Ser Leu Ser Phe Arg Arg
450 455 460

10 Leu Asp Glu Arg Val Met Gly Ser His Met Met Ser Pro His Ser Pro
465 470 475

Met Ala Ser Pro Leu Val Arg Ala Thr
480 485

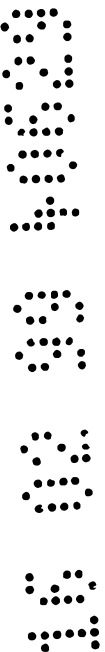


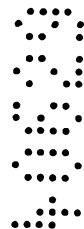
Fig 2.: Coffee fruit-expressed ACC synthase gene sequence. (SEQ. ID. NO. 11)

	GTAATCTCTT CTAAATCAA CCATTCTCTT CATTCTTCAC TTGACAAGGC CACTGCATTC	60
	TTCATTCTTT CTTGATATAT AGCCATTTTT TTCATTCTTT CTTGATATAT AGCCATTTTT	120
5	TTCATTCTTT CTTCAATCAT TGTCTGGAGA AGTTGGTTGA GTTTCTTGA AAATTCAAGC	180
	AAAACA ATG GAG TTC AGT TTG AAA AAC GAA CAA CAA CAA CTC TTG TCG AAG	231
	ATG GCA ACC AAC GAT GGA CAT GGC GAA AAC TCG CCT TAT TTT GAT GGT	279
10	TGG AAG GCA TAT GAT AGT GAT CCT TAC CAT CCC ACC AGA AAT CCT AAT	327
	GGT GTT ATA CAG ATG GGA CTC GCA GAA AAT CAG TTA TGC TTT GAT TTG	375
15	ATC GAG GAA TGG GTT CTG AAC AAT CCA GAG GCT TCC ATT TGC ACA GCA	423
	GAA GGA GCG AAC AAA TTC ATG GAA GTT GCT ATC TAT CAA GAT TAT CAT	471
	GGC TTG CCA GAG TTC AGA AAT GCT GTA GCA AGG TTC ATG GAG AAG GTG	519
20	AGA GGT GAC AGA GTC AAG TTC GAT CCC AAC CGC ATT GTG ATG AGT GGT	567
	GGG GCA ACC GGA GCT CAT GAA ACT CTG GCC TTC TGT TTA GCT GAC CCT	615
25	GAA GAT GCG TTT TTG GTA CCC ACA CCA TAT TAT CCA GGA TTT GAT CGG	663
	GAT TTG AGG TGG CGA ACA GGG ATG CAA CTT CTT CCA ATT GTT TGT CGC	711
	AGC TCC AAT GAT TTT AAG GTC ACT AAA GAA TCC ATG GAA GCT GCT TAT	759
30	CAG AAA GCT CAA GAA GCC AAC ATC AGA GTA AAG GGG TTC CTC TTA AAT	807

	AAT CCA TCA AAT CCA TTG GGA ACT GTT CTT GAC AGG GAA ACT TTG ATT	855
	GAT ATA GTC ACA TTC ATC AAT GAC AAA AAT ATC CAC TTG ATT TGT GAT	903
5	GAG ATA TAT TCT GCC ACC GTC TTC AGC CAG CCC GAA TTC ATC AGC ATC	951
	TCT GAA ATA ATT GAG CAT GAT GTT CAA TGC AAC CGT GAT CTC ATA CAT	999
	CTT GTG TAT AGC CTG TCC AAG GAC TTG GGC TTC CCT GGA TTC AGA GTT	1047
10	GGC ATT TTG TAT TCA TAT AAT GAC GCT GTT GTC AGC TGT GCT AGA AAA	1095
	ATG TCG AGT TTC GGC CTT GTT TCA ACA CAA ACT CAG CAT CTG ATT GCA	1143
15	TCA ATG TTA TCG GAC GAA GCA TTT ATG GAC AAA ATC ATT TCC ACG AGC	1191
	TCA GAG AGA TTA GCT GCA AGG CAT GGT CTT TTC ACA AGA GGA CTT GCT	1239
	CAA GTA GGC ATT GGC ACC TTA AAA AGC AGT GCG GGC CTT TAT TTC TGG	1287
20	ATG GAC TTA AGG AGA CTC CTC AGG GAG TCC ACA TTT GAG GCA GAA ATG	1335
	GAA CTT TGG AGG ATC ATA ATA CAT GAA GTC AAG CTC AAT GTT TCA CCA	1383
25	GGC TTA TCT TTC CAT TGC TCA GAA CCA GGA TGG TTC AGA GTT TGC TTT	1431
	GCC AAC ATG GAC GAC GAA AGT GTG AGA GTT GCT CTC AGA AGA ATC CAC	1479
	AAA TTT GTG CTT GTT CAG GGC AAG GCA ACA GAG CCA ACA ACT CCA AAG	1527
30	AGT CGC TGC GGA AGC AGC AAA CTT CAA CTC AGC TTA TCT TTC CGC AGA	1575

	TTG GAC GAA AGG GTG ATG GGA TCG CAT ATG ATG TCC CCT CAC TCC CCG	1623
	ATG GCT TCA CCT TTG GTT CGG GCT ACA TAAATCATTT CTTGATCAGA TCATATAGCA	1680
5	AAGATTCCTG AGTAAATACT CGAAACCCCTT TCTGGATAAC TGAAAAGAGA GTTGTGATT	1740
	CTTTGCTGTA TCATACAAAC ACGTTACAGG CATTTTTTGG CCATCTGATG CGTGCAAATT	1800
	GCATCAAATG CTTTATTAT TGTATATTC ATTTGTGTAC CTTGGTTTTT CTTGCCCTTC	1860
	AGTCCTCCTT GTTTTTGTT TCTTGTAT TATTTCTTC CAGTTGATCA GTTAAACGAA	1920
	GGAAGCTCAA TTGTTCAAG CTATTAGTAA CAGATCATTT TGTAATAGCA ATAGTTTCAG	1980
10	GATTCTGAAA TGAAAGTTTA TCATTTTCC ATCATTTTAA AAAAAAAAAA AAAAAAAAAA	2040

Note: The coding portion of this sequence is shown by grouping the bases as codons.



115

[illegible]

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Phe Ala Leu Gln Leu Glu Lys Leu Ala Glu Leu Leu Leu Asp Leu Leu
120 125 130

5 Cys Glu Asn Leu Gly Leu Glu Lys Gly Tyr Leu Lys Lys Ala Phe Tyr
135 140 145

Gly Thr Lys Gly Pro Thr Phe Gly Thr Lys Val Ser Asn Tyr Pro Pro
150 155 160

10 Cys Pro Arg Pro Glu Leu Ile Lys Gly Leu Arg Ala His Thr Asp Ala
165 170 175 180

15 Gly Gly Ile Ile Leu Leu Phe Gln Asp Asp Lys Val Ser Gly Leu Gln
185 190 195

Leu Leu Lys Asp Gly Glu Trp Val Asp Val Pro Pro Met Arg His Ser
200 205 210

20 Ile Val Ile Asn Ile Gly Asp Gln Leu Glu Val Ile Thr Asn Gly Lys
215 220 225

Tyr Lys Ser Val Met His Arg Val Ile Ala Gln Pro Asp Gly Asn Arg
230 235 240

25 Met Ser Leu Ala Ser Phe Tyr Asn Pro Gly Ser Asp Ala Val Ile Tyr
245 250 255 260

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Pro Ala Pro Ala Leu Val Glu Lys Glu Ala Glu Asp Lys Gln Ile Tyr
265 270 275

Pro Lys Phe Val Phe Glu Asp Tyr Met Lys Leu Tyr Ala Gly Leu Lys
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Phe Gln Ala Lys Glu Pro Arg Phe Glu Ala Met Lys Ala Val Glu Ser
295 300 305

10 Thr Val Asn Leu Gly Pro Ile Ala Thr Val
310 315

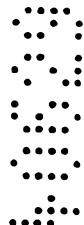


Fig 4: DNA SEQUENCE OF THE COFFEE FRUIT-EXPRESSED ACC OXIDASE CDNA.
(SEQ. ID. NO. 13)

	TGTAACGAA GCATAAGCAC AAGCAAACAC AACTAGAAA GAGAG ATG GCT ACA TTC	57
5	CCC CTA ATC GAC ATG GAG AAG CTT GAC GGT GAA GAG AGG GCT GCC ACT	105
	ATG GGA GTC ATA AAA GAT GCT TGT GAA AGC TGG GGC TTC TTT GAG GTG	153
	TTG AAT CAT GGG ATA TCT AAT GAG CTC ATG GAC ACA GTG GAG AGG CTA	201
10	ACA AAG GAG CAT TAC AAG AAA TGT ATG GAA CTA AAG TTC AAG GAA ATG	249
	GTG GAG AGC AAG GAA TTG GAA GCT GTT CAG ACT GAG ATC AAT GAT TTG	297
15	GAC TGG GAA AGT ACC TTC TTC TTG CGC CAT CTT CCT GTT TCC AAC ATC	345
	TCA GAA GTC CCT GAT CTT GAT GAT GAA TAC AGA AAG GTT ATG AAG GAA	393
	TTT GCG TTG CAA CTT GAG AAA CTA GCA GAG CTC CTG TTG GAC TTG CTA	441
20	TGC GAG AAC CTT GGC CTA GAG AAA GGC TAT CTG AAG AAA GCC TTC TAT	489
	GGC ACC AAA GGA CCA ACC TTT GGC ACC AAA GTC AGC AAT TAC CCT CCA	537
25	TGC CCT CGT CCA GAA CTG ATC AAG GGC CTC CGG GCA CAC ACC GAT GCC	585
	GGC GGC ATC ATC CTG CTG TTC CAG GAT GAC AAG GTC AGC GGT CTC CAG	633
	CTC CTC AAG GAT GGT GAA TGG GTG GAT GTT CCG CCT ATG CGC CAC TCC	681
30	ATT GTA ATC AAC ATC GGC GAC CAA CTT GAG GTA ATC ACA AAT GGA AAA	729

	TAC AAG AGT GTG ATG CAC CGG GTG ATA GCT CAA CCA GAT GGG AAC AGA	777
	ATG TCA CTA GCA TCA TTC TAC AAT CCA GGA AGT GAT GCA GTG ATC TAT	825
5	CCA GCA CCG GCA TTG GTT GAG AAA GAG GCA GAG GAC AAG CAG ATA TAT	873
	CCC AAG TTT GTG TTC GAG GAC TAC ATG AAG CTC TAT GCT GGC CTT AAG	921
	TTC CAA GCT AAA GAG CCC AGG TTT GAA GCC ATG AAG GCC GTG GAA AGC	969
10	ACC GTA AAC TTG GGT CCA ATC GCA ACT GTT TGAGATAATA CACGCTTTGA	1019
	TCTGCTGCTG TCTTATAATG CGCGTTTGCG TAATCATATC CTAGCATAGT ATATCTGAGA	1079
15	TCTGAGTCTG TATTGTGGTG TGAGTTTGGT TTAGCCCCCTT GTTAATGCTT GGATTGGACT	1139
	AGTTAAATGT GGAGCTGGTT TGTTAGATAA GATAGTCTTG CCAGGATCTT TGAGTAAATA	1199
	TGATTCTGCG GAAGTCTGCG GTGAATGATA ACGTGTAAG CAATCCGAAA GTTACCTTTC	1259
20	TGGGGCTTTG TCATATGCAA TGGAGAAGGA ATCTTCCAAA AAAAAAAAAA AAAAAAAAAA	1319
	A	1320
25	Note: The coding portion of this sequence is shown by grouping the bases as codons.	

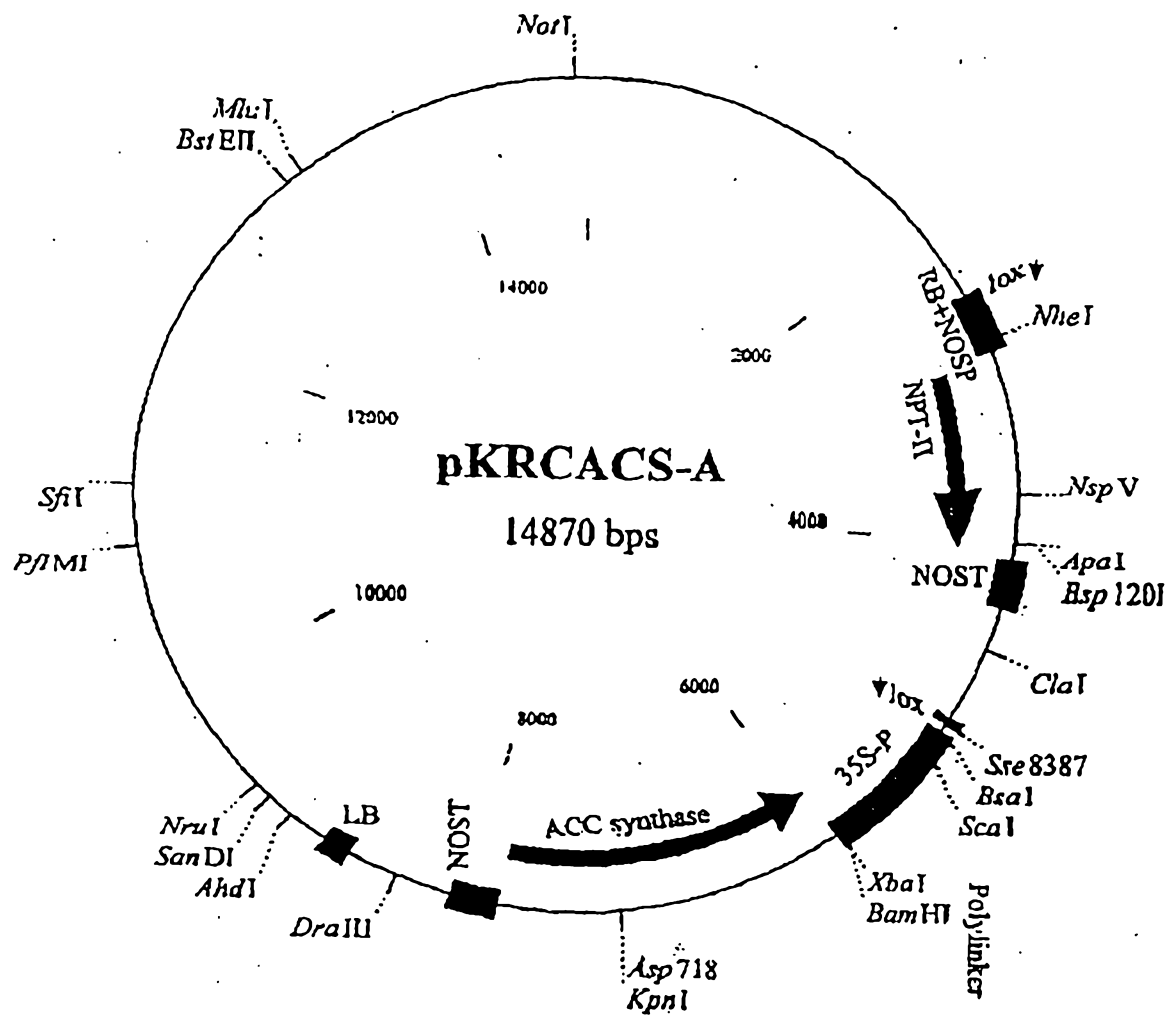


Fig. 5 Coffee ACC synthase antisense cDNA in pKR1

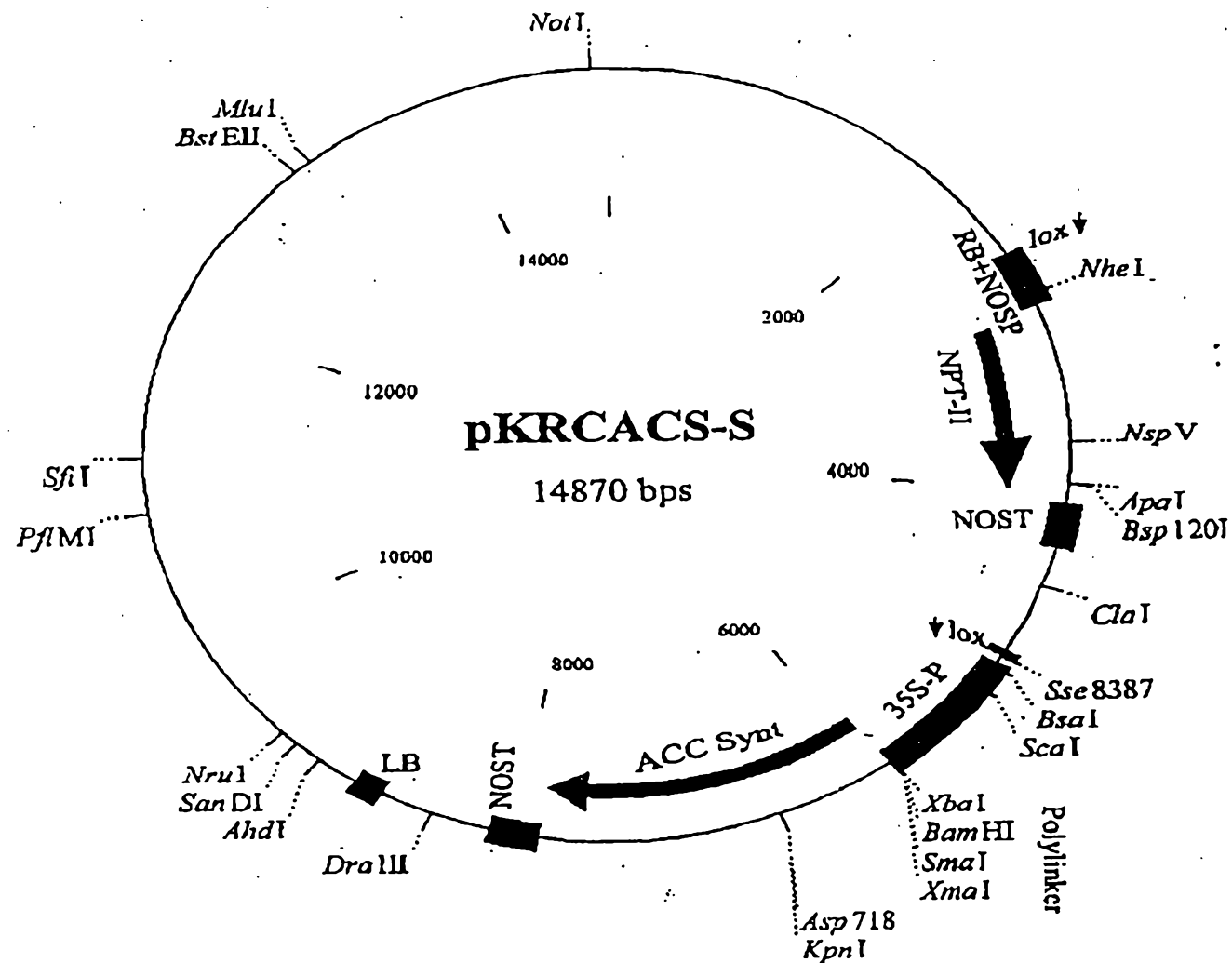


Fig. 6 Coffee ACC synthase sense cDNA in pKR1

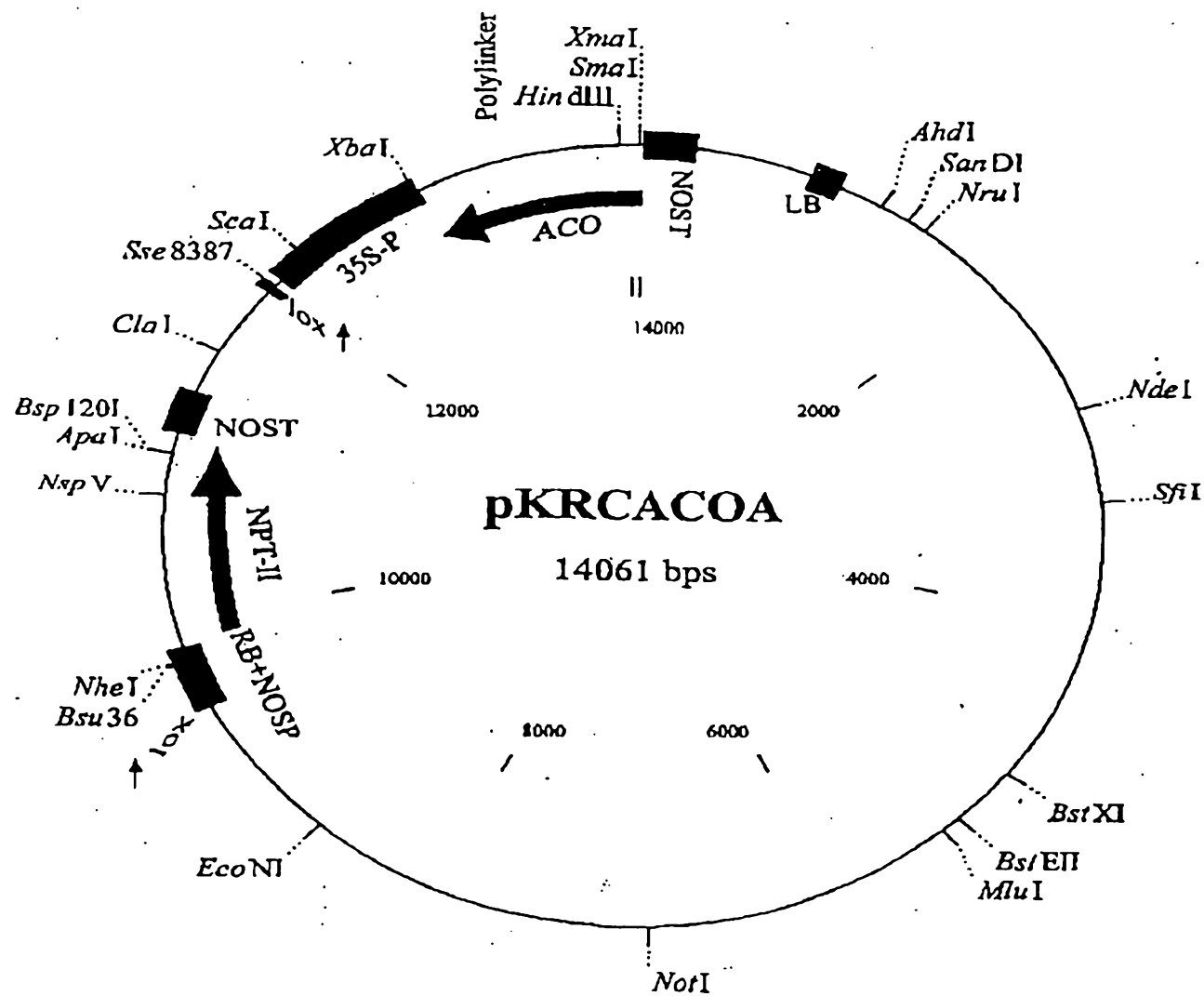


Fig. 7 Coffee ACC oxidase antisense cDNA in pKR1

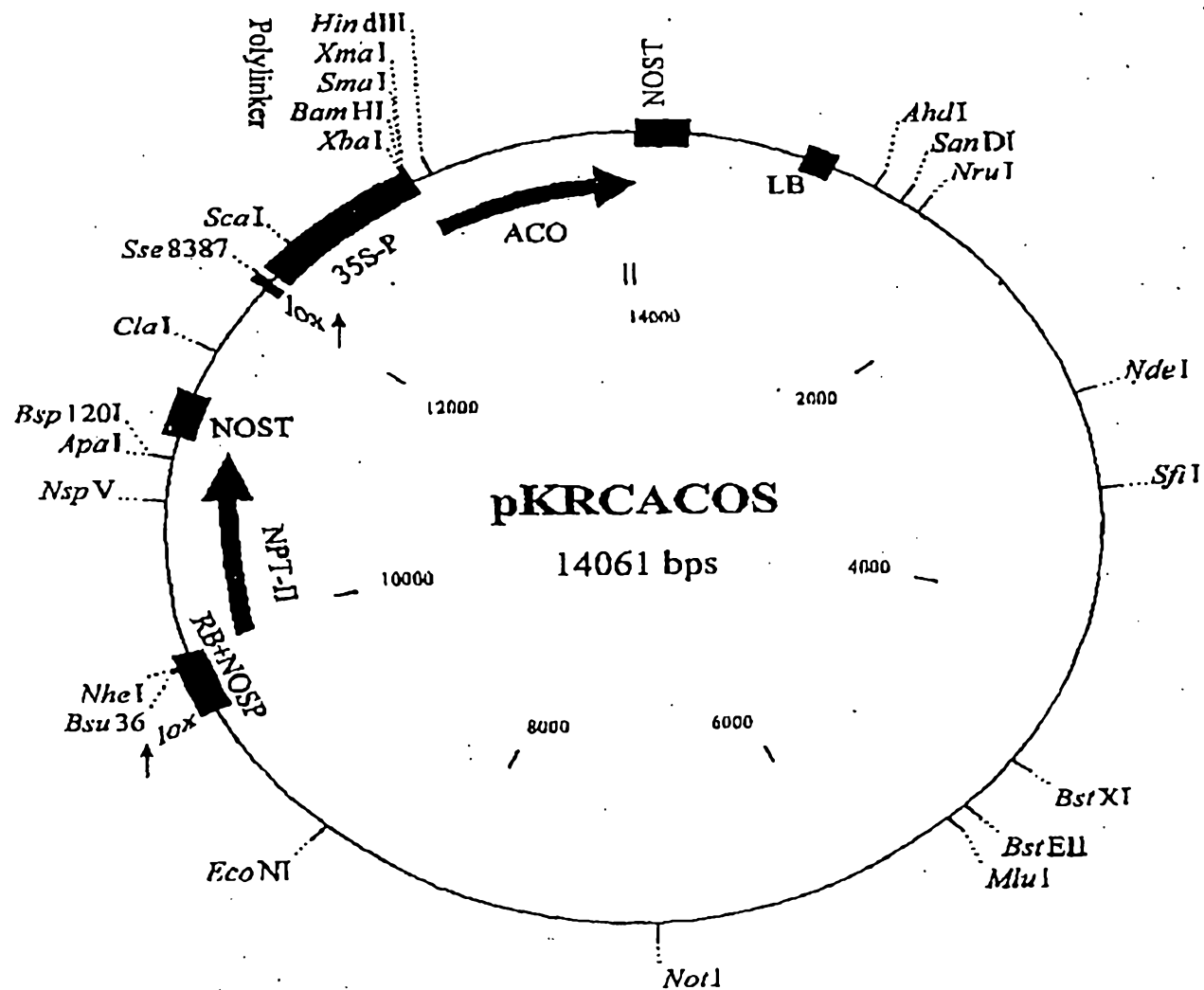


Fig. 8 Coffee ACC oxidase sense cDNA in pKR1