

**REGULATED EXPRESSION OF HETEROLOGOUS GENES IN PLANTS
AND TRANSGENIC FRUIT WITH A MODIFIED RIPENING PHENOTYPE**

FIELD OF THE INVENTION

5 The present invention relates the regulated
expression of heterologous genes in transgenic fruit-
bearing plants, where fruit produced by the these
plants has a modified ripening phenotype. The
invention also relates to the fruit produced by such
10 transgenic plants, to methods of producing the
transgenic plants and to the transgenic plants
themselves.

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BACKGROUND OF THE INVENTION

15 Ethylene is a plant hormone which is a powerful regulator of plant metabolism, acting, and interacting with other plant hormones in trace amounts. Ethylene is a gas under normal physiological conditions. Even at low concentrations, ethylene has profound hormonal
20 effects on plants.

The effects of ethylene, whether produced by the plant itself or applied exogenously, are numerous, dramatic, and of considerable commercial importance. Among the diverse physiological effects are the
25 following: leaf abscission; fading in flowers; flower wilting; leaf yellowing; leaf epinasty; and stimulation of ripening in fruits and vegetables. Ethylene promotes senescence in plants, both in selected groups of cells and in whole organs, such as, fruits, leaves,
30 or flowers. Senescence is the natural, genetically controlled degenerative process which usually leads to death in plants.

Normally, ethylene production from plant tissue is low. Large quantities of ethylene, however, are
35 produced during ripening and senescence processes. A

large amount of ethylene is also produced following trauma caused by chemicals, temperature extremes, water stress, ultraviolet light, insect damage, disease, or mechanical wounding. Ethylene produced by plants under such trauma conditions is referred to as "wound ethylene" or "stress ethylene". In fruits and vegetables, the stimulation of ethylene production by cuts or bruises may be very large and bear considerably on storage effectiveness. Ethylene-induced leaf browning is a common basis for loss in many plants, including lettuce and tobacco. In some tissues, exposure to only a small amount of ethylene may cause an avalanche of ethylene production in adjacent plants or plant tissues such as fresh produce. This autocatalytic effect can be very pronounced and lead to loss of fruit quality during transportation and storage.

Current technologies that specifically address post-harvest storage life have been in existence for decades and are hampered by such problems as high cost, side effects, and an inability to completely shut off ethylene production. Included in this group are controlled atmosphere (CA) storage, chemical treatment, packaging, and irradiation.

CA facilities slow ethylene biosynthesis through: (i) low temperature, (ii) reducing the oxygen level below 3%, and (iii) elevating the carbon dioxide level in the storage area to the 3%-5% range. Expensive scrubbers are sometimes added which reduce ethylene already respired to the atmosphere. Drawbacks are that CA facilities are expensive to construct, have a high utility cost, and are unable to completely eliminate ethylene production and side effects. Also, CA storage techniques can only control external ethylene and not that which resides inside the plant tissue. CA storage

can also lead to undesirable side effects: injury can result from high CO₂ levels, low O₂ levels, or low temperature.

Another treatment is to limit the ethylene biosynthesis in the plant tissue through chemical treatment. Aminoethoxyvinylglycine (AVG), an analog of the antibiotic rhizobitoxine, is one such inhibitor. However, AVG cannot be used as a chemical additive in foods due to its high toxicity. Silver thiosulfate (STS) is also effective in slowing fruit ripening and flower fading, but is also toxic and cannot be used on foods. Further, STS only works with certain flowers and often causes black spotting.

Recently, molecular genetic approaches leading to transgenic plants with impaired biosynthesis of ethylene have been reported. Hamilton, et al., identified a cDNA clone for tomato EFE (pTOM13) by inhibiting ethylene synthesis with an antisense gene expressed in transgenic plants. Oeller, et al., showed that expression of antisense RNA to the rate-limiting enzyme in the biosynthetic pathway of ethylene, 1-aminocyclopropane-1-carboxylate synthase, inhibits fruit ripening in tomato plants. Klee, et al., cloned the gene encoding ACC deaminase, from soil bacteria, and introduced it into tomato plants. Reduction in ethylene synthesis in transgenic plants did not cause any apparent vegetative phenotypic abnormalities. However, fruits from these plants exhibited significant delays in ripening, and the mature fruits remained firm for at least 6 weeks longer than the non-transgenic control fruit.

SUMMARY OF THE INVENTION

The present invention relates to plant transformation vectors, chimeric genes and related DNA

constructs. In one embodiment the invention relates to a transgenic fruit-bearing plant having (i) a DNA sequence encoding a product that is effective to reduce ethylene biosynthesis in fruit from the plant, and (ii) a promoter whose expression is induced during fruit ripening, by a plant cell cytokine, or by ethylene synthesis by the fruit. The DNA sequence is heterologous to the promoter and is operably linked to the promoter to enable expression of said product. The DNA sequence may encode any of the following products: S-adenosylmethionine hydrolase, aminocyclopropane-1-carboxylic acid (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC oxidase cosuppression molecule, and ACC synthase cosuppression molecule.

Promoters useful in the present invention may be selected from a variety of plant sources, including, but not limited to, genes homologous to a tomato E4 or E8 genes, including tomato and raspberry E4 and E8 genes. Exemplary other promoters may be obtained from the avocado cellulase gene, tomato ACC oxidase gene, tomato polygalacturonase gene, or homologs of any of these genes in other plants.

The present invention also includes a method for modifying ripening fruit of a fruit bearing plant. In the method, the transgenic plants described above are grown to produce a transgenic plant bearing fruit. Fruit produced by the plant has an initial burst of ethylene production, followed by a reduction in the level of ethylene synthesis by the fruit, resulting in a fruit having a modified ripening phenotype. Such phenotypes include the delay and/or suspension of fruit ripening, typically relative to wild-type (i.e., non-transgenic) fruit.

In another aspect, the present invention includes the above described transgenic fruit and fruit cells.

Further, the invention includes a method for producing a transgenic fruit-bearing plant, where fruit
5 produced by the plant has a modified ripening phenotype. In this method, the following chimeric gene is introduced (e.g., by transformation) into progenitor cells of the plant: (i) a DNA sequence encoding a product effective to reduce ethylene biosynthesis in
10 fruit from the plant, and (ii) a promoter whose expression is induced during fruit ripening, by a plant cytokine, or by ethylene synthesis by the fruit. As above, the DNA sequence is heterologous to the promoter and is operably linked to said promoter to enable
15 expression of the product. The transformed progenitor are grown cells to produce a transgenic plant bearing fruit. The method further includes transforming progenitor cells of the plant with a selectable vector containing chimeric gene. The DNA sequences and
20 promoters may be as described above.

In another aspect, this method includes isolating useful promoters, typically, employing the following steps: (i) selecting a probe DNA molecule containing a sequence homologous to a region of the promoter of
25 interest, such as from the tomato E4 or E8 genes;
(ii) contacting the probe with a plurality of target DNA molecules derived from the genome of a selected fruit-bearing plant under conditions favoring specific hybridization between the probe molecule and a
30 target molecule homologous to the probe molecule;
(iii) identifying a target molecule having a DNA sequence homologous to the probe; and
(iv) isolating promoter sequences associated with the target molecule.

The invention also includes expression vectors containing the chimeric genes described above. These vectors are useful for transformation of plant cells and may be included in commercial kits.

5 Another embodiment of the present invention is a polypeptide in a plant having (i) a DNA sequence encoding a product that is effective to reduce ethylene biosynthesis in fruit from the plant, and (ii) a promoter whose expression is induced during fruit
10 ripening or by ethylene synthesis by said fruit, where said DNA sequence is heterologous to said promoter and said DNA sequence is operably linked to said promoter to enable expression of said product. In another aspect, the invention includes plant and fruit cells
15 containing such chimeric genes, as well as vectors containing such genes.

One embodiment of the invention is a method for delaying wound-induced ripening of fruit of a fruit-bearing plant. This method includes transforming
20 progenitor cells of the plant with a selectable vector containing (i) a promoter that has the sequence of the tomato E4 gene promoter or the sequence of a promoter for a gene homologous to the tomato E4 gene, and (ii) a heterologous gene, whose product is effective to reduce
25 ethylene biosynthesis in fruit from the plant, which is under the control of the promoter, growing the transformed progenitor cells to produce a transgenic plant bearing fruit, and subjecting the fruit to a wound. The method also includes subjecting the fruit
30 to a wound by picking the fruit from a transgenic plant. In the method, an exemplary heterologous gene is S-adenosylmethionine hydrolase.

The transgenic plant may be selected from the group consisting of, but not limited, to tomato,
35 raspberry, strawberry and melon. The promoter may be

selected from the same group. Further plant species are described in the specification. In one embodiment the promotor is a tomato E4 promoter.

5 In another aspect of the invention, the promoter is isolated by the steps of (i) selecting a probe DNA molecule containing a sequence homologous to a region of tomato E4 gene DNA, (ii) contacting the probe with a plurality of target DNA molecules derived from the genome of a selected fruit-bearing plant under condi-
10 tions favoring specific hybridization between the probe molecule and a target molecule homologous to the probe molecule, (iii) identifying a target molecule having a DNA sequence homologous to tomato E4 gene, and (iv) isolating promoter sequences associated with the target
15 molecule.

The probe molecule may carry a reporter moiety; the identifying step then includes detecting the moiety.

The method also describes isolating the promotor
20 by (i) selecting first and second oligonucleotide primers corresponding to an upstream and a downstream region, respectively, of an E4 gene, (ii) amplifying a region of the E4 gene DNA between the first and second primers to generate probe molecules, (iii) contacting
25 the probe molecules with a plurality of target DNA molecules derived from the genome of a selected fruit-bearing plant under conditions favoring specific hybridization between the probe molecule and a target molecule homologous to the probe molecule, (iv) identifying
30 a target molecule having a DNA sequence homologous to tomato E4 gene, and (v) isolating promoter sequences associated with the target molecule.

The invention also describes a delayed-ripening fruit, containing (i) a heterologous S-adenosylmeth-
35 ionine hydrolase gene, and (ii) a promoter effective to

produce transient expression of the gene when the fruit is picked.

A method is described for inducing transient, wound-induced expression of a heterologous gene in
5 fruit of a fruit-bearing plant, comprising transforming progenitor cells of the plant with a selectable vector containing (i) a promoter that has the sequence of the tomato E4 gene promoter or the sequence of a promoter for a gene homologous to the tomato E4 gene, and (ii) a
10 heterologous gene which is under the control of the promoter, growing the transformed progenitor cells to produce a transgenic plant bearing fruit, and subjecting the fruit to a wound. In this method, expression of the heterologous gene typically reduces ethylene
15 biosynthesis. In one embodiment, the heterologous gene encodes S-adenosylmethionine hydrolase.

In one embodiment, the present invention includes, a method for delaying ripening of the fruit of a fruit-bearing plant. The method includes transforming plant
20 progenitor cells, or host cells, with a selectable vector containing a plant E4 gene promoter and a heterologous gene, such as S-adenosylmethionine hydrolase, whose product reduces ethylene biosynthesis in the plant. The transformed cells are grown to
25 produce a transgenic plant bearing fruit. In a related embodiment, the method is used, essentially as above, to delay senescence in flowers and vegetables. The E4 promoter, and/or the transformed plant, may be selected from a variety of plants, including fruit-bearing
30 plants, such as tomato, eggplant, legumes, raspberry, strawberry, melon, avocado, cherry, apricot, citrus fruits, etc.; flowers, such as roses and carnations; and vegetables, such as cauliflower, and lettuce.

In another aspect, the invention includes a method
35 of isolating E4 promoters from plants. The method

includes selecting an E4 probe, hybridizing it with genomic DNA from selected plant species, identifying DNA or clones from positively-hybridizing targets, and isolating promoter sequences associated with the positive target molecule. Positively-hybridizing targets can be identified by either primary or secondary detection of reporter moieties attached to the probe. The probe can be obtained by a number of methods, including primer-based DNA amplification or isolation of restriction digest fragments.

In another embodiment, the invention includes a delayed-ripening fruit, containing a heterologous gene, such as the S-adenosylmethionine hydrolase gene, and a promoter, such as the E4 promoter, effective to produce transient expression of the heterologous gene when the fruit is picked. The promoter may be obtained from a variety of plants, such as outlined above, using methods of the present invention.

Also included in the present invention is an E4 promoter molecule and a DNA construct containing the promoter molecule operably linked to a heterologous gene, where expression of the heterologous gene is under the control of the promoter DNA molecule.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A schematically illustrates the metabolic reactions for the synthesis of ethylene from methionine under both normal and stress conditions. Figure 1B schematically illustrates the effect of the enzyme SAMase (AdoMetase) on ethylene biosynthesis.

Figure 2 schematically illustrates the steps described for the genetic engineering of the AdoMetase-encoding gene in vector pAG-111.

Figure 3 illustrates the elements of the tomato E8 promoter and the primers used to amplify and isolate the promoter sequences.

Figure 4 outlines the steps involved in the construction of pGA-SESKN from pGA-ESKN and shows the elements of the E8 gene adjacent the AdoMetase (SAMase) coding sequences which are followed by *nosT* transcription termination sequences.

Figure 5A schematically represents the structure of the pGA-ESKN vector. Figure 5B schematically represents the structure of the pGA-SESKN vector.

Figure 6 shows the photograph of an autoradiogram which demonstrates the AdoMetase mRNA levels in fruit derived from two different transgenic plants.

Figure 7 shows a quantitation of the results presented in Figure 6. These results illustrate the effect of variations of the E8 promoter on AdoMetase mRNA levels in ripening tomatoes.

Figure 8 is a graph representing the relative levels of AdoMetase activity in ripening tomatoes at different stages.

Figures 9A to 9D presents the data for ethylene production in the fruit of 4 different transgenic plants (Figure 9A, ES 19-2; Figure 9B, LS 4-2; Figure 9C, ES 35-1; and Figure 9D, ES22 A-1) over a ten day period after entry of the fruit into breaker stage.

Figure 10 illustrates the post-harvest shelf life of tomatoes obtained from SESKN transgenic plants.

Figure 11 presents the sequence of the SAM-K modification of the AdoMetase gene derived from bacteriophage T3.

Figure 12A shows a diagram of the steps followed in constructing vector pAG-114 (pESKN).

Figure 12B shows a diagram of the steps followed in constructing vector pAG-5321 (pGA-ESKN).

5 Figure 13 presents the sequence of the upstream minus 2216 base pair region of the tomato E8 gene (SEQ ID NO:24).

Figure 14 shows a photograph of an autoradiogram of a Southern blot of tomato, raspberry, strawberry,
10 melon, carnation and cauliflower DNA probed with a fragment containing the coding sequence from the tomato E4 gene.

Figure 15 shows a DNA sequence of an isolated E4 gene from a raspberry genomic DNA library.

15 Figure 16 shows a schematic of the tomato E4 gene, and the primers used to isolate a 1.18 kb tomato E4 promoter fragment.

Figures 17A-D show a diagram of the steps followed in constructing vectors pAG-111 (Figures 17A-17B), pAG-
20 117 (Figures 17B-17C) and pAG-5520 (Figures 17C-17D).

Figures 18A and 18B show the results of an RNase protection assay to detect SAMase mRNA in fresh and wounded leaves of normal and E4/SAMase transgenic tomato plants.

25 Figure 19 shows a bar graph of ethylene production in wounded leaves of normal and E4/SAMase transgenic tomato plants.

Figures 20 and 21 show the results of an RNase protection assay to detect SAMase mRNA in E4/SAMase
30 transgenic tomato ripening fruit at four stages of ripening.

Figure 22 shows the results of a Western blot, of E4/SAMase transgenic tomatoes at four stages of ripening, probed with an antibody to SAMase.

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Figure 23 shows a chart of ethylene production in normal and E4/SAMase transgenic tomatoes as a function of time after harvest.

5 Figure 24 shows the DNA sequence of the tomato E4 gene.

Figure 25 shows the DNA sequence of a HindIII/KpnI fragment containing the tomato E4 promoter and the SAMase gene, and the translated amino acid sequence of the SAMase gene.

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DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

"Homologous" DNA refers to DNA not introduced into a host organism by recombinant means.

15 "Heterologous" DNA refers to DNA which has been transfected into a host organism. Typically, heterologous DNA refers to DNA that is not originally derived from the transfected or transformed cells' genomic DNA (e.g., CAT and β -galactosidase gene sequences).

20 However, any DNA introduced into an organism by recombinant means is referred to as heterologous DNA (e.g., introduction into a tomato of a vector carrying the tomato E4 promoter).

25 A "chimeric gene," in the context of the present invention, typically comprises a promoter sequence operably linked to non-homologous DNA sequences that encode a gene product (e.g., a tomato E8 promoter adjacent DNA sequences encoding S-adenosylmethionine cleaving enzyme).

30 Two nucleotide sequences are considered to be "functionally homologous" if they hybridize with one another under moderately stringent conditions, i.e. 0.1% SSC at room temperature. Examples of such hybridization conditions are given in Examples 6 and 7.

Typically, two homologous nucleotide sequences are greater than or equal to about 60% identical when optimally aligned using the ALIGN program (Dayhoff).

Two amino acid sequences are considered
5 "homologous" if their amino acids are greater than or equal to about 60% identical when optimally aligned using the ALIGN program mentioned above.

A "modified ripening" phenotype typically refers to an alteration of the rate of ripening of a
10 transgenic fruit relative to corresponding wild-type fruit. For example, delayed ripening fruit (i.e., ripening takes longer than corresponding wild-type fruit) or suspension of the fruit's ability to complete the ripening process.

15 A "product" encoded by a DNA molecule includes, for example, RNA molecules and polypeptides.

II. Regulated Promoters for Expression of Genes in Plants.

20 The present invention provides a method to regulate plant cell expression of any gene in a tissue or development stage-specific manner, in particular, genes whose products reduce ethylene synthesis in plant cells. In one embodiment, the invention teaches the
25 use of tissue/stage-specific promoters whose expression is induced during fruit ripening or by the presence of ethylene. To regulate cellular production of ethylene, a gene whose product results in a reduction of ethylene synthesis is operably linked to such promoter (creating
30 a chimeric gene). In one aspect of the invention, an initial burst of ethylene synthesis occurs, expression of the chimeric gene is induced and production of ethylene by the cell is subsequently reduced. When the chimeric gene is present in fruit cells, the result is

fruit having a modified ripening phenotype relative to wild-type (non-transgenic) fruit.

The initial burst of ethylene allows fruit to initiate ripening, but the subsequent down regulation
5 of ethylene production delays the course of fruit ripening, i.e., the fruit are suspended in their ability to complete the ripening process. Climateric or non-climateric fruit may be produced by this method.

Several examples of regulated promoters are
10 described below, including two embodiments of the tomato E8 promoter, the tomato E4 promoter and the raspberry E4 promoter. Further useful promoters include, but are not limited to, the avocado cellulase promoter (Cass, et al.), the tomato polygalacturonase
15 promoter (Bird, et al.), and the tomato ACC oxidase (ACCO) promoter, as well as promoters from homologous genes obtained from other plants.

Exemplary gene products that result in reduction of ethylene synthesis include, but are not limited to
20 the following: S-adenosylmethionine hydrolase; 1-aminocyclopropane-1-carboxylate deaminase (Klee, et al.; Sheehy, et al.); the ACC synthase gene in an antisense or cosuppression configuration (Oeller, et al.; Van der Straeten, et al.); and the ACC oxidase
25 gene in either an antisense or cosuppression configuration (Hamilton, et al.; Holdsworth, et al.). Cosuppression has been described by Jorgensen, et al. (1991, 1993), both herein incorporated by reference.

Other gene products that may be useful in the
30 reduction of ethylene biosynthesis include catalytic antibodies and ribozyme molecules.

The present invention provides, in one aspect, nucleic acid constructs suitable for transforming plants with heterologous genes under the control of an
35 E4 promoter. (originally isolated from tomato; Cordes,

et al.) or an E8 promoter. In one embodiment, the plant is a fruit-bearing plant, and the heterologous gene is a gene effective to reduce ethylene biosynthesis in fruit from the plant. In another
5 embodiment, the plant is a flowering plant, and the heterologous gene is a gene effective to reduce ethylene biosynthesis in flowers of the plant. In still another embodiment, the plant is a vegetable, and the heterologous gene is a gene effective to reduce
10 ethylene biosynthesis in the vegetable.

Experiments performed in support of the present invention demonstrate that an exemplary heterologous gene effective to reduce ethylene biosynthesis in tissues of a plant is the AdoMetase gene, isolated from
15 bacteriophage T3 (Hughes, et al., 1987a). These experiments show that ethylene production in ripe transgenic tomatoes containing the AdoMetase gene under the control of a tissue/stage specific promoter is significantly lower.

20 In addition, the tomatoes develop color to a light red stage and then cease further color development, and remain firm longer than control tomatoes. Further, the experiments show that the E4 promoter coupled to a heterologous gene can be activated by wounding plant
25 tissue containing the construct, and that the activation of the promoter is typically transient in nature.

A. Tomato E4 Promoter

30 The tomato E4 promoter is both stage and tissue specific (Cordes, et al.). Typically, E4 mRNA is abundant in ripening fruit and is not detected in leaf, root, stem, or unripe fruit. E4 gene expression can, however, be activated by ethylene, and the ethylene-
35 induced expression can be detected in a variety of

plant tissues (Lincoln, et al., Lincoln and Fischer). Further, the *rin* (ripening inhibited) mutation, that blocks many aspects of ripening, including softening, ethylene production, and color development (Giovannoni, et al.), reduces the concentration of E4 mRNA by greater than 10-fold. The sequence of the E4 promoter has been published (Cordes, et al.) and the DNA sequence of the minus 1173 base pair region is presented in the first portion of Figure 25.

10 The tomato E4 promoter may be employed in vector constructs used to produce transgenic plants, such as transgenic tomatoes. For example, a vector engineered according to methods of the present invention (detailed below), containing the tomato E4 promoter connected to
15 the AdoMetase gene (e.g. vector pAG-5520), may be used to produce transgenic raspberries, strawberries, melons, carnations, cauliflower, and the like. The AdoMetase gene will be expressed in the fruit of these transgenic plants will delay ripening. An advantage of
20 this method is a savings of time and resources involved in vector construction, since the same vector can be used to transform many different plant types. Experiments performed in support of the present invention have demonstrated the ability to the tomato
25 E4 promoter to facilitate DNA coding sequence expression in raspberries.

 Alternatively, E4 promoter sequences may be isolated from the same type of plant that is to be transformed, and incorporated into the vector
30 constructs used to perform the transformations. For example, a raspberry E4 promoter may be connected to a heterologous gene, such as the AdoMetase gene, and used to transform raspberries. This method is typically preferable, because a promoter from the same type of
35 plant as is transformed is more likely to contain all

of the regulatory elements required for appropriate stage and tissue specificity of expression. For example, isolation of a genomic copy of a raspberry E4 gene with 5' regulatory sequences is described below.

5 E4 promoters may isolated from other plants using a number of methods, including those described below.

B. Identification of Plant E4 Promoters

The present invention provides for the use E4

10 promoters from species other than tomato in vector constructs containing heterologous genes. Southern blot experiments performed in support of the present invention demonstrate the presence of DNA molecules having high sequence homology with the tomato E4 gene

15 in raspberry, strawberry, melon, carnation and cauliflower. Similar Southern blot analyses may be performed on other fruit-bearing plants to identify additional E4 genes.

A Southern blot analysis used herein is detailed

20 in Example 6. E4 homologues are identified in a Southern blot of the genomic DNA of the plants listed above probed with a labelled DNA fragment containing the coding sequence of the tomato E4 gene.

The probe is selected to contain the coding

25 sequence of tomato E4, rather than the promoter sequence, because coding sequences are typically more conserved from species to species than are promoter sequences. In the experiments detailed in Example 6, probe molecules are generated from tomato genomic DNA

30 using primer-specific amplification (Mullis; Mullis, et al.). The oligonucleotide primers are selected such that the amplified region included the entire coding sequence of the tomato E4 gene. Primers may also be selected to amplify only a selected region of the E4

35 gene.

Alternatively, a probe can be made by isolating restriction-digest fragments containing the sequence of interest from plasmid DNA.

The probe is labeled with a detectable moiety to enable subsequent identification of homologous target molecules. Exemplary labeling moieties include radioactive nucleotides, such as ³²P-labeled nucleotides, digoxigenin-labeled nucleotides, biotinylated nucleotides, and the like, available from commercial sources.

In the case of primer-amplified probe, labeled nucleotides may be directly incorporated into the probe during the amplification process. Probe molecules derived from DNA that has already been isolated, such as restriction-digest fragments from plasmid DNA, are typically end-labeled (Ausubel, et al.).

Target molecules, such as *HindIII* DNA fragments from the genomes of the above-listed plants, are electrophoresed on a gel, blotted, and immobilized onto a nylon or nitrocellulose filter. Labeled probe molecules are then contacted with the target molecules under conditions favoring specific hybridization between the probe molecules and target molecules homologous to the probe molecules.

Conditions favoring specific hybridization are referred to as moderately to highly stringent, and are affected primarily by the salt concentration and temperature of the wash buffer (Ausubel, et al., Sambrook, et al.). Conditions such as those used in the final wash in Example 6 are typically classified as moderately stringent, due to the low salt concentration, and are expected to preserve only specific hybridization interactions, allowing the identification and isolation of homologous genes in different plant species.

Following contacting, hybridization, and washing, target molecules with sequences homologous to the probe are identified by detecting the label on the probe. The label may be detected directly, for example, as in
5 radioactive label detected on autoradiograms, or it may be detected with a secondary moiety, for example, fluorescently-labeled streptavidin binding to a biotinylated probe.

10 C. Isolation of Other E4 Promoters

Following the identification of plants containing E4 genes, the DNA encoding the genes, including the promoter regions, may be isolated from the respective species, by, for example, screening a genomic DNA
15 library. Experiments performed in support of the present invention, detailed in Example 7, demonstrate the isolation of a genomic copy of a raspberry E4 gene from a raspberry genomic DNA library.

The library of interest is screened with a probe
20 containing sequences corresponding to the coding sequence of a known E4 gene, such as the tomato E4 gene. The screening is done using known methods (Ausubel, et al., Sambrook, et al.), essentially as described above.

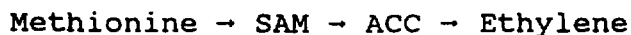
25 Positive plaques or colonies are isolated, and the insert DNA is sequenced and compared to known E4 sequences. Clones containing inserts with sequences corresponding to genes homologous to tomato E4 are identified and, if necessary, used to obtain additional
30 clones until the promoter region of interest is isolated. The sequence of the raspberry E4 gene is presented in Figure 15 (SEQ ID NO:26).

III. Heterologous Genes

According to methods of the present invention, heterologous genes are linked to the promoters of the present invention. Exemplary heterologous gene for the transformation of plants include genes whose products are effective to reduce ethylene biosynthesis in specific tissues of those plants, e.g. the fruits, flowers or leaves. One of these genes, AdoMetase, is discussed in detail below.

A. Ethylene Synthesis

The amino acid methionine has been shown to be a precursor of ethylene (C_2H_4) in plant tissues (reviewed by Imaseki). Methionine, however, is not the immediate precursor but first must be converted to the sulfonium compound S-adenosylmethionine (SAM) and, subsequently, aminocyclopropane-1-carboxylic acid (ACC) prior to conversion to ethylene. The metabolic reactions for the synthesis of ethylene from methionine under both normal and stress conditions are presented in Figure 1A, and summarized as follows:



ACC synthase catalyzes the degradation of SAM to ACC and 5'-methylthioadenosine (MTA). This enzymatic reaction appears to be the rate limiting step in ethylene formation. For example, the natural plant hormone indoleacetic acid (IAA or auxin) stimulates ethylene production by inducing the synthesis of ACC synthase. Conversely, the synthesis of SAM from methionine and the production of ethylene from ACC do not require auxin induction.

In addition, wounding and fruit ripening induces the formation of ACC synthase and, therefore, the

conversion of SAM to ACC. The other product of the ACC synthase reaction, MTA, must be recycled back into methionine so as to provide an adequate supply of methionine for continual ethylene production. This recycling pathway from MTA to methionine, also presented in Figure 1A, has been shown to exist in plant tissue (Adams, et al.; Kushad, et al.). The degradation of MTA has added significance in light of the finding that MTA is a potent inhibitor of ACC synthase. The importance of the degradation and recycling of MTA in normal plant tissues is, therefore, twofold: 1) to prevent the direct inhibition of ethylene synthesis by MTA, and 2) to provide adequate methionine for continual ethylene synthesis.

The first step in the degradation of MTA in plant tissue is the hydrolysis of this nucleoside to 5-methylthioribose (MTR) by a specific MTA nucleosidase. MTR not only provides its methylthio moiety for the formation of methionine, but also contributes four carbons from its ribose towards the synthesis of this amino acid. Therefore, the methylthio group is conserved by recycling. It should be noted that this pathway merely maintains a methionine supply for ethylene biosynthesis, but does not result in a net increase in methionine synthesis.

1. AdoMet hydrolase

One approach to reduce ethylene biosynthesis in plants reported here utilizes a gene that encodes the enzyme S-adenosylmethionine hydrolase. This approach has been described in the PCT International Application US90/07175. This enzyme, encoded by the *E. coli* bacteriophage T3, hydrolyses AdoMet to homoserine and MTA. The enzyme is known as its recommended name, AdoMet hydrolase (AdoMetase), or by its other name, S-

adenosylmethionine cleaving enzyme (SAMase) (Studier, et al.). Both products of the reaction (i.e., homoserine and MTA) are recycled to methionine; MTA as previously shown (Figure 1A) and homoserine via a
5 metabolism pathway known to exist in plant tissues.

The AdoMetase gene has been identified, isolated, cloned, and sequenced (Hughes, et al., 1987a; Hughes, et al., 1987b). The gene contains two in-frame reading sequences that specify polypeptides of 17105 and 13978
10 daltons. Both polypeptides terminate at the same ochre codon. This results in the 14 kd polypeptide being identical to 82% of the 17kd polypeptide starting at the carboxyl end of the longer polypeptide. Both polypeptides are present in partially purified cells
15 and from *E. coli* expressing the cloned gene (Hughes, et al., 1987b; Studier, et al., 1976). Other bacteriophages that encode the AdoMetase or SAMase genes are coliphage BA14, *Klebsiella* phage K11, and *Serratia* phage IV (Mertens, et al.; Horsten, et al.).

20 The effect AdoMetase expression in plant cells has on the plant methionine recycling pathway is shown schematically in Figure 1B. Experiments performed in support of the present invention, using transgenic tomatoes expressing an AdoMetase gene and monitoring
25 ethylene production, have demonstrated that the effect of AdoMetase on the pathway is to "short circuit" the branch that produces ethylene: ethylene production is reduced in such transgenic plants.

Different bacteriophages may be expected to
30 contain AdoMetase genes with variations in their DNA sequences. The isolation of AdoMetase coding sequences from bacteriophage coding sequences can be accomplished as previously described for AdoMetase from bacteriophage T3. Alternatively, degenerative
35 hybridization probes for AdoMetase coding sequences can

be generated and used to screen plasmids carrying fragments of a selected bacteriophage's genome for the presence of homologous sequences. AdoMetase enzymatic activity can be evaluated by standard biochemical tests
5 (see for example, Example 13).

Furthermore, the amino acid sequence of AdoMetase may be modified by genetic techniques to produce enzymes with altered biological activities (see below). An increase in the biological activity could permit the
10 use of lower amounts of the enzyme to control ethylene biosynthesis in plants.

IV. Vector Construction

Plant transformation vectors are constructed
15 according to methods known in the art (see, for example, Houck, et al., and Becker, et al.)

A series of recombinant DNA manipulations are performed in the AdoMetase gene prior to placement in an Agrobacterium expression vector. Initially, a
20 MaeIII to BamHI fragment from M13HB1 (Hughes, et al., 1987a) is subcloned into the pUC19 plasmid vector to produce pAG-110 (Example 1, pUC19-SAMase Figure 2). To increase the translational efficiency of the AdoMetase gene in plants, site directed mutagenesis of the
25 nucleic acid sequences surrounding the ATG start codon is performed. A synthetic double stranded 39 base pair oligonucleotide is synthesized and substituted for the BamHI to XmnI fragment at the 5' end of the gene (Figure 2). The net effect of this substitution is to
30 change the CACCAAATGA (SEQ ID NO:14) in the native T3 sequence to GCCACCATGG (SEQ ID NO:15) which is an optimal eukaryotic translational initiation sequence (Kozak, et al.; Lutcke, et al.).

The change also introduces an NcoI site (CCATGG)
35 at the AdoMetase start codon which facilitates fusions

to different promoters. The only alteration to the AdoMetase coding sequence is the amino acid at amino acid position two which is changed from isoleucine to valine: this is a highly conservative amino acid
5 change. The altered form of AdoMetase was named SAM-K (Figure 11).

A recombinant vaccinia vector with SAM-K (vv:SAM-K) was constructed. Expression of this vector in African green monkey cells or T3-infected bacterial
10 cells was compared with the gene to the native T3 gene when expressed in the same cells. The specific activity of AdoMetase was higher in the vv:SAM-K infected cells than in the T3 infected bacterial cells demonstrating that SAM-K encodes a fully functional
15 version of AdoMetase.

Experiments performed in support of the present invention have demonstrated constitutive expression of AdoMetase in transgenic plants. In these plants there is a significant reduction in the ability of these
20 plants to synthesize ethylene.

Using the sequence shown in Figure 24 primers are prepared for use in the polymerase chain reaction (PCR) to amplify a 1177 base pair region of the E4 promoter from tomato genomic DNA (Example 8). The primers are
25 designed with unique restriction sites at each end and were used to place the promoter in the proper orientation 5' of the SAMase gene in pAG-111 (Figures 17A, 17B). The 5' end of the promoter fragment has a *HindIII* site, while the 3' end has an *NcoI* site
30 (CCATGG) placed such that the ATG start codon of the E8 gene product is used as the ATG in the *NcoI* site. This allows precise placement of the entire E4 promoter directly in front of the SAMase amino acid coding sequences with no intervening sequences (Example 8).

A selectable vector expressing AdoMetase under the control of the E4 promoter (pAG-5520) is constructed as detailed in Example 8 and schematized in Figures 17A-17D. For selection, the vector contains the neomycin phosphotransferase II gene, providing aminoglycoside antibiotic (e.g. kanamycin) resistance.

V. Plant Transformation

A. Methods of Transforming Plants

pAG-5520 is transferred to tomato plants (Example 9) to generate transgenic plants expressing AdoMetase. Tomato progenitor cells (tomato cotyledon tissue explants) are transformed with EHA101 bacteria containing pAG-5520 and grown in tissue culture in the presence of kanamycin for 8 to 10 weeks to produce plants.

A number of methods, in addition to Agrobacterium-based methods, may be employed to elicit transformation of plant progenitor cells, such as electroporation, microinjection, and microprojectile bombardment. These methods are well known in the art (Comai, et al., Klein, et al.; Miki, et al.; Bellini, et al.) and provide the means to introduce selected DNA into plant genomes: such DNA may include a DNA cassette which consists of the E4 gene promoter functionally adjacent, for example, AdoMetase coding sequences.

B. Identifying and Evaluating Transformants

Several transgenic plants are assayed for their ability to synthesize AdoMetase mRNA, AdoMetase protein, and their ability to inhibit the biosynthesis of ethylene. The assays are performed after the plant tissue being assayed has been subjected to a wound, and are carried out both on leaves of the plant, as well as the fruit. Leaf wounds are typically cuts on the leaf,

performed either with a dull knife or with a circular bore. Fruit "wounds" can be as mild as picking the fruit from the plant.

Leaf-based assays can be informative if the promoter driving the heterologous gene (transgene) is at least somewhat active in leaf tissue, as is the case for the E4 promoter. In such cases, leaf-based assays are useful for initial screens of the expression level of a transgene, since they can be performed much earlier than fruit-based assays. Fruit-based assays, on the other hand, provide more accurate data on transgene expression in the target tissue itself (fruit). The results of both types of assays are detailed in Example 13.

AdoMetase mRNA levels are determined using, for example, an RNAase protection assay (RPA) (Example 10). Figures 18A and 18B show the results of an RPA using fresh or wounded leaves from normal and transformed tomato plants. Plants showing detectable transgene mRNA expression in leaves are grown to produce fruit, and the fruit is then tested mRNA expression. Figures 20 and 21 shows the results of an RPA using the fruit from one transgenic plant at different stages of fruit ripening. While the absolute level of expression varies considerably among different transformed lines, the relative level of expression as a function of ripening stage is consistently transient, typically peaking at the orange stage, with expression at low levels in fully ripe fruit in all but one case.

Ethylene biosynthesis is measured in leaves and fruit of transgenic tomatoes. Figure 19 shows the level of ethylene synthesis in wounded leaves from normal (M) and four transgenic tomato plants. The experiments are performed as detailed in Example 11. Some of lines show a reduction in ethylene

biosynthesis, but the values are not well correlated with those obtained in the RNase assay presented in Figures 18A and 18B. The assay is nevertheless useful for screening, as suggested above, since plants
5 negative for AdoMetase expression in leaf wound assays are also negative for AdoMetase expression in fruit.

Ethylene biosynthesis in transgenic fruit, is typically reduced relative to control fruit, as is shown in Figure 23. Whereas control tomatoes show an
10 increasing rate of ethylene synthesis during the 5 days following harvest, fruit from transgenic line E4-05 shows a decreasing rate of synthesis, which bottoms out approximately one week post-harvest. This result demonstrates that constructs of the present invention
15 are effective at reducing the level of ethylene biosynthesis in the fruit of fruit-bearing plants.

After these time-points, the rates of ethylene synthesis reverse directions for both normal and transgenic plants. This characteristic
20 demonstrates the physiological effects of the transient nature of heterologous gene expression under control of an E4 promoter, and that activation of the E4 promoter, for example, by wounding, can transiently inhibit ethylene biosynthesis.

25 To correlate the effects of AdoMetase mRNA expression, Western Blots were performed, as detailed in Examples 12 and 13. The results are shown in Figure 22. These results demonstrate that transformation of tomato progenitor cells with a construct containing the
30 E4 promoter coupled to the AdoMetase gene is effective to reduce ethylene biosynthesis in the fruit of the transgenic plants, and further, that this reduction can have a transient time-course.

The biological effects of AdoMetase gene
35 expression include a cessation of color development

beyond the light red stage, and tomatoes that remain firm much longer than untransformed controls.

VI. E8 Promoter Regulated Gene Expression.

5 Regulatable promoters have been employed in the method of the present invention. One exemplary regulatable promoter is the tomato E8 gene promoter. Expression of the E8 gene has been shown to be induced (i) at the onset of ripening, and (ii) by treatment of
10 tomatoes with ethylene (Deikman, et al., 1988; Lincoln, et al.; Giovannoni, et al.). The sequence of the E8 promoter has been published (Deikman, et al., 1988; Deikman, et al., 1992) and the DNA sequence of the minus 2216 base pair region is presented in Figure 13.

15 Using the sequence shown in Figure 13 primers were prepared for use in the polymerase chain reaction (PCR) to amplify the 1124 base pair promoter from tomato genomic DNA (Example 1). The primers were designed with unique restriction sites at each end and were used
20 to place the promoter in the proper orientation 5' of the SAM-K gene in pUC19 (Figure 3). The 3' end of the promoter fragment had an *NcoI* site (CCATGG) placed such that the ATG start codon of the E8 gene product was used as the ATG in the *NcoI* site. This allowed precise
25 placement of the entire E8 promoter directly in front of the SAM-K amino acid coding sequences with no intervening sequences (Example 1, Figure 12A).

 Two AdoMetase expressing vectors were constructed (Example 1), the pAG-5321 (pGA-ESKN) vector (Figures
30 12A, 12B and Figure 5A) and the pGA-SESKN vector (Figure 4 and Figure 5B). The pAG-5321 vector contains a portion of the E8 promoter (Figure 4, lower E8 promoter) adjacent the AdoMetase coding sequences. A lambda EMBL-3 clone containing genomic tomato sequences
35 that hybridize to the -1124 E8 region was isolated and

used as the source for a region upstream of the -1124 E8 (lower E8) promoter. Restriction mapping analysis and subcloning allowed identification of an approximately 1200 bp *HindIII* to *XbaI* fragment as the region immediately upstream of the original -1124 bp E8 promoter (Figure 4). This region was added to the pAG-5321 construct to yield pGA-SESKN, which contained the approximately -2254 bp E8 promoter fused to the AdoMetase gene (Figure 4, SE8).

Both of these vectors were transferred to tomato plants (Example 2) to generate transgenic plants expressing AdoMetase. A number of methods, in addition to *Agrobacterium*-based methods, may be employed to elicit transformation of the plant host, such as electroporation, microinjection, and microprojectile bombardment. These methods are well known in the art (Klein, et al.; Miki, et al.; Bellini, et al.). Further, these methods provide the means to introduce selected DNA into plant genomes: such DNA may include a DNA cassette which consisting of the E8 gene promoter functionally adjacent AdoMetase coding sequences.

Several transgenic plants were assayed for their ability to synthesize AdoMetase mRNA using a sensitive RNAase protection assay (RPA) (Example 3). Figures 6 and 7 show the results of an RPA using the fruit from two transgenic plants (ESKN, transformed with pAG-5321, and SESKN, transformed with pGA-SESKN) at different stages of fruit ripening. Other tissues from these plants including immature and mature leaves, flowers and stems were negative for the presence of AdoMetase RNA. Although the expression of AdoMetase in ESKN transgenic plants was regulated to the post mature green fruit, it was repeatedly observed (as shown in Figures 6 and 7) that the expression of AdoMetase turned off in the fully ripe fruit. On the other hand,

the SESKN transgenic fruit maintained AdoMetase mRNA expression in ripe fruit.

To determine whether the presence of AdoMetase enzyme activity correlated with the level of AdoMetase mRNA, an AdoMetase assay was performed using extracts from four fruit obtained at different stages from an ESKN transgenic plant (Example 5). Figure 8 shows the level of AdoMetase activity in mature green, breaker, orange, and ripe fruit from a single pAG-5321 transgenic plant. These data demonstrate that AdoMetase activity follows roughly the same expression pattern in ripening fruit as the AdoMetase mRNA levels.

The data presented above suggest that inclusion of the upstream region of the native E8 promoter in a chimeric gene construct enhances long-lived expression of the chimeric gene in ripening transgenic tomatoes. Figure 6 shows the RPA results from pGA-SESKN line 22A-1 and from pAG-5321 line 18. ESKN line 18 had one of the highest levels of AdoMetase expression of all the ESKN transgenic lines. Quantitative measurement of AdoMetase mRNA is shown in Figure 7. The results show that the -2254 bp E8 promoter expression is maintained through the fully ripe stage of fruit development. This expression pattern is in sharp contrast to the -1124 bp E8 promoter (ESKN) mRNA levels also shown in Figure 6.

Ethylene evolution measurements from fruit picked at breaker and analyzed daily are shown in Figure 9. The rate at which fruit from SESKN lines 22A and 35-1 produced lycopene was reduced as evidenced by the time required for orange fruit development. Furthermore, the total amount of ethylene produced from these tomatoes was reduced by approximately 80%. The expression of AdoMetase and a reduction in ethylene

biosynthesis was strictly correlated in the 25 SESKN transgenic plants analyzed.

The SESKN tomatoes that synthesized less ethylene were assessed for their shelf life properties when
5 stored at room temperature (22°C) (Example 5). Three fruit each from SESKN lines 22A-1 and 35-1 were compared with untransformed normal tomatoes. Senescence was determined by visually observing contraction and wrinkles on the tomato skin. Firmness
10 was not measured but was noted to be much greater in the transgenic lines. The results of these senescence assessments are shown in Figure 10. Even at 55 days post-breaker, the 22A-1 tomatoes remained firm and appeared to be suffering more from dehydration than
15 from the softening-induced senescence of the normal tomatoes.

These results demonstrate the ability to provide tissue specific regulation to DNA sequences encoding a gene product, e.g., AdoMetase enzyme, in transgenic
20 plants. In addition, the results obtained with the two different E8 promoters (lower E8 and SE8) suggest the use of these promoters for similar tissue specific expression of any desired gene product. Further, the two regions of the E8 promoter (lower E8 and upper E8,
25 Figure 4) can be used as hybridization probes against libraries of DNA representative of the genomes of other plant species. Homologous sequences to the E8 promoter are then tested for tissue specific expression in the plant species from which they were isolated. Such
30 promoters, as well as the E8 promoter itself, can be tested for regulatable expression in heterologous plant systems using the methods described herein. A reporter gene, such as GUS (β -glucuronidase), can be used to test tissue specific regulatable expression from these

promoters. Expression of GUS protein can be easily measured by fluorometric, spectrophotometric or histochemical assays (Jefferson, 1987).

5 Variants of the E8 promoter may be isolated from different tomato cultivars or other plant species by standard recombinant manipulations such as primer specific amplification (Mullis; Mullis, et al.) or oligonucleotide hybridization (Ausubel, et al.; Sambrook, et al.).

10

VII. Advantages of the Invention

These results demonstrate the ability to provide tissue and stage specific regulation of gene expression in transgenic plants. A tissue or stage specific promoter is a region of DNA that regulates transcription of the immediately adjacent (downstream) gene to a specific plant tissue or developmental stage of the plant or plant tissue. Other gene products which may be useful to express using these promoters include genes encoding (i) flavor (e.g., thaumatin) or color modification (e.g., products that modify lycopene synthesis), (ii) enzymes or other catalytic products (such as, ribozymes or catalytic antibodies) that modify plant cell processes, and (iii) gene products that affect ethylene production, such as antisense molecules, enzymes that degrade precursors of ethylene biosynthesis, catalytic products or cosuppression molecules. Further, it is useful to restrict expression of some genes to specific tissues, such as the fruit -- for example, any gene that would be deleterious to the plant if it were expressed constitutively. Such genes would include genes which encoded degradative enzymes that deplete necessary metabolites.

As can be seen from the results described above, derivatives of the E8 and E4 promoter regions can be used as on/off switches for the tissue and stage specific expression of genes whose expression is under
5 their control.

The constructs and methods of the present invention are applicable to all higher plants including, but not limited to, the following:

Solanaceae. *Lycopersicon* (tomato) and *Capsicum*
10 (peppers);

Cucurbitaceae. *Cucurbita* (squashes), *Cucumis* (melons, cantaloupe) or *Citrullus* (watermelon);

Rosaceae. *Malus* (apple), *Prunus* (peaches, plums, nectarines), *Rubus* (raspberry), *Fragaria* (strawberry),
15 and *Pyrus* (pears);

Annonaceae. *Annona* (sweetsop, cherimoya);

Musaceae. *Musa* (banana);

Lauraceae. *Persea* (avocado);

Saxifragaceae. *Ribes* (currents);

20 Ebenaceae. *Diospyros* (persimmon);

Caricaceae. *Carica* (papaya);

Anacardiaceae. *Mangifera* (mango);

Myrtaceae. *Psidium* (guava);

Actinidiaceae. *Actinidia* (kiwifruit).

25 Variants of the E8 and E4 promoter may be isolated from different tomato cultivars and from other plants by the methods described above. A reporter gene, such as GUS (β -glucuronidase), can be used to test tissue specific regulatable expression from such promoters.
30 Expression of GUS protein can be easily measured by fluorometric, spectrophotometric or histochemical assays (Jefferson, 1987).

A further advantage of the present invention is the ability to produce fruit that initiate ripening,

but the subsequent down regulation of ethylene production delays the overall time course of fruit ripening, i.e., the fruit are suspended or delayed in their ability to complete the ripening process.

5

VIII. Utility

A. The Vectors of the Present Invention.

The present invention provides vectors suitable for the transformation of plants. The vectors,
10 chimeric genes and DNA constructs of the present invention are also useful for the expression of heterologous genes. Transgenic plants, and their fruit products, carrying the chimeric genes of the present invention, may be a useful source of recombinantly-
15 expressed material.

In one embodiment, the chimeric genes of the present invention have two components: (i) a DNA sequence encoding a product that is effective to reduce ethylene biosynthesis in fruit from the plant, and (ii)
20 a promoter whose expression is induced during fruit ripening or in response to ethylene.

The vectors of the present invention may be constructed to carry an expression cassette containing and insertion site for DNA coding sequences of
25 interest. The transcription of such inserted DNA is then under the control of a suitable promoter (i.e., a promoter whose expression is induced during fruit ripening, in response to ethylene, or in response to a plant cytokine). Exemplary of such promoters are
30 promoters obtained from tomato E4 or E8 genes, or homologs thereof (e.g., raspberry E4), and avocado cellulase gene or tomato polygalacturonase gene.

Such expression cassettes may have single or multiple transcription termination signals at the
35 coding-3'-end of the DNA sequence being expressed. The

expression cassette may also include, for example, DNA sequences encoding (i) a leader sequence (e.g., to allow secretion or vacuolar targeting), and (ii) translation termination signals.

5 Further, the vectors of the present invention may include selectable markers for use in plant cells (such as, the nptII kanamycin resistance gene). The vectors may also include sequences that allow their selection and propagation in a secondary host, such as, sequences
10 containing an origin of replication and a selectable marker. Typical secondary hosts include bacteria and yeast. In one embodiment, the secondary host is *Escherichia coli*, the origin of replication is a *colE1*-type, and the selectable marker is a gene encoding
15 ampicillin resistance. Such sequences are well known in the art and are commercially available as well (e.g., Clontech, Palo Alto, CA; Stratagene, La Jolla, CA).

The vectors of the present invention may also be
20 modified to intermediate plant transformation plasmids that contain a region of homology to an *Agrobacterium tumefaciens* vector, a T-DNA border region from *Agrobacterium tumefaciens*, and chimeric genes or expression cassettes (described above). Further, the
25 vectors of the invention may comprise a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens*.

The vectors of the present invention are useful for stage and/or tissue specific expression of nucleic acid coding sequences in plant cells. For example, a
30 selected peptide or polypeptide coding sequence can be inserted in an expression cassette of a vector of the present invention. The vector transformed into host cells, the host cells cultured under conditions to allow the expression of the protein coding sequences,
35 and the expressed peptide or polypeptide isolated from

the cells. Transformed progenitor cells can also be used to produce transgenic plants bearing fruit.

In one aspect of the invention, fruit produced by such transgenic plants has an initial burst of ethylene
5 production, followed by a reduction in the level of ethylene synthesis by the fruit. The fruit then demonstrates a modified ripening phenotype.

The vectors, chimeric genes and DNA constructs of the present invention can be sold individually or in
10 kits for use in plant cell transformation and the subsequent generation of transgenic plants.

B. Transgenic Plants and Fruit.

Experiments performed in support of the present
15 invention demonstrate that plants carrying chimeric genes of the present invention (comprising, a DNA sequence encoding a product that is effective to reduce ethylene biosynthesis in fruit from the plant and a promoter whose expression is induced during fruit
20 ripening or in response to ethylene) exhibit significantly lower levels of ethylene production following harvest. Examples of this result have been described herein, for example, using expression of the AdoMetase gene under the control of a E4 and E8
25 promoters. Due to the deleterious effects of ethylene biosynthesis on the handling and storage of commercially-important plants and plant products (such as fruits, vegetables and flowers) plants in which ethylene synthesis is reduced are of substantial value.

30 Similarly, flowering plants containing heterologous genes effective to reduce ethylene biosynthesis will retain a fresh appearance longer than untransformed counterparts. Reduced ethylene biosynthesis in leafy vegetables, such as lettuce,
35 would reduce leaf browning and lead to a longer shelf-

life. The transgenic tomatoes described herein are illustrative of present invention. These transgenic tomatoes remain firm much longer after harvest than normal tomatoes, such transgenic tomatoes may be
5 harvested at a later, vine-ripened, stage and still retain the transportability previously associated with, for example, green tomatoes.

Further, the expression constructs of the present invention allows an initial burst of ethylene synthesis
10 to begin the ripening process. The promoters of the present invention can be manipulated to suspend further ripening or to increase the time-course of the ripening process (relative to wild-type fruit). The constructs of the present invention demonstrate the first example
15 of tissue-type or developmental stage-specific control over ethylene production.

C. Expression in Heterologous Plant Systems

Experiments performed in support of the present
20 invention demonstrate the versatility of the chimeric gene constructs of the invention. The vector constructs of the present invention can be used for transformation and expression of heterologous sequences in transgenic plants independent of the original plant
25 source for the promoter sequence. For example, the tomato E4-Adometase chimeric gene was introduced into raspberries. The transgenic raspberries were propagated under green house conditions. Proteins were prepared from the transgenic raspberry fruit, the
30 proteins size-fractionated and transferred to nylon membranes. The bound proteins were then probed with a monoclonal antibody specifically immunoreactive with the Adometase protein. Results from these experiments demonstrate the expression of Adometase in the
35 raspberry fruit.

These data suggest that the tomato E4 promoter is useful for the promotion of gene expression in tomato and heterologous systems, i.e., plant cells other than tomato. Further, the expression mediated by the promoter appears to be tissue/developmental-stage specific even in heterologous plants. These findings support the usefulness of the vectors, chimeric genes and DNA constructs of the present invention for transformation of species of fruit-bearing plants, where such plants are different species than the plant source of the promoter sequences.

The following examples illustrate, but in no way are intended to limit the present invention.

Materials and Methods

Tomato seed (*Lycopersicon esculentum* Mill. var. *cerasiforme* (Dunal) Alef. cv. Large Red Cherry) were obtained from Peto Seed, Inc. (Saticoy, CA) and were grown under standard greenhouse conditions. Harvested fruit were stored at room temperature (22°C).

Standard recombinant DNA techniques were employed in all constructions (Adams, et al.; Ausubel, et al., Sambrook, et al.).

EXAMPLE 1

Cloning of the AdoMetase Gene

A. Isolation of the AdoMetase Gene.

The AdoMetase gene was identified on an *AluI*-*HaeIII* restriction fragment from purified T3 DNA (Hughes, et al., 1987a). Bacteriophage T3 is available under ATCC No. 11303-B3 (American Type Culture Collection, 12301 Parklawn Dr., Rockville MD 20852). The DNA fragment was first cloned into the bacteriophage M13 MP8 vector (Pharmacia LKB

Biotechnology, Inc., Piscataway, NJ). A MaeIII to BamHI fragment was subcloned into the pUC19 plasmid vector (Pharmacia) to produce pUC19-AdoMetase (pAG110 or pUC19-SAMase; Figure 2). This vector was
5 transformed into *E. coli* and used as a source of DNA for further construction experiments and for DNA sequence determination.

10 B. Modification of the Amino-Terminal Sequence of the Cloned AdoMetase Gene.

The cloned AdoMetase gene was further engineered to contain a consensus eukaryotic translation initiation site (Kozak; Lutcke, et al.) by altering the nucleotide sequence surrounding the SAMase ATG start-
15 codon using a synthetic double-stranded oligonucleotide.

The plasmid pUC19-AdoMetase (pAG110) was digested with *XmaI* and *BamHI* and the 1.9 kb and 1.3 kb fragments were purified by electro-elution after agarose gel
20 electrophoresis. A double stranded synthetic oligonucleotide linker having the sequence indicated in Figure 3 was ligated to the 1.9 kb fragment and this ligated DNA subjected to *XmaI* digestion to remove excess linkers.

25 The linkered 1.9 kb fragment was then re-purified by electrophoresis on low melting temperature agarose and ligated to the 1.3 kb fragment to form the plasmid pUC19-SAM-K (pAG-111). The altered gene region was subjected to DNA sequence analysis. The gene sequence
30 is given in Figure 11. This gene was designated SAM-K and used to construct the following plant expression vectors. This plasmid DNA can also be used to directly transform the plant host via electroporation, microinjection, or microprojectile bombardment.

C. Vector Constructions using the Tomato E8 Promoter.

Two different forms of the E8 promoter were used to construct SAM-K-containing vectors. The first (-
5 1124 bp) was isolated from tomato (*Lycopersicon
esculentum* var. *cerasiform*) DNA using polymerase chain
reaction (PCR) (Mullis; Mullis, et al.; Perkin-Elmer
Cetus, Norwalk CT). The primers used in the PCR
10 reaction were based on the sequence described by
Deikman, et al. (1988). The sequences of the
oligonucleotide primers are given in Figure 3. The
oligonucleotides were designed to incorporate
restriction endonuclease sites (*XbaI* and *NcoI*) at the
5' and 3' ends, respectively, of the amplified E8-
15 promoter fragment. These restriction endonuclease
cleavage sites facilitated subcloning into the pUC19-
SAM-K vector (see Figure 2): an *NcoI* site is present
near the ATG start codon region in the synthetic
oligonucleotide.

20 Figure 12A outlines the generation of the vector
pAG-5321 (pGA-ESKN) starting from vector pNCN
(Pharmacia, Inc., Piscataway, NJ) and pUC-SAM-K
(described above). The sequence of the E8 promoter
(the lower E8 promoter) is similar to the sequence
25 presented as bases 1189 to 2214 in Figure 13.

Figure 12B outlines one approach to the generation
of *Agrobacterium* vectors for use in the present
invention. However, the E8/AdoMetase cassette, present
in, for example, pAG-5321, can be incorporated in a
30 number of vectors useful for plant transformation.

Agrobacterium binary vectors were developed from
pGA482 (An, et al., 1985), a pBIN19 derivative
(Clontech Laboratories) containing the neomycin
phosphotransferase II gene fused to the nopaline
35 synthesis gene promoter (An, et al., 1988). The

resulting vector, designated pAG-5321 is shown in Figure 5A.

The second E8 promoter (-2254 bp) was isolated from a lambda EMBL-3 clone that contained the entire E8 gene. The E8 gene clone was selected from a tomato (*Lycopersicon esculentum* var. VFN8) genomic library obtained from Clontech Laboratories (Palo Alto, CA) using the PCR-derived E8 promoter fragment (described above) as a hybridization probe in plaque-lift filter hybridizations. The lambda clone carrying the E8 gene was identified by a positive hybridization signal. The E8-bearing phage was plaque purified and the lambda phage DNA isolated.

The lambda E8 genomic clone was used as a source of the *HindIII* to *XbaI* fragment that is the approximately -2254 to -1124 bp upstream region of the E8 promoter. This fragment was inserted 5' of the approximately -1124 bp E8 promoter in pAG-5321 at the *HindIII* and *XbaI* sites (Figure 4). The resulting plasmid was named pGA-SESKN. Figure 13 shows the nucleotide sequence of the -2216 bp region from one cultivar (Deikman, et al., 1988, 1992). The *HindIII* to *XbaI* fragment (used for construction of the approximately -2254 promoter) contains additional sequences 5' to the end of this -2216 bp sequence.

Figure 4 shows the relationship of the two portions of the E8 promoter that are present in pGA-SESKN.

Standard recombinant DNA techniques were employed in all constructions (Adams, et al.; Ausubel, et al.). Another lambda vector, pGEM7Zf(+)SAM-K, was constructed by cloning the *BamHI* to *KpnI* AdoMetase fragment from pUC19-SAM-K into the same sites of pGEM7Xf(+) (Promega, Inc., Madison, WI).

Other plant cloning vectors, such as pBI121 (Clontech Laboratories, Inc., Palo Alto, CA), can also be used to practice the present invention. The plant promoter upstream of the AdoMetase gene sequence can be varied to obtain tissue specific expression, temperature dependent expression, or light dependent expression in the transgenic plants. Another useful plant promoter, in addition to the E8 promoter described above, is the constitutive Cauliflower Mosaic Virus (CaMV) promoter (Pharmacia).

EXAMPLE 2

Plant Transformation

The pAG-5321 and pGA-SESKN AdoMetase plasmids were separately introduced into *Agrobacterium* using a direct transformation method.

Agrobacterium tumefaciens strain EHA101 (Hood, et al.), a disarmed derivative of *Agrobacterium tumefaciens* strain C58, was used to introduce coding sequences into plants. This strain contains a T-DNA-less Ti plasmid. The pAG-5321 and pGA-SESKN AdoMetase plasmids were transferred into EHA101 using electroporation essentially as described by Nagel, et al. Briefly, an *Agrobacterium tumefaciens* culture was grown to mid-log phase (OD 600 0.5 to 1.0) in YEP media (10 g yeast extract, 10 g peptone, and 5 g NaCl per liter). After chilling on ice, 50 mls of these cells were pelleted, resuspended in 1 ml of ice cold 20 mM CaCl₂ and split into 1 ml aliquots.

Typically, one μ g of plasmid DNA was added to an aliquots and incubated on ice for 30 minutes. The aliquot was then frozen in liquid nitrogen and thawed at 37°C for 5 minutes. One ml of YEP media was added and incubated at 28°C for 2 hours. The cells were pelleted, resuspended in 50 μ l of YEP, and plated on

YEP agar plates containing 20 μ g/ml kanamycin. Kanamycin-resistant transformed colonies appear within 2 days.

Tomato cotyledon tissue explants were excised from both the tip and base of the cotyledon. Cotyledon explants were pre-conditioned for 2 days on tobacco feeder plates (Fillatti, et al.). The pre-conditioned explants were inoculated with EHA101 containing the pAG-5321 or pGA-SESKN AdoMetase plasmid of interest and finally placed in a 10 ml overnight culture of EHA101/[pAG-5321 or pGA-SESKN] for 5 minutes. The explants were then co-cultivated with the EHA101 strains for 2 days on tobacco feeder plates as described by Fillatti, et al.

The explants were grown in tissue culture media (Fillatti, et al.) containing 2Z media, MS salts, Nitsch and Nitsch vitamins, 3% sucrose, 2 mg/l seatin, 500 mg/l carbenicillin, 100 mg/l kanamycin and 0.7% agar. The explants were grown in tissue culture for 8 to 10 weeks. The carbenicillin treatments were kept in place for 2 to 3 months in all media. The explants and plants were kept on carbenicillin until they were potted in soil as a counter-selection to rid the plants of viable *Agrobacterium tumefaciens* cells.

EXAMPLE 3

RNAase Protection Assays for the Detection of SAMase mRNA

Tomato fruits at various stages of development from transgenic plants and wild-type plants were used as mRNA sources. mRNA was extracted from tomato cells and purified using the "QUICK PREP RNA" kit from Pharmacia, Inc. RNAse Protection Assays (RPA) were performed following the manufacturer's instructions using an "RPAII" kit from Ambion, Inc. (Hialeah, FL).

This method has been previously described by Lee, et al.

pGEM7Zf(+)SAM-K was used to generate ^{32}P -UTP-labeled RNA probe using bacteriophage T7 RNA polymerase as contained in the "RIBOPROBE IT T7 RNA POLYMERASE SYSTEM" from Promega, Inc. The radiolabeled probe was purified on a preparative polyacrylamide gel and used for up to one week.

One microgram of isolated mRNA was hybridized to approximately 10,000 CPM of the RNA probe and further processed as per the instructions in the "RPA II" kit. Briefly, one microgram of the purified mRNA was mixed with 10,000 CPM of the RNA probe in a total volume of 15 μl . 20 μl of a hybridization buffer that allows hybridization of complementary sequences (Ausubel, et al.; Maniatis, et al.; Sambrook, et al.) is then added. The hybridization solution is provided in the "RPAII" kit from Ambion. The solution was heated to 90°C for 3-4 minutes to denature all the RNA and incubated at 45°C overnight to allow hybridization of complementary sequences. The solution was cooled to 37°C and RNase (provided in the Ambion kit) was added which degrades all un-hybridized probe.

Protected probe was resolved on a denaturing polyacrylamide gel, dried, and exposed to film for up to 16 hours. Quantitative analysis of the RPA signals was accomplished by excising each band from the gel, dissolving the band in a liquid fluor, and determining the radioactivity present in the sample using liquid scintillation counting. A standard curve was generated using various amounts of unlabeled RNA synthesized from a AdoMetase fragment cloned into pGEM5Z(+) in the sense orientation. The linear range of the assay was dependent on the amount of input ^{32}P -labeled RNA probe

in the RNAase protection assay but typically ranged from 10 pg to 1 ng of mRNA.

EXAMPLE 4

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Ethylene Measurements

The assay for tomato ethylene evolution is performed over a 0.5 to 1.0 hour period by sealing glass jars containing individual fruit and sampling 2 ml aliquots for gas chromatographic analysis. A Hewlett Packard 5890 (Palo Alto, CA) gas chromatograph with a flame ionization detector and a 6ft Porapak N column was used for ethylene measurements (Adams, et al.). This system combined with an HP Vectra computer and the current version of "CHEMSTATION" (Hewlett Packard) allows measurement of ethylene concentrations as low as 0.2 nl of ethylene in a 2 ml sample (0.1 ppm). After measurement of the ethylene in the headspace, the values are converted to nl of ethylene per gram of tissue per hour.

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EXAMPLE 5

Characterization of Transgenic Tomatoes

A. Promoter Effect on SAMase mRNA Levels in Ripening Transgenic Fruit.

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Transgenic fruit were selected from two transgenic plants, ESKN #18 and SESKN #22A, at three stages of ripening, breaker (Br), Orange (Or) and Ripe (Ri). Transgenic plant ESKN #18 contained the lower E8 promoter (Figure 4) adjacent the Sam-K AdoMetase gene. Transgenic plant SESKN #22A contained the entire SE8 promoter (Figure 4) adjacent the Sam-K AdoMetase gene. The AdoMetase mRNA level in ripening transgenic fruit was determined as described in Example 3.

The products of the RNA protection assay were resolved on polyacrylamide gels and exposed to X-ray

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film. A representative autoradiogram of the RNA protection assay is presented in Figure 6. As can be seen in the figure, AdoMetase mRNA was present in both transgenic plants at the breaker stage of fruit ripening. However, the levels of AdoMetase mRNA drop in the ESKN transgenic plant, relative to the SESKN transgenic plant, at the orange and ripe stages of fruit ripening.

The level of AdoMetase mRNA was quantitated as described in Example 3 by liquid scintillation counting and determination of mRNA concentrations relative to a standard curve. Figure 7 presents the results of this analysis. The results are consistent with those shown in Figure 6. AdoMetase mRNA was present in both transgenic plants at the breaker stage of fruit ripening with the concentrations lower in ESKN #18. At the orange and ripe stages of fruit ripening the levels of AdoMetase mRNA drop in the ESKN transgenic plant, relative to the level at breaker stage and the levels in the fruit from the SESKN transgenic plant. The AdoMetase mRNA levels stay relatively constant in the SESKN transgenic plant.

B. Relative Levels of SAMase Activity in Ripening Transgenic Tomatoes.

To determine whether the presence of AdoMetase enzyme activity correlated with the level of AdoMetase mRNA, a ¹⁴C-SAM-based AdoMetase assay was performed using extracts from four different fruit stages from a single pAG-5321 transgenic plant (ESKN).

Plant tissues to be assayed for AdoMetase enzyme activity were frozen and ground to a powder in liquid nitrogen. The ground tissue was then suspended in 1.5 volumes of 200 mM Tris-HCl (pH 7.5), 10 mM DTT, and 10 mM EDTA. The suspension was vortexed vigorously then

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subjected to centrifugation at 40,000 × g at 4°C for 20 minutes. The following was added to 50 µl of extract: 5 µl of ¹⁴C-SAM (DuPont-New England Nuclear, NEC-363) at 20 µCi/ml and a specific activity of 58.0 mCi/mmol.

- 5 The reaction was incubated at 37°C for 1 hour then 40 µl of the reaction was spotted on a cellulose thin layer chromatography (TLC) plate (J.T. Baker, Inc., Phillipsburg, N.J., Baker-Flex Cellulose F) and resolved for 3 hours in 70:70:20:40,
10 butanol:acetone:acetic acid:water. The MTA and MTR spots were identified using autoradiography, excised, and counted using liquid scintillation.

Figure 8 shows the level of AdoMetase activity in mature green, breaker, orange, and ripe fruit. The
15 level of AdoMetase activity is defined as the percent conversion of SAM (S-adenosylmethionine) to MTA (5'-Methylthioadenosine) and MTR (5'-Methylthioribose). The decreasing level of AdoMetase activity from breaker to ripe fruit in the ESKN transgenic plant is
20 consistent with the AdoMetase mRNA levels shown in Figure 7.

Untransformed tomato fruit extracts do not degrade SAM to MTA or MTR at any stage of ripening when used in this assay.

25

C. Ethylene Production in Ripening Transgenic Fruit.

Ethylene produced from transgenic tomatoes carrying the AdoMetase gene under the regulation of the
30 SE8 promoter (Figure 4) was determined as described in Example 4. Greenhouse grown tomatoes from 4 transgenic lines were tested. The results of the analysis are presented in Figures 9A to 9D. Each of the four graphs shown in Figure 9 represent the comparison of fruit
35 from one pGA-SESKN transgenic line (Es 19-2, LS 4-2, ES

35-1 and ES 22A-1) with the fruit from untransformed controls. The control values (open squares) are the same in each of the four graphs and represent the average of six fruit from two different plants. The values from each transgenic line (closed symbols) are the average of ethylene determinations for three fruit. Error bars represent one standard deviation of the data.

The data represent a time period of ten days after the breaker stage of fruit ripening (post-breaker). These data demonstrate a reduction in the amount of ethylene production in transgenic tomatoes versus normal fruit over the ten day period.

D. Post-Harvest Shelf-life of SESKN Tomatoes.

Tomatoes from the SESKN transgenic plants that synthesized less ethylene were assessed for their shelf life properties when stored at 22°C. Three fruit from each from SESKN lines 35-1, 22A-1 and LS4-2 were compared with tomatoes from two untransformed, normal plants (M16 and M15). Senescence was determined each day by visual examination of the fruit for the occurrence of contraction and wrinkles on the tomato skin. The results of these senescence assessments are shown in Figure 10.

As can be seen from the results in the figure, the bar graph shows the time for the fruit to achieve each stage: all fruit were picked at the breaker stage. For instance, line 35-1 took 18 days to ripen (Ripe stage) but then senescence developed at day 27. Line 22A-1 took 7 days to turn orange, 13 days to turn red, then 52 days to senescence. Even at 55 days post-breaker, the 22A-1 tomatoes remained firm and appeared to be suffering more from dehydration than from the softening-induced senescence of the normal tomatoes.

Firmness was not measured for the tomatoes from the five plants described above, however, the firmness was noted to be much greater in the fruit from the transgenic lines.

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EXAMPLE 6

Southern Blot Analysis of E4 Homologues in Several Species of Plants

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A Southern blot analysis was conducted to determine if sequences homologous to the tomato E4 gene were present in other plant species. The blot consisted of *Hind*III digests of six genomic plant DNAs: tomato, raspberry, strawberry, melon, carnation and cauliflower, along with size standards. This blot was hybridized with a probe following standard methods (Maniatis, et al.). The probe was a ~740 bp polymerase chain reaction (PCR; Mullis, Mullis, et al.) product amplified from genomic tomato DNA using PCR primers flanking the coding sequence of the E4 gene. The probe was labeled by incorporating ³²P-labeled nucleotides into the PCR reaction.

The primers were designed according to Cordes, et al. (1989). The 5' primer sequence, corresponding to the region between nucleotides 1439 and 1452 of the E4 gene (SEQ ID NO:8), is represented as SEQ ID NO:6 (ACG CAT GGA GGG TAA CAA). Positions 5-7 of this primer correspond to the ATG start codon of the E4 gene. The 3' primer sequence, corresponding to the region between nucleotides 2160 and 2177 of the E4 gene (SEQ ID NO:8), is represented as SEQ ID NO:7 (GAA GCA AGA CAG CAA ATG).

An autoradiograph of the blot is shown in Figure 14. Several bands are apparent in each lane, with the

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lane corresponding to tomato DNA showing the strongest signal.

EXAMPLE 7

5 Isolation of DNA Fragments Homologous to Tomato E4 from a Raspberry Genomic Library

A. Screening of the Library.

A raspberry genomic library in lambda GEM-11 was
10 obtained from Novagen (Madison, WI) and screened by
standard methods with the tomato E4 gene probe
described above. Three lambda clones which hybridized
to the probe were identified. The clones were purified
by 3 rounds of plaque purification. One of the clones
15 was selected for further analysis.

B. Analysis of a Positive Clone.

The clone was digested with several enzymes (Apa
I, Bam HI, Eco RI, Hind III, Nco I, Sac I, and Sal I),
20 run on a gel, and transferred to a "SUREBLOT" nylon
membrane (Oncor, Gaithersburg, MD). The blot was
hybridized overnight at 42°C with the tomato E4 probe
in "HYBRISOL I" hybridization cocktail (Oncor,
Gaithersburg, MD). The final (most stringent) wash was
25 0.1% SSC, 0.1% SDS for 30 minutes at room temperature
(22°C).

A 1.6kb Sac I fragment which hybridized to the
probe was subcloned into pGEM5Zf(+) (Promega, Madison,
WI) and further characterized. A 225 bp region in that
30 fragment was found to be highly homologous to the
tomato E4 gene at both the DNA level (74%) and the
amino acid level (80%). The sequence of this region
(SEQ ID NO:12) was compared to the sequence of a
portion of the tomato E4 gene (SEQ ID NO:8).

35 Additional raspberry E4 gene sequences were
obtained by further hybridization screening of

raspberry genomic library clones. The sequence of a genomic copy of a raspberry E4 gene is presented in Figure 15 (nucleotide sequence: SEQ ID NO:25; polypeptide sequence: SEQ ID NO:26).

5

EXAMPLE 8

Cloning of the AdoMetase Gene

A. Isolation of the AdoMetase Gene.

The AdoMetase (SAMase) gene was identified on an
10 *AluI*-*HaeIII* restriction fragment from purified T3 DNA
(Hughes, et al., 1987a). Bacteriophage T3 is available
under ATCC No. 11303-B3 (American Type Culture
Collection, 12301 Parklawn Dr., Rockville MD 20852).
The DNA fragment was first cloned into the
15 bacteriophage M13 MP8 vector (Pharmacia LKB
Biotéchnology, Inc., Piscataway, NJ). A *MaeIII* to
BamHI fragment was subcloned into the pUC19 plasmid
vector (Pharmacia) to produce pAG-110 (pUC19-SAMase,
Figure 2). This vector was transformed into *E. coli*
20 and used as a source of DNA for further construction
experiments, detailed below.

B. Modification of the Amino-Terminal Sequence of the Cloned AdoMetase Gene.

25 The cloned AdoMetase gene was further engineered
to contain a consensus eukaryotic translation
initiation site (Kozak; Lutcke, et al.) by altering the
nucleotide sequence surrounding the AdoMetase ATG
start-codon using a synthetic double-stranded
30 oligonucleotide.

Plasmid pAG-110 was digested with *XmnI* and *BamHI*
and the 1.9 kb and 1.3 kb fragments were purified by
electro-elution after agarose gel electrophoresis. A
double stranded synthetic oligonucleotide linker formed
35 by annealing oligonucleotides represented by SEQ ID

NO:1 and SEQ ID NO:3 (Figure 2) was ligated to the 1.9 kb fragment. This ligated DNA was subjected to *XmnI* digestion to remove excess linkers.

The linkered 1.9 kb fragment was then re-purified
5 by electrophoresis on low melting temperature agarose and ligated to the 1.3 kb fragment to form the plasmid pAG-111. The altered gene region was sequenced to confirm its identity. pAG-111 was used in subsequent recombinant DNA manipulations, including the
10 construction of plant expression vectors, detailed below. This plasmid DNA can also be used to directly transform the plant host via electroporation, microinjection, or microprojectile bombardment.

15 C. Vector Constructions using the Tomato E4 Promoter.

1. pAG-110, pAG-111, pAG-117

A 1.18kb E4 promoter was isolated from tomato (*Lycopersicon esculentum* var. *cerasiform*) DNA using the
20 polymerase chain reaction (PCR; Mullis; Mullis, et al.; Perkin-Elmer Cetus, Norwalk CT). The primers used in the PCR reaction were based on the sequence described by Cordes, et al. The sequences of the 5' and 3' oligonucleotide primers, shown in Figure 16, are
25 represented as SEQ ID NO:4 and SEQ ID NO:5, respectively. The oligonucleotides were designed to incorporate restriction endonuclease sites (*HindIII* and *NcoI*) at the 5' and 3' ends, respectively, of the amplified E4-promoter fragment. These restriction
30 endonuclease cleavage sites were used to subclone the E4-promoter fragment (Figure 17A) into the pAG-111 vector (Figure 17B), which contains an *NcoI* site at the ATG start codon in the region modified by the synthetic oligonucleotide (see Figure 2). The resulting vector,
35 containing an E4:SAMase chimeric construct in the

region between the *HindIII*/*KpnI* sites, was termed pAG-117.

2. pAG-5321

5 Agrobacterium binary vectors were developed from pGA482 (An, et al., 1985), a pBIN19 derivative (Clontech Laboratories) containing the neomycin phosphotransferase II gene (providing kanamycin resistance) fused to the nopaline synthesis (NOS) gene promoter (An, et al., 1988).

10 Figure 12B outlines the generation of vector pAG-5321 starting from vectors pGA482 and pAG-114. Figure 12A outlines the generation of the vector pAG-114 from vectors pAG-111 (described above) and pNCN (Pharmacia, Inc., Piscataway, NJ).

3. pAG-5520

25 The E4:SAMase chimera was excised from pAG-117 with a *HindIII*/*KpnI* digest. The resulting 1.7 kb fragment was purified as above, and cloned upstream of the nopaline synthase polyA addition site in pAG-5321, resulting in pAG-5520 (Figure 17D). The identity of the *HindIII*/*KpnI* insert was confirmed by DNA sequence analysis. This vector was used to generate the transgenic plants described herein.

30 Figures 17A-D and 12B-B outline one approach to the generation of Agrobacterium vectors for use in the present invention. However, the E4/SAMase cassette, present in, for example, pAG-117, can be incorporated in a number of vectors useful for plant transformation, such as pBI121 (Clontech Laboratories, Inc., Palo Alto, CA).

4. pAG-924

Vector pAG-924 was constructed by cloning the *Bam*HI/*Kpn*I SAMase fragment from pAG-111 into the same sites of pGEM7Zf(+) (Promega, Inc., Madison, WI). This
5 plasmid was used to make the RNA probe for RNase protection assays, described in Example 8.

5. Other Constructs

DNA constructs may be made using genes other than
10 the AdoMetase gene under the control of an E4 promoter, for example, other genes effective to reduce ethylene biosynthesis. Preferably, the E4 promoter is isolated from the same species of plant into which the construct is being introduced. For example, a tomato E4 promoter
15 may be used to direct the expression of a heterologous gene, such as AdoMetase, in tomatoes, while a raspberry E4 promoter may be used to direct the expression of a heterologous gene in raspberries.

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EXAMPLE 9

Plant Transformation

Agrobacterium tumefaciens strain EHA101 (Hood, et al.), a disarmed derivative of *Agrobacterium tumefaciens* strain C58, was used to introduce coding
25 sequences into plants. This strain contains a T-DNA-less Ti plasmid. The pAG-5520 construct was transferred into EHA101 using electroporation essentially as described by Nagel, et al. Briefly, an *Agrobacterium tumefaciens* culture was grown to mid-log
30 phase (OD 600 0.5 to 1.0) in MG/L media (5 gm tryptone, 2.5 g yeast extract, 5 gm NaCl, 5 gm mannitol, 1.17 gm sodium glutamate, 0.25 gm K₂HPO₄, 0.1 g MgSO₄, 2 µg biotin per liter, pH adjusted to 7.2 with NaOH). After chilling on ice 250 ml of the culture were pelleted,
35 resuspended in sterile, chilled 1 mM Hepes/KOH pH 7.0,

pelleted and resuspended as before, pelleted again, resuspended in sterile, chilled 10% glycerol, pelleted again, resuspended in 500 μ l sterile, chilled 10% glycerol and split into 80 μ l aliquots which were
5 frozen on dry ice/ethanol and stored at -80°C .

Typically, 0.1-1 μ g of plasmid DNA was added to a 40 μ l aliquot of cells and incubated on ice 30-60 seconds. The mix was then transferred to a 0.1 cm gap electroporation cuvette (Invitrogen) and pulsed at 1.25
10 kV (BioRad Gene Pulser at 25 μ F, BioRad Pulse controller at 200 Ω). One ml of MG/L media was added quickly after the pulse. The mixture was transferred to a microfuge tube and allowed to incubate at 28°C for 1 hour. The cells were diluted 1:100, and 10 and
15 100 μ l were plated on two MG/L plates containing 20 μ g/ml kanamycin, respectively. Kanamycin-resistant transformed colonies appeared within 2 days.

Seven to eight day-old tomato cotyledon tissue explants were excised from both the tip and base of the
20 cotyledon. Cotyledon explants were pre-conditioned for 2 days on tobacco feeder plates (Fillatti, et al.). The pre-conditioned explants were inoculated with EHA101 containing the pAG-5520 plasmid and placed in a 10 ml overnight culture of EHA101/pAG-5520 for 5
25 minutes. The explants were then co-cultivated with the EHA101 strain for 2 days on tobacco feeder plates as described by Fillatti, et al.

The explants were grown in tissue culture media (Fillatti, et al.) containing 2Z media, MS salts,
30 Nitsch and Nitsch vitamins, 3% sucrose, 2 mg/l seatin, 500 mg/l carbenicillin, 100 mg/l kanamycin and 0.7% agar for 8 to 10 weeks. Carbenicillin was used for 2 to 3 months (until the plants were potted in soil) in all media as a counter-selection to rid the plants of
35 viable *Agrobacterium tumefaciens* cells.

EXAMPLE 10RNAase Protection Assays for the Detection of AdoMetase mRNA

5 Tomato fruits at various stages of development from transgenic plants and wild-type plants were used as mRNA sources. mRNA was extracted from tomato cells and purified using the "QUICK PREP RNA" kit from Pharmacia, Inc (Piscataway, NJ). Alternatively, total
10 RNA was isolated using a LiCl precipitation procedure. Tissues were frozen in liquid nitrogen and ground to a fine powder. 550 μ l phenol/buffer (1:1, Tris-saturated phenol, pH 6.9 : Extraction buffer (100 mM LiCl, 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS)) at 80°C was added
15 for every 100 mg of the powder. The mixture was vortexed for 60 seconds, 250 μ l chloroform was added, the mixture vortexed for another 30 seconds, and spun to separate the phases. The aqueous phase was removed to a new tube, an equal volume of 4 M LiCl was added
20 and mixed, and the sample was placed at -20°C for 2 to 24 hours. The RNA was pelleted, washed and resuspended in water.

RNAse Protection Assays (RPA) were performed following the manufacturer's instructions using an
25 "RPAII" kit from Ambion, Inc. (Hialeah, FL), as previously described by Lee, et al.

Plasmid pAG-924 was used to generate ^{32} P-UTP- labeled RNA probe using bacteriophage T7 RNA polymerase as contained in the "RIBOPROBE II T7 RNA POLYMERASE
30 SYSTEM" from Promega, Inc. The radiolabeled probe was purified on a preparative polyacrylamide gel and used for up to one week.

One microgram of isolated mRNA was hybridized to approximately 10,000 CPM of the RNA probe and further
35 processed as per the instructions in the "RPA II" kit. Briefly, between 0.7 and 1.0 nanogram of the purified

mRNA was mixed with 10,000 CPM of the ^{32}P -RNA probe in a total volume of 15 μl . 20 μl of a hybridization buffer that allows hybridization of complementary sequences (Ausubel, et al.; Maniatis, et al.; Sambrook, et al.) is then added. The hybridization solution is provided in the "RPAII" kit from Ambion (Hialeah, FL). The solution was heated to 90°C for 3-4 minutes to denature all the RNA and incubated at 45°C overnight to allow hybridization of complementary sequences. The solution was cooled to 37°C and RNase (provided in the Ambion kit), which degrades all un-hybridized probe, was added.

Protected probe was resolved on a denaturing polyacrylamide gel, dried, and exposed to film for up to 3 hours. Quantitative analysis of the RPA signals was accomplished by excising each band from the gel, dissolving the band in a liquid fluor, and determining the radioactivity present in the sample using liquid scintillation counting. A standard curve was generated using various amounts of unlabeled RNA synthesized from a AdoMetase fragment cloned into pGEM5Z(+) in the sense orientation. The linear range of the assay was dependent on the amount of input ^{32}P -labeled RNA probe in the RNAase protection assay but typically ranged from 10 pg to 1 ng of mRNA.

EXAMPLE 11

Ethylene Measurements

A. Leaf disks

Measurement of ethylene from leaf discs was performed by excising five one-centimeter leaf discs from mature tomato leaves and placing them in a 25 ml Erlenmeyer flask on top of filter paper saturated with Murishige and Skoog (MS) medium or MS medium

supplemented with 10 μ M of the auxin naphthalene acetic acid (NAA).

B. Fruit

5 The assay for tomato ethylene evolution was performed by sealing glass jars containing individual fruit for a 0.5 to 1.0 hour period and sampling 2ml aliquots for gas chromatographic analysis.

10 B. Ethylene measurements

Ethylene evolution was measured by gas chromatography/flame ionization after 20 hours and recorded as nanoliters of ethylene/gram fresh weight/hour. A Hewlett Packard 5890 (Palo Alto, CA) 15 gas chromatograph with a flame ionization detector and a 6ft Porapak N column was used for all ethylene measurements (Adams, et al., Ward, et al.). This system combined with an HP Vectra computer and the current version of "CHEMSTATION" (Hewlett Packard) 20 allows measurement of ethylene concentrations as low as 0.2 nl of ethylene in a 2ml sample (0.1ppm).

Following measurement of the ethylene in the headspace, the values were converted to nanoliters of ethylene per gram of tissue per hour.

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EXAMPLE 12

Western Blot Analysis

Frozen tomato tissues were ground in liquid nitrogen, extracted directly into Lammeli sample buffer 30 (50mM Tris, pH 6.8, 1% SDS, 5% betamecaptoethaon, 10% glycerol, and .005% bromophenol blue), heated to 95°C for 5 minutes and centrifuged to remove debris. Total soluble protein in the supernatants was measured using the Coomassie Plus protein assay (Pierce, Rockford, 35 IL). Eight micrograms of soluble protein from each

sample, or known quantities of purified AdoMetase (positive control) were resolved on a polyacrylamide gel and electrophoretically transferred to Immobilon-P membrane using standard procedures. The blot was
5 incubated with 2 μ g/ml of the SAM10-9A3.1.3 monoclonal antibody to SAMase (Goding) in PBS-Tween (phosphate-buffered saline, 0.05% Tween 20), 1% bovine serum albumin (BSA) buffer for 60 minutes at 25°C. The blot was then washed 4 times in PBS-Tween buffer and
10 incubated for 60 minutes with a goat antimouse HRP-conjugate suspended in PBS-Tween, 1% BSA buffer (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Bound antibody was detected using the Renaissance chemiluminescence reagent (DuPont NEN, Boston, MA)
15 according to the manufacturer's instructions.

EXAMPLE 13

Characterization of Transgenic Tomato Plants

20 A. Promoter Effect on AdoMetase mRNA Levels in Wounded Leaves.

Fresh and wounded leaves from six independent transgenic plants, E4-1, E4-2, E4-3, E4-4, E4-5 and E4-6, were assayed for AdoMetase mRNA levels. All transgenic lines contained the E4 promoter adjacent the
25 AdoMetase gene.

Single, freshly detached leaves were wounded by cutting 6-7 times with a dull knife. Two hours after wounding the total RNA was extracted, reacted and analyzed as described in Example 10. Fresh leaves were
30 dropped directly into liquid nitrogen immediately after picking to halt RNase activity, and processed as above.

The products of the RNA protection assay were resolved on polyacrylamide gels and exposed to X-ray film. A representative autoradiogram of the RNA
35 protection assay is presented in Figure 18A. As can be

seen in the figure, expression of SAMase is silent in normal tomato plant leaves (fresh and wounded) and in fresh leaves isolated from transgenic plants.

Expression of AdoMetase RNA is clearly evident,
5 however, in wounded leaves from four of the six transgenic lines.

The level of AdoMetase mRNA was quantitated as described in Example 10 by liquid scintillation counting. Figure 18B presents the results of this
10 analysis, shown as a ratio of wound inducibility, for four of the lines shown in Figure 18A (E4-2, E4-4, E4-5 and E4-6), along with four other lines (E4-8, E4-10, E4-12 and E4-13). The results are consistent with those shown in Figure 18A, and indicate that a
15 transgene driven by an E4 promoter can be activated in plant tissues by the wounding of those tissues, in other words, that the E4 promoter is wound-inducible.

20 B. Promoter Effect on Ethylene Production in Wounded Leaves.

A leaf disc ethylene production assay was conducted, as detailed in Example 11, to measure the impact of wound-induced AdoMetase expression on wound-induced ethylene synthesis expected from these tissues.
25 Leaves from a normal (M) and four transgenic (E4-2, E4-4, E4-5 and E4-12) tomato plants were cut with a cork bore into one cm discs that were measured for their ability to release ethylene.

Table 1 summarizes ethylene synthesis in leaves
30 from control and four transgenic lines 4.5 and 19 hours after wounding. Leaves from two of the pAG-5520 transgenic lines were significantly reduced in their ability to produce ethylene. Lines E4-4 and E4-5 produced 69.6% and 84.8%, respectively, of the
35 ethylene produced by the control plants during the

first 4.5 hours. The ethylene reduction in those two lines were greater from 4.5 to 19 hours during which they produced 54.4% and 45.6%, respectively, of the controls. The data in this table are also presented in Figure 19 in graphical form.

TABLE 1

WOUND-INDUCED ETHYLENE SYNTHESIS IN
PAG-5520 TRANSGENIC PLANTS

Plant ID	4.5 hr. (nl/g/hr)	4.5 to 19 hr. (nl/g/hr)
Control	17.1 ± 2.7	5.7 ± 1.6
E4-2	20.2 ± 0.42	5.1 ± 1.5
E4-4	11.9 ± 1.3	3.1 ± 0.6
E4-5	14.5 ± 0.5	2.6 ± 0.4
E4-12	21.1 ± 5.1	5.4 ± 1.4

Error values are one standard deviation of the data (n=3).

C. Adometase mRNA Expression in Ripening Transgenic Fruit.

Expression of AdoMetase in ripening pAG-5520 tomato fruit was measured using an RNase protection assay (RPA), as detailed in Example 10. Ripening fruit were harvested at four different stages, mature green (MG), breaker (Br), orange (Or) and ripe (Ri).

Figure 20 displays the AdoMetase RNA expression level at each of these stages. Figure 21 is a

graphical representation of the same data, quantitated as described in Example 10 by liquid scintillation counting. Two of the transgenic lines assayed showed little or no expression of AdoMetase. Six other lines
5 showed significant AdoMetase RNA expression, with the orange stage being predominant. In five of the six lines expressing AdoMetase, the expression level at the ripe stage was substantially diminished, demonstrating the transient nature of the E4-directed expression.

10

D. Western Blot Analysis of AdoMetase Expression.

Western blot analysis was carried out on protein extracts from E4-5 transgenic ripening tomatoes as
15 detailed in Example 12. The results are shown in Figure 22.

Figure 22 shows the level of AdoMetase at four stages of fruit ripening. The pattern of expression matches that of AdoMetase transcription, including a
20 decline in AdoMetase at the ripe stage. Known quantities of purified recombinant AdoMetase were run in the control lanes and used to establish a standard curve based on signal intensity. This allowed
estimation of the relative amount of AdoMetase in these
25 tomatoes and was calculated to be approximately 0.05% of the total soluble protein at the orange stage of ripening.

30 E. Ethylene Production in Ripening Transgenic Fruit.

Daily ethylene production by control and pAG-5520 transgenic fruit picked at the breaker stage was measured by sealing glass jars containing individual fruit and sampling 2 ml aliquots for gas
35 chromatographic analysis, as detailed in Example 11.

Measurements were made over a period of 15 days post-harvest.

The results of the analysis are presented in Figure 23, which shows a comparison of fruit from one pAG-5520 transgenic line (E4-05) with the fruit from untransformed controls. The control values are represented as open squares, whereas the values from the transgenic line are represented as diamonds. The values are the average of ethylene determinations for three fruit. Error bars represent one standard deviation of the data.

In the transgenic line, the rate of ethylene production declines steeply immediately after harvest, reaching a minimum of approximately 1.0 nl/g/h at 7 days post-harvest. After this point, the rate increases to a level of approximately 2 to 3-fold the minimum and remains relatively constant. The kinetics of ethylene production from these transgenic tomatoes correlates with the observed AdoMetase RNA transcription and AdoMetase accumulation in the corresponding fruit. When AdoMetase expression is high the level of ethylene production is low, as expected.

The data represent a time period of fifteen days after the breaker stage of fruit ripening (post-breaker), and demonstrate a reduction in the amount of ethylene production in transgenic tomatoes versus normal fruit over the fifteen day period. Further, the data graphically illustrate the biological consequence of the transient nature of E4-driven Adometase expression described above.

The effect of AdoMetase gene expression on ripening has several dimensions, including (i) pAG-5520 tomatoes (transgenic line E4-12-D) develop color to a light red stage and then cease further color

development, and (ii) the transgenic tomatoes remain firm for much longer than controls.

While the invention has been described with
5 reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

68

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:
 (A) NAME: Epitope, Inc.
 (C) CITY: Beaverton
 (D) STATE: OR
 (E) COUNTRY: USA
10 (F) POSTAL CODE: 97005

 (ii) TITLE OF INVENTION: Regulated Expression of Heterologous
 Genes in Plants and Transgenic Fruit
 with a Modified Ripening Phenotype
15

 (iii) NUMBER OF SEQUENCES: 26

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 (E) COUNTRY: USA
 (F) ZIP: 94306
25

 (v) COMPUTER READABLE FORM:
 (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
30 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

 (vi) CURRENT APPLICATION DATA:
 (A) APPLICATION NUMBER:
 (B) FILING DATE:
35 (C) CLASSIFICATION:

 (vii) PRIOR APPLICATION DATA:
 (A) APPLICATION NUMBER: US 08/261,677
 (B) FILING DATE: 17-JUN-1994
40

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69

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(B) TELEFAX: (415) 324-0960

(2) INFORMATION FOR SEQ ID NO:1:
10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO
20

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Figure 2 - top strand of synthetic
25 oligo

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 12..38
30

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

GATCCGCCAC C ATG GTT TTC ACT AAA GAG CCT GCG AAC G
Met Val Phe Thr Lys Glu Pro Ala Asn
35 1 5

39

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 9 amino acids
(B) TYPE: amino acid

SUBSTITUTE SHEET (RULE 26)

70

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met Val Phe Thr Lys Glu Pro Ala Asn
1 5

10 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

15 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

20 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

25 (C) INDIVIDUAL ISOLATE: Figure 2, bottom strand of synthetic
oligo

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

30 CGTTCGCAGG CTCTTAGTG AAAACCATGG TGGCG

35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA

71

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Figure 16 - E4 promoter, 5' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

10 AGCCCATGGA AGCTTAAAGT AAACCT

26

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Figure 16 - E4 promoter, 3' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

30

TACCCTCCAT GGCTCAATCT CT

22

(2) INFORMATION FOR SEQ ID NO:6:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: DNA

72

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: E4 gene 5' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10 ACGCATGGAG GGTAACAA

18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: tomato E4 gene 3' primer

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

30

GAAGCAAGAC AGCAAATG

18

(2) INFORMATION FOR SEQ ID NO:8:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2796 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: DNA (genomic)

73

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Figure 24 - E4 tomato gene DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

10 (B) LOCATION: 1439..1774

(ix) FEATURE:

(A) NAME/KEY: exon

15 (B) LOCATION: 1439..1774

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1859..2113

20 (ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 1859..2113

(ix) FEATURE:

25 (A) NAME/KEY: intron

(B) LOCATION: 1775..1858

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

30

GAATTCTCAA TTGAGCCCAA TTCAATCTCC AATTTCACC CGTTTTAAAA CTTTTTATTA 60

AGATATGTTT CTATATTGAA AGTATGAATT ATTATCTATT TAACATCTTT TAGGATTTAT 120

35 CTATCCATTT GCTACTTTTT TAACAAAAAA TTCTTGAGTG AAAATTCAAA TTGTGATTAT 180

AAAAGTTAAA TATCAATATG TTAAATTATT AAGATTAATC GGGTCAAATT GCGGGGTCAA 240

GGCCCAATTC TTTTITAGCC CATTTAAGCT CAAAGTAAAC TTGGGTGGGT CAAGACCCAA 300

40

CTCGATTCTT GTTCAACCCA TTTTAATATT TCTATTTTCA ACCTAACCCG CTCATTTGAT 360

74

	ACCCCTACAA ATATCATATT TGTGTGTGAA ATATTTTTTG GGCTGGAGAG AGAGGCCCCG	420
	AGGGGAGTGG AGGGGTGGGG TGGGGAGAGA GAGCGAGAAA GAGTGGAGAG AGAAATTTGA	480
5	TATGAAATCC TACATATATT ACAGATTGTA ATGTTCTAAA CTATAACGAT TTGTCATAAA	540
	CACATATCAT GGATTTGTCT TTTTGTGTAA TTTTCCCAAT TGTAATAGG ACTTCGTTAT	600
	TTGAAACTTG AAAGTGAAGT CACATAGATT AAGTACAAAC ATTAATTAAA GACCGTGGTG	660
10	GAATGATAAA TATTTATTTA TCTTTAATTA GTTATTTTTT TGGGAGCTCT TTATTCCAAT	720
	GTGAGACTTT TGCGACATAT ATTCAAATTT AATCGAATCA CAATATGTAT TAGATTGATA	780
15	AAAAAATAAT TTTTTTACAA TGTTAGTTGA GACTCATAAC TTAATGCCTA TTGGTAATCT	840
	ATGACTCCTA ATTCCTTAAT TATTTAAATA TATCATCTTG ATCGTTAACA AAGTAATTTT	900
	GAAAGACCAC GAGTAAGAAG ACAAACGAGA ATACCAAAAA ATTCAAAAAT TTAATGTGAT	960
20	TTGGTCAATC GATCTACGTC CATAAAGGAG ATGAGTAATC TACTATAAAT ATGAGAGTAC	1020
	AAAATACAGA GAGAAACAAC CTCAACTAAT TCACTCGGAA TACATGAGAA GTTCACACAA	1080
25	GTGATAACGT ATCAAACCTG TGACCCACAC TTTTCCCTCT AACCAGCT CTTAAACTA	1140
	TATTGTGAAT GCTGATTAAG TTAAACGAAA CAGTCCTAAA TCTTTCCGT CCTATGAGAA	1200
	ACAAGATTAA TCAATTCACA ATTTTTTTAA AAAGAAAAAC CTGTAAGAAA TTTAGGCAAA	1260
30	CAAAACCTAA CACAAGTTTG TTTTGTGTTT TACTACCAAC AAGAAATTCA AATGGCAAAT	1320
	GTATAACGCA TCTTAGCTAA TTATATGACC AGATTCAGAT TAATATACAT CTTACCCAT	1380
35	GCAATCCATT TCTATATAAA GAAACATACA CGAAGTCAT ATTATTAGAG ATTGAGCA	1438
	ATG GAG GGT AAC AAC AGC AGT AGC AAG TCA ACC ACC AAT CCA GCA TTG	1486
	Met Glu Gly Asn Asn Ser Ser Ser Lys Ser Thr Thr Asn Pro Ala Leu	
	1 5 10 15	
40	GAT CCG GAT CTG GAC AGC CCG GAT CAG CCG GGT CTG GAG TTT GCC CAA	1534

75

	Asp	Pro	Asp	Leu	Asp	Ser	Pro	Asp	Gln	Pro	Gly	Leu	Glu	Phe	Ala	Gln		
				20					25						30			
	TTT	GCT	GCC	GGC	TGC	TTT	TGG	GGA	GTC	GAA	TTG	GCT	TTC	CAG	AGG	GTT	1582	
5	Phe	Ala	Ala	Gly	Cys	Phe	Trp	Gly	Val	Glu	Leu	Ala	Phe	Gln	Arg	Val		
			35					40					45					
	GGA	GGA	GTA	GTG	AAG	ACG	GAG	GTT	GGG	TAC	TCT	CAG	GGG	AAT	GTC	CAT	1630	
10	Gly	Gly	Val	Val	Lys	Thr	Glu	Val	Gly	Tyr	Ser	Gln	Gly	Asn	Val	His		
		50					55					60						
	GAC	CCG	AAC	TAC	AAG	CTT	ATT	TGC	TCC	GGA	ACA	ACC	GAA	CAT	GCC	GAG	1678	
	Asp	Pro	Asn	Tyr	Lys	Leu	Ile	Cys	Ser	Gly	Thr	Thr	Glu	His	Ala	Glu		
15		65				70				75			80					
	GCC	ATT	CGG	ATC	CAG	TTT	GAC	CCG	AAT	GTC	TGC	CCG	TAT	TCC	AAT	CTC	1726	
	Ala	Ile	Arg	Ile	Gln	Phe	Asp	Pro	Asn	Val	Cys	Pro	Tyr	Ser	Asn	Leu		
				85					90				95					
20	CTT	TCT	CTA	TTT	TGG	AGT	CGC	CAT	GAC	CCG	ACC	ACT	CTA	AAT	CGC	CAG	1774	
	Leu	Ser	Leu	Phe	Trp	Ser	Arg	His	Asp	Pro	Thr	Thr	Leu	Asn	Arg	Gln		
				100				105					110					
25	GTATCAAATT	CCTTTGGTGT	TTCATTTTAT	GTGATTAATA	TTAAAAATTT	TTTATATAAA	1834											
	TGTCATGATG	ATGTTTGTG	CTAG	GGT	AAT	GAT	GTG	GGA	AAG	CAA	TAC	CGC	1885					
							Gly	Asn	Asp	Val	Gly	Lys	Gln	Tyr	Arg			
							1				5							
30	TCA	GGA	ATA	TAT	TAC	TAT	AAT	GAT	GCT	CAG	GCT	CAA	CTG	GCA	AGG	GAG	1933	
	Ser	Gly	Ile	Tyr	Tyr	Tyr	Asn	Asp	Ala	Gln	Ala	Gln	Leu	Ala	Arg	Glu		
		10				15				20			25					
	TCG	TTA	GAA	GCT	AAG	CAG	AAG	GAA	TTT	ATG	GAT	AAG	AAA	ATT	GTC	ACT	1981	
35	Ser	Leu	Glu	Ala	Lys	Gln	Lys	Glu	Phe	Met	Asp	Lys	Lys	Ile	Val	Thr		
				30					35				40					
	GAA	ATT	CTT	CCT	GCT	AAG	AGA	TTT	TAT	AGA	GCT	GAA	GAG	TAT	CAC	CAG	2029	
40	Glu	Ile	Leu	Pro	Ala	Lys	Arg	Phe	Tyr	Arg	Ala	Glu	Glu	Tyr	His	Gln		
				45				50					55					

76

CAA TAT CTA GAG AAG GGT GGG GGC AGA GGT TGT AAG CAG TCG GCT GCA 2077
Gln Tyr Leu Glu Lys Gly Gly Gly Arg Gly Cys Lys Gln Ser Ala Ala
60 65 70

5 AAG GGC TGC AAT GAC CCA ATA AGG TGC TAC GGT TGACAGCAGA TCTTTGAATG 2130
Lys Gly Cys Asn Asp Pro Ile Arg Cys Tyr Gly
75 80 85

10 TCATAGCAAC TACAAAGAA CTTGTTAGAC ATTTGCTGTC TTGCTTCTTT AAATTGAAT 2190
AAACATGACA ATGATTCTTA TAACTACTTG CTCTCTTGGA TGAATAACT AGTTGTCGTA 2250
AAGTATTCTC CTCTTGCTAA TTATTATCTC TCTTTATATG GTACCTGCAA TTTGTTGCTT 2310

15 TAGTTACAGA ATAATGGACG TCAATTCTAT ATCTTAATTT GTTTTAAGTC TTAAATGAGG 2370
TGGTTTGTGT TTGAAAGCAA TATCAAGCAT AGTAATACCA ATGATTTAGT AGATGAACTT 2430
AATCAATCA AATTCCAAA TGCAGTCTAC AAATTGACAA CATGAAGTTA AGTGTATCTT 2490

20 ATGTAAATTG ACATCTTTCC TAGTAGATGC CTAATACTTT TGTAAAGACT AAAATAAGCA 2550
CAGATGAGGC TTGTGCATTT AACTTAGAGT TCATCCTTAG GTGTGGCTGC AGGAGACCCT 2610

25 GTAGGGTTGC TTGAAGTCTT GATGGGGTAG GAGGGTTGCA TTGCTATACC ACACAACCCC 2670
TCTTCAGCGT CAACCTTGCG CTGCATTCTA ATGTATCCTT TTTCTCCCCA TTCAGCTCCC 2730
CATGAGTTCT TCACAATCCA GTATTGGTT CCATCGACGG TTGTGCCATA CCCCACAATA 2790

30 GCCACA 2796

(2) INFORMATION FOR SEQ ID NO:9:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 196 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: protein

77

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

Met Glu Gly Asn Asn Ser Ser Ser Lys Ser Thr Thr Asn Pro Ala Leu
 1 5 10 15
 5 Asp Pro Asp Leu Asp Ser Pro Asp Gln Pro Gly Leu Glu Phe Ala Gln
 20 25 30
 Phe Ala Ala Gly Cys Phe Trp Gly Val Glu Leu Ala Phe Gln Arg Val
 10 35 40 45
 Gly Gly Val Val Lys Thr Glu Val Gly Tyr Ser Gln Gly Asn Val His
 50 55 60
 15 Asp Pro Asn Tyr Lys Leu Ile Cys Ser Gly Thr Thr Glu His Ala Glu
 65 70 75 80
 Ala Ile Arg Ile Gln Phe Asp Pro Asn Val Cys Pro Tyr Ser Asn Leu
 85 90 95
 20 Leu Ser Leu Phe Trp Ser Arg His Asp Pro Thr Thr Leu Asn Arg Gln
 100 105 110
 Gly Asn Asp Val Gly Lys Gln Tyr Arg Ser Gly Ile Tyr Tyr Tyr Asn
 25 115 120 125
 Asp Ala Gln Ala Gln Leu Ala Arg Glu Ser Leu Glu Ala Lys Gln Lys
 130 135 140
 30 Glu Phe Met Asp Lys Lys Ile Val Thr Glu Ile Leu Pro Ala Lys Arg
 145 150 155 160
 Phe Tyr Arg Ala Glu Glu Tyr His Gln Gln Tyr Leu Glu Lys Gly Gly
 165 170 175
 35 Gly Arg Gly Cys Lys Gln Ser Ala Ala Lys Gly Cys Asn Asp Pro Ile
 180 185 190
 Arg Cys Tyr Gly
 40 195

78

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 1678 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15 (vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Figure 25 - E4 tomato promoter /
AdoMetase gene DNA

20 (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1174..1629

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

25 AAGCTTAAAG TAAACTTGGG TGGGTCAAGA CCCAACTCGA TTTCTGTTCA ACCCATTTTA 60
ATATTTCTAT TTTCAACCTA ACCCGCTCAT TTGATACCCC TACAAATATC ATATTTGTGT 120
30 GTGAAATATT TTTTGGGCTG GAGAGAGAGG CCCCAGAGGG AGTGGAGGGG TGGGGTGGGG 180
AGAGAGAGCG AGAAAGAGTG GAGAGAGAAA TTTGATATGA AATCCTACAT ATATTACAGA 240
TTGTAATGTT CTAAACTATA ACGATTTGTC ATAAACACAT ATCATGGATT TGTCTTTTTG 300
35 TGTAATTTTC CCAATTGTAA ATAGGACTTC GTTATTTGAA ACTTGAAAGT GAAGTCACAT 360
AGATTAAGTA CAAACATTAA TTAAAGACCG TGGTGAATG ATAAATATTT ATTTATCTTT 420
40 AATTAGTTAT TTTTTTGGGA GCTCTTTATT CCAATGTGAG ACTTTTGC GA CATATATTCA 480

79

	AATTTAATCG AATCACAATA TGTATTAGAT TGATAAAAAA ATAATTTTTT TACAATGTTA	540
	GTTGAGACTC ATAACCTACT GCCTATTGGT AATCTATGAC TCCTAATTCC TTAATTATTT	600
5	AAATATATCA TCTTGATCGT TAACAAAGTA ATTTGAAAG ACCACGAGTA AGAAGACAAA	660
	CGAGAATACC AAAAAATTCA AAAATTTAAT GTGATTGGT CAATCGATCT ACGTCCATAA	720
	AGGAGATGAG TAATCTACTA TAAATATGAG AGTACAAAAT ACAGAGAGAA ACAACCTCAA	780
10	CTAATTCACCT CGGAATACAT GAGAAGTTCA CACAAGTGAT AACGTATCAA ACTTGTGACC	840
	CACACTTTTC CCTCTAACCA AAGCTCTTAA AACTATATTG TGAATGCTGA TTAAGTTAAA	900
15	CGAAACAGTC CTAAATCTTT TCCGTCCTAT GAGAAACAAG ATTAATCAAT TCACAATTTT	960
	TTTAAAAAGA AAAACCTGTA AGAAATTTAG GCAAACAAAA CCTAACACAA GTTTGTTTTT	1020
	GTCTTTTACTA CCAACAAGAA ATTCAAATGG CAAATGTATA ACGCATCTTA GCTAATTATA	1080
20	TGACCAGATT CAGATTAATA TACATCTTCA CCCATGCAAT CCATTTCTAT ATAAAGAAAC	1140
	ATACACGAAC TTGATATTAT TAGAGATTGA GCC ATG GTT TTC ACT AAA GAG CCT	1194
25	Met Val Phe Thr Lys Glu Pro	
	1 5	
	GCG AAC GTC TTC TAT GTA CTG GTT TCC GCT TTC CGT TCT AAC CTC TGC	1242
	Ala Asn Val Phe Tyr Val Leu Val Ser Ala Phe Arg Ser Asn Leu Cys	
	10 15 20	
30	GAT GAG GTG AAT ATG AGC AGA CAC CGC CAC ATG GTA AGC ACT TTA CGT	1290
	Asp Glu Val Asn Met Ser Arg His Arg His Met Val Ser Thr Leu Arg	
	25 30 35	
35	GCC GCA CCG GGT CTT TAT GGC TCC GTT GAG TCA ACC GAT TTG ACC GGG	1338
	Ala Ala Pro Gly Leu Tyr Gly Ser Val Glu Ser Thr Asp Leu Thr Gly	
	40 45 50 55	
	TGC TAT CGT GAG GCA ATC TCA AGC GCA CCA ACT GAG GAA AAA ACT GTT	1386
40	Cys Tyr Arg Glu Ala Ile Ser Ser Ala Pro Thr Glu Glu Lys Thr Val	
	60 65 70	

80

CGT GTA CGC TAC AAG GAC AAA GCG CAG CCA CTC AAT GTT GCA CGC CTA 1434
 Arg Val Arg Tyr Lys Asp Lys Ala Gln Pro Leu Asn Val Ala Arg Leu
 75 80 85

5 GCT TCT AAT GAG TGG GAG CAA GAT TGC GTA CTG GTA TAC AAA TCA CAG 1482
 Ala Ser Asn Glu Trp Glu Gln Asp Cys Val Leu Val Tyr Lys Ser Gln
 90 95 100

ACT CAC ACG GCT GGT CTG GTG TAC GCT AAA GGT ATC GAC GGG TAT AAG 1530
 10 Thr His Thr Ala Gly Leu Val Tyr Ala Lys Gly Ile Asp Gly Tyr Lys
 105 110 115

GCT GAA CGT CTG CCG GGT AGT TTC CAA GAG GTT CCT AAA GGC GCA CCG 1578
 Ala Glu Arg Leu Pro Gly Ser Phe Gln Glu Val Pro Lys Gly Ala Pro
 15 120 125 130 135

CTG CAA GGC TGC TTC ACT ATT GAT GAG TTC GGT CGC CGC TGG CAA GTA 1626
 Leu Gln Gly Cys Phe Thr Ile Asp Glu Phe Gly Arg Arg Trp Gln Val
 140 145 150

20 CAA TAACGTGTTA AACTCAAGGT CATGCACGAT GCGTGGCGGA TCGGGTACC 1678
 Gln

25

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 152 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: protein

35

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

Met Val Phe Thr Lys Glu Pro Ala Asn Val Phe Tyr Val Leu Val Ser
 1 5 10 15

40 Ala Phe Arg Ser Asn Leu Cys Asp Glu Val Asn Met Ser Arg His Arg
 20 25 30

82

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..213

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

GAG CTC AGG TTT CAG CGA GTG GCC GGT GTG GTC AAG ACC GAG GTT GGG      48
Glu Leu Arg Phe Gln Arg Val Ala Gly Val Val Lys Thr Glu Val Gly
  1             5             10             15
10 TAC TCC CAG GGC CAC GTC CAC GAT CCG AAT TAC AAA CTG GTC TGC TCC      96
Tyr Ser Gln Gly His Val His Asp Pro Asn Tyr Lys Leu Val Cys Ser
             20             25             30

15 GGA ACT ACC AAC CAT TCG GAG GTC GTT CGG GTC CAG TTC GAC CCG CAA      144
Gly Thr Thr Asn His Ser Glu Val Val Arg Val Gln Phe Asp Pro Gln
             35             40             45

GTC TAC CCA TAC TCG GAC CTG CTT TCC GTC TTT TGG TCT CGT CAT GAT      192
20 Val Tyr Pro Tyr Ser Asp Leu Leu Ser Val Phe Trp Ser Arg His Asp
             50             55             60

CCA ACG ACT GTC AAT CGC CAG GTATGGGGAT TG      225
Pro Thr Thr Val Asn Arg Gln
25 65             70

```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

```

30 (A) LENGTH: 71 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

```

(ii) MOLECULE TYPE: protein

35

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

```

Glu Leu Arg Phe Gln Arg Val Ala Gly Val Val Lys Thr Glu Val Gly
  1             5             10             15
40 Tyr Ser Gln Gly His Val His Asp Pro Asn Tyr Lys Leu Val Cys Ser

```

81

His Met Val Ser Thr Leu Arg Ala Ala Pro Gly Leu Tyr Gly Ser Val
 35 40 45

Glu Ser Thr Asp Leu Thr Gly Cys Tyr Arg Glu Ala Ile Ser Ser Ala
 5 50 55 60

Pro Thr Glu Glu Lys Thr Val Arg Val Arg Tyr Lys Asp Lys Ala Gln
 65 70 75 80

10 Pro Leu Asn Val Ala Arg Leu Ala Ser Asn Glu Trp Glu Gln Asp Cys
 85 90 95

Val Leu Val Tyr Lys Ser Gln Thr His Thr Ala Gly Leu Val Tyr Ala
 100 105 110

15 Lys Gly Ile Asp Gly Tyr Lys Ala Glu Arg Leu Pro Gly Ser Phe Gln
 115 120 125

Glu Val Pro Lys Gly Ala Pro Leu Gln Gly Cys Phe Thr Ile Asp Glu
 20 130 135 140

Phe Gly Arg Arg Trp Gln Val Gln
 145 150

25 (2) INFORMATION FOR SEQ ID NO:12:

 (i) SEQUENCE CHARACTERISTICS:

 (A) LENGTH: 225 base pairs

 (B) TYPE: nucleic acid

30 (C) STRANDEDNESS: double

 (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: DNA (genomic)

35 (iii) HYPOTHETICAL: NO

 (iv) ANTI-SENSE: NO

 (vi) ORIGINAL SOURCE:

40 (C) INDIVIDUAL ISOLATE: Figure 25 - raspberry E4 gene DNA

83

20 25 30

Gly Thr Thr Asn His Ser Glu Val Val Arg Val Gln Phe Asp Pro Gln
 35 40 45

5 Val Tyr Pro Tyr Ser Asp Leu Leu Ser Val Phe Trp Ser Arg His Asp
 50 55 60

Pro Thr Thr Val Asn Arg Gln
 10 65 70

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
 15 (A) LENGTH: 10 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA

 (iii) HYPOTHETICAL: NO

 (iv) ANTI-SENSE: NO

25 (vi) ORIGINAL SOURCE:
 (C) INDIVIDUAL ISOLATE: original T3 sequence

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

30 CACCAAATGA

10

(2) INFORMATION FOR SEQ ID NO:15:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA

84

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

5 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Kozak sequence

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

10 GCCACCATGG

10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 81 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

25

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Fig. 2, first DNA sequence

(ix) FEATURE:

30

(A) NAME/KEY: CDS

(B) LOCATION: 49..81

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

35

AAGCTTGCAT GCCTGCAGGT CGACTCTAGA GGATCCCCGT AACACCAA ATG ATT TTC

57

Met Ile Phe

1

40 ACT AAA GAG CCT GCG AAC GTC TTC

81

Thr Lys Glu Pro Ala Asn Val Phe

85

5

10

(2) INFORMATION FOR SEQ ID NO:17:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

15 Met Ile Phe Thr Lys Glu Pro Ala Asn Val Phe

1

5

10

(2) INFORMATION FOR SEQ ID NO:18:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

30

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Fig. 2, T3 SAMase

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CACCAAATGA TT

12

40 (2) INFORMATION FOR SEQ ID NO:19:

86

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Fig. 2, SAM-K

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCCACCATGG TT

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Fig. 3, E8 5' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGTCTAGAAG GAATTCACG

87

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: Fig. 3, E8 3' primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

20

ATTACAGTG CAAAAGACCA TGGAA

25

25 (2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 586 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

35 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

40

(C) INDIVIDUAL ISOLATE: Fig. 11, pUC19-SAM-K

88

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 66..521

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

	ACAGCTATGA CCATGATTAC GCCAAGCTTG CATGCCTGCA GGTCCGACTCT AGAGGATCCG	60
10	CCACC ATG GTT TTC ACT AAA GAG CCT GCG AAC GTC TTC TAT GTA CTG Met Val Phe Thr Lys Glu Pro Ala Asn Val Phe Tyr Val Leu	107
	1 5 10	
15	GTT TCC GCT TTC CGT TCT AAC CTC TGC GAT GAG GTG AAT ATG AGC AGA Val Ser Ala Phe Arg Ser Asn Leu Cys Asp Glu Val Asn Met Ser Arg	155
	15 20 25 30	
20	CAC CGC CAC ATG GTA AGC ACT TTA CGT GCC GCA CCG GGT CTT TAT GGC His Arg His Met Val Ser Thr Leu Arg Ala Ala Pro Gly Leu Tyr Gly	203
	35 40 45	
25	TCC GTT GAG TCA ACC GAT TTG ACC GGG TGC TAT CGT GAG GCA ATC TCA Ser Val Glu Ser Thr Asp Leu Thr Gly Cys Tyr Arg Glu Ala Ile Ser	251
	50 55 60	
30	AGC GCA CCA ACT GAG GAA AAA ACT GTT CGT GTA CGC TAC AAG GAC AAA Ser Ala Pro Thr Glu Glu Lys Thr Val Arg Val Arg Tyr Lys Asp Lys	299
	65 70 75	
35	GCG CAG GCA CTC AAT GTT GCA CGC CTA GCT TGT AAT GAG TGG GAG CAA Ala Gln Ala Leu Asn Val Ala Arg Leu Ala Cys Asn Glu Trp Glu Gln	347
	80 85 90	
40	GAT TGC GTA CTG GTA TAC AAA TCA CAG ACT CAC ACG GCT GGT CTG GTG Asp Cys Val Leu Val Tyr Lys Ser Gln Thr His Thr Ala Gly Leu Val	395
	95 100 105 110	
40	TAC GCT AAA GGT ATC GAC GGG TAT AAG GCT GAA CGT CTG CCG GGT AGT Tyr Ala Lys Gly Ile Asp Gly Tyr Lys Ala Glu Arg Leu Pro Gly Ser	443
	115 120 125	

89

TTC CAA GAG GTT CCT AAA GGC GCA CCG CTG CAA GGC TGC TTC ACT ATT 491
 Phe Gln Glu Val Pro Lys Gly Ala Pro Leu Gln Gly Cys Phe Thr Ile
 130 135 140

5 GAT GAG TTC GGT CGC CGC TGG CAA GTA CAA TAAGTGTTAA ACTCAAGGTC 541
 Asp Glu Phe Gly Arg Arg Trp Gln Val Gln
 145 150

10 ATGCACGATG CGTGGCGGAT CGGGTACCGA GCTCGAATTC ACTGG 586

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 152 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Val Phe Thr Lys Glu Pro Ala Asn Val Phe Tyr Val Leu Val Ser
 1 5 10 15

25 Ala Phe Arg Ser Asn Leu Cys Asp Glu Val Asn Met Ser Arg His Arg
 20 25 30

30 His Met Val Ser Thr Leu Arg Ala Ala Pro Gly Leu Tyr Gly Ser Val
 35 40 45

Glu Ser Thr Asp Leu Thr Gly Cys Tyr Arg Glu Ala Ile Ser Ser Ala
 50 55 60

35 Pro Thr Glu Glu Lys Thr Val Arg Val Arg Tyr Lys Asp Lys Ala Gln
 65 70 75 80

Ala Leu Asn Val Ala Arg Leu Ala Cys Asn Glu Trp Glu Gln Asp Cys
 85 90 95

40

Val Leu Val Tyr Lys Ser Gln Thr His Thr Ala Gly Leu Val Tyr Ala

90

100	105	110
Lys Gly Ile Asp Gly Tyr Lys Ala Glu Arg Leu Pro Gly Ser Phe Gln		
115	120	125

5 Glu Val Pro Lys Gly Ala Pro Leu Gln Gly Cys Phe Thr Ile Asp Glu

130	135	140
-----	-----	-----

10 Phe Gly Arg Arg Trp Gln Val Gln

145	150	
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(2) INFORMATION FOR SEQ ID NO:24:

- 15 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2216 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- 20 (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
- 25 (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
- (C) INDIVIDUAL ISOLATE: Fig. 13, E8 promoter
- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

	GAATTCATTT TTGACATCCC TAATGATATT GTTCACGTAA TTAAGTTTTG TGGAAGTGAG	60
35	AGAGTCCAAT TTTGATAAGA AAAGAGTCAG AAAACGTAAT ATTTTAAAAG TCTAAATCTT	120
	TCTACAAATA AGAGCAAATT TATTTATTTT TTAATCCAAT AAATATTAAT GGAGGACAAA	180
	TTCAATTCAC TTGGTTGTAA AATAAACTTA AACCAATAAC CAAAGANCTA ATAAATCTGA	240
40	AGTGGAATTA TTAAGGATAA TGTACATAGA CAATGAAGAA ATAATAGGTT CGATGAATTA	300

91

	ATAATAATTA AGGATGTTAC AATCATCATG TGCCAAGTAT ATACACAATA TTCTATGGGA	360
	TTTATAATTT CGTTACTTCA CTTAACTTTT GCGTAAATAA AACGAATTAT CTGATATTTT	420
5	ATAATAAAAC AGTTAATTAA GAACCATCAT TTTAACAAC ATAGATATAT TATTTCTAAT	480
	AGTTTAATGA TACTTTTAAA TCTTTTAAAT TTTATGTTTC TTTTAGAAAA TAAAAATTCA	540
	AAAAAATTAA ATATATTTAC AAAAATAACA ATCAAACACA ACTTCATATA TTAAAGCAA	600
10	AATATATTTT GAAAATTTC AAGTGCCTAA CAAATAAGAC AAGAGGAAAA TGTACGATGA	660
	GAGACATAAA GAGAACTAAT AATTGAGGAG TCCTATAATA TATAATAAAG TTTATTAGTA	720
15	AACTTAATTA TTAAGGACTC CTAAATATA TGATAGGAGA AAATGAATGG TGAGAGATAT	780
	TGGAAACTT AATAATTAAG GATNTTAAAA TATATGGTAA AAGATAGGCA AAGTATCCAT	840
	TATCCCCTTT TAACTTGAAG TCTACCTAGG CGCATGTGAA AGGTTGATT TTTGTCACGT	900
20	CATATAGCTA TAACGTAAAA AAAGAAAGTA AAATTTTAA TTTTTTTAA TATATGACAT	960
	ATTTTAAACG AAATATAGGA CAAAATGTAA ATGAATAGTA AAGGAAACAA AGATTAATAC	1020
25	TTACTTTGTA AGAATTTAAG ATAAATTTAA AATTTAATAG ATCAACTTTA CGTCTAGAAA	1080
	GACCCATATC TAGAAGGAAT TTCACGAAAT CGGCCCTTAT TCAAAAATAA CTTTTAAATA	1140
	ATGAATTTTA AATTTTAAGA AATAATATCC AATGAATRAA TGACATGTAG CATTTTACCT	1200
30	AAATATTTCA ACTATTTTAA TCCAATATTA ATTTGTTTTA TTCCAACAA TAGAAAGTCT	1260
	TGTGCAGACA TTTAATCTGA CTTTCCAGT ACTAAATATT AATTTTCTGA AGATTTTCGG	1320
35	GTTTAGTCCA CAAGTTTTAG TGAGAAGTTT TGCTCAAAAT TTAGGTGAG AAGGTTTGAT	1380
	ATTTATCTTT TGTAAATTA ATTTATCTAG GTGACTATTA TTTATTTAAG TAGAAATTCA	1440
	TATCATTACT TTTGCCAACT TGAGTCATA ATAGGAGTAG GTGTATATGA TGAAGGAATA	1500
40	AACAAGTTCA GTGAAGTGAT TAAAATAAAA TATAATTTAG GTGTACATCA AATAAAAACC	1560

92

TTAAAGTTTA GAAAGGCACC GAATAATTTT GCATAGAAGA TATTAGTAAA TTTATAAAAA 1620
TAAAGAAAT GTAGTTGTCA AGTTGTCTTC TTTTTTTTGG ATAAAAATAG CAGTTGGCTT 1680
5 ATGTCATTCT TTTACAACCT CCATGCCACT TGTCCAATTG TTGACACTTA ACTAATTAGT 1740
TTGATTCATG TATGAATACT AAATAATTTT TTAGGACTGA CTCAAATATT TTTATATTAT 1800
CATAGTAATA TTTATCTAAT TTTTAGGACC ACTTATTACT AAATAATAAA TTAECTACTA 1860
10 CTATATTATT GTTGTGAAAC AACAAAGTTT TGGTTGTTAT GATGAAACGT AACTATATC 1920
AGTATGAAAA ATTCAAAACG ATTAGTATAA ATTATATTGA AAATTGATA TTTTCTATT 1980
15 CTTAATCAGA CGTATTGGGT TTCATATTTT AAAAAGGGAC TAACTTAGA AGAGAAGTTT 2040
GTTTGAAACT ACTTTTGTCT CTTTCTTGTT CCCATTCTC TCTTAGATT CAAAAAGTGA 2100
ACTACTTTAT CTCTTTCTTT GTTCACATTT TATTTTATTC TATTATAAAT ATGGCATCCT 2160
20 CATATTGAGA TTTTAGAAA TTATTCTAAT CATTACAGT GCAAAGACC ATGGAA 2216

(2) INFORMATION FOR SEQ ID NO:25:

- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2708 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
30 (ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
35 (iv) ANTI-SENSE: NO
(v) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: RASPBERRY E4 GENE
40 (ix) FEATURE:
(A) NAME/KEY: misc_feature

93

(B) LOCATION: 1468..1469

(D) OTHER INFORMATION: /note= "small sequencing gap of
unknown size"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

	AAGCTTAATT GAGATGATTA GCCCAGACCC AGCAGGATTA GGCTTAATGG TGGTCCATTT	60
10	GAGAAAAAGA TTAAAAATGA TGTCATAAAA AAACNTGGTC GBCAGGATTC NAACCTGCCGC	120
	GGGCAAAGCC ACATGATTTC TAGTCATGCC CGATAACCAC TCCGGCACGA CCACAATGAT	180
	GCTACAATTG CTTTGGTGTG ATCATTAACT TATGGTTGAG TTTGATGCTG ATTAATACTA	240
15	TTATGTTTCC ATTAATACT TTTGAAGTAT ACAAATTAC GAATTTATAA CCAAATTTGA	300
	GGTATAATAT GCGAGAGCTA CCTAAATTTT TCTTACTTAA TTTTAAAGTA CATTCAAATT	360
20	CTGAATTTAT ATTGTGTATA GTCAGAAAAC AATCTACATA TTTAAACACA TAAATTTCTC	420
	ACGTTTATAA TCAATTTTGT CGGTTCTGT AATTTTCTA AAATAAAAAG CAACCAAAT	480
	TGTGCATCAA CTTATTACAT ACCATGGGAA ATGCAACTT CAAACTTAT GGAATCAAAG	540
25	GGTACATATC TAACTACAT ATTGTCAGAT TCTTCACTCT TATTTCTTGA GGGCCTCGAG	600
	GCATTACCAA CCAAATCCAA AAATTGCTTT CGAATCTCAA TAAAAAGGAT AACCCCATGA	660
30	AAAAGACGTG GACGGCAGGA TTCGAACCTG CGCGCAGAGC CCACATGATT TCTAGTCATG	720
	CCCATAACC ACTCCGGCAC GTCCACTTCA CTGTTAACGT TTACAGTAAC AAGTCACTAA	780
	CTACTAATCA ACATTAGCTC AGGAAATCAA AACTAGATTA TTTACATTTA CAACGACATG	840
35	TCGTTTGAAG TAGTTGGTCT GTATCTGAGT AGCTTTGGCG GGTAGATTCA ATCGCATTTT	900
	TGCATATAAA ACTGATCCTC CCTCTATCGC CAAAGTCAA CTGAAAATGG CTTCCACCAC	960
40	CACCAACAAC CCAGCTCTAG ACCCAGATTC GGACACTCCG GATAATCCGG GTCACGAGTT	1020

94

	TGCTCAATTC GGATCCGGGT GCTTCTGGGG AGCCGAGCTC AGGTTTCAGC GAGTGGCCGG	1080
	TGTGGTCAAG ACCGAGGTTG GGTACTCCCA GGGCCACGTC CACGATCCGA ATTACAACT	1140
5	GGTCTGCTCC GGAAC TACCA ACCATTCCGA GGTGTTCCG GTCCAGTTCG ACCCGCAAGT	1200
	CTACCCATAC TCGGACCTGC TTTCCGTCTT TTGGTCTCGT CATGATCCAA CGACTGTCAA	1260
	TCGCCAGGTA TGGGGATTGG GGA CTTCTGT TTTCATTGA ATTTTGATGC TAAAAAATTT	1320
10	CTTGCTTTTT CATACTACAC AGTACACACA AAAAGTTGTG TTTTTTTTCA TTCTTTTAAA	1380
	TAGTAGTTGG AAAAGTGCTC TTGGAGTTGA AGAGTACTTC AGTATTGCAT ATGGTCTCAG	1440
15	TGAAATGATA GTGATTATCA TAAGGAGTTT AAAGGCAGGA TGCATTTTGT GTATGANTGA	1500
	TTTTGGGTAG AATATTTTGT GAACAGTTAA AATTTATGGG CTGCTGCACA CTGGCTATGA	1560
	ACAAATGTAT AGCATTAAAG TGCTTATGAC AAATTCACAA TTGTATATTA GCAGCAGAGA	1620
20	CATTAAAGTT TCTAAATGCC TTTTAAGTAG ATTGGAAAA AGTGCTTTTT TTGGTTGAAG	1680
	AAGCACATTC ACTATTTGCC TGTTAATGGA ATTGGTAATG ATGAATCACA AGGATATTTG	1740
25	TGAATACAAG CAGGATGCTT TTAGTGTGCA AGTGATCTTT CGGAACATTT AAAATCGTCA	1800
	TAACAAAGGT GTAACATAAG AAGGCTTTGA AATATTCTCA ATTTCTCATT GATTGAATGA	1860
	ATTATGTGTT AGGGTGGAGA TGTGGGTACT CAATATCGAT CTGGAATATA CTA CTAAC	1920
30	GAAACGCAGG CCCGTCTAGC ACAGGAATCA AAGGAAGCAA AGCAACTGGA GTTTAAGGAT	1980
	AAGAAGGTGG TGACAGAGAT TCTTCCAGCA AAGAGGTTTT ACAGGGCAGA GGAGTACCAT	2040
35	CAGCAATATC TCGCAAAGGG AGGAGGTAAT GGCAACAAAC AATCTGCTGA AAAAGGTTGC	2100
	AATGATCCTA TTCGATGCTA TGGTTGAGAA ACTAATGCAT TATGCCATTA TTAAACTCT	2160
	ACTGGTTTAC TATGCAGAAA CACCTATGTC AGTTCAATTA TACTGAAGGC ACCAAAGTGT	2220
40	CATCTTAAAT TATATGGCAA TGTTTTACTC GTTATGAATA AAGGAGGTCC AAGTCGACCA	2280

95

GATATGAACA AATGAAATAT TGCCATGTTA ATTGGAATCC AGTAGTAATT AGGATTTGTT 2340

TTGGTGTATG TACTCCGATA TCAAGATATG CAAATGATGC ATTGTGTTTT TATATATTGA 2400

5 CAAGTTCCAA ATTATAGTAC TTCGTATGTG TTATGCGGTT TAATTAGTGT TGCTTACTTG 2460

AATGGTATAT TACTATTATG CTTAGTAGGA ACTAGGAACT AGGGAATATG TTGTGATAGA 2520

GTTGTCCAAC GAAATTTTGG ACCAAAGTTA TTTCATTGAA TAAAACTAC AGTCTTAGAG 2580

10 ATACATCCAA TTCTATAAAG TGAAGAAGC AAATATTATT TGTTTCATGAG GCTATGAGTC 2640

ATGAACTTTA TGCTATAACC GAAGCAACCT CAGAAAAGTC GAAGTAAATT GTGTATTGTT 2700

15 TAGAGCTC 2708

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 191 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

30 (vi) ORIGINAL SOURCE:

(C) INDIVIDUAL ISOLATE: RASPBERRY E4 PROTEIN

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Met Ala Ser Thr Thr Thr Asn Asn Pro Ala Leu Asp Pro Asp Ser Asp

1 5 10 15

40 Thr Pro Asp Asn Pro Gly His Glu Phe Ala Gln Phe Gly Ser Gly Cys

20 25 30

96

	Phe Trp Gly Ala Glu Leu Arg Phe Gln Arg Val Ala Gly Val Val Lys
	35 40 45
5	Thr Glu Val Gly Tyr Ser Gln Gly His Val His Asp Pro Asn Tyr Lys
	50 55 60
	Leu Val Cys Ser Gly Thr Thr Asn His Ser Glu Val Val Arg Val Gln
	65 70 75 80
10	Phe Asp Pro Gln Val Tyr Pro Tyr Ser Asp Leu Leu Ser Val Phe Trp
	85 90 95
	Ser Arg His Asp Pro Thr Thr Val Asn Arg Gln Gly Gly Asp Val Gly
	100 105 110
15	Thr Gln Tyr Arg Ser Gly Ile Tyr Tyr Tyr Asn Glu Thr Gln Ala Arg
	115 120 125
	Leu Ala Gln Glu Ser Lys Glu Ala Lys Gln Leu Glu Phe Lys Asp Lys
20	130 135 140
	Lys Val Val Thr Glu Ile Leu Pro Ala Lys Arg Phe Tyr Arg Ala Glu
	145 150 155 160
25	Glu Tyr His Gln Gln Tyr Leu Ala Lys Gly Gly Gly Asn Gly Asn Lys
	165 170 175
	Gln Ser Ala Glu Lys Gly Cys Asn Asp Pro Ile Arg Cys Tyr Gly
	180 185 190

IT IS CLAIMED:

1. A transgenic fruit-bearing plant, comprising
(i) a DNA sequence encoding a product that is
effective to reduce ethylene biosynthesis in fruit from
the plant, and (ii) a promoter whose expression is
induced during fruit ripening or by ethylene synthesis by
said fruit, where said DNA sequence is heterologous to
said promoter and said DNA sequence is operably linked to
said promoter to enable expression of said product.
2. A transgenic plant of claim 1, wherein said DNA
sequence encodes S-adenosylmethionine hydrolase.
3. A transgenic plant of claim 1, wherein said DNA
sequence encodes a product selected from the group
consisting of aminocyclopropane-1-carboxylic acid (ACC)
deaminase, ACC oxidase antisense molecule, ACC synthase
antisense molecule, ACC oxidase cosuppression molecule,
and ACC synthase cosuppression molecule.
4. A transgenic plant of claim 1, wherein the
promoter is obtained from a gene homologous to a tomato
E4 or E8 gene.
5. A transgenic plant of claim 4, wherein the
promoter is from a tomato E4 or E8 gene.
6. A transgenic plant of claim 4, wherein the
promoter is from a raspberry E4 gene.
7. A transgenic plant of claim 1, wherein the
promoter is obtained from a gene selected from the group
consisting of avocado cellulase gene and tomato
polygalacturonase gene.



8. A transgenic plant of claim 1, wherein the promoter is obtained from a gene homologous to a gene selected from the group consisting of avocado cellulase gene and tomato polygalacturonase gene.

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9. A method for modifying ripening fruit of a fruit bearing plant, comprising,

growing the plant of claim 1, to produce a transgenic plant bearing fruit, wherein fruit produced by said plant has an initial burst of ethylene production, followed by a reduction in the level of ethylene synthesis by said fruit, resulting in a fruit having a modified ripening phenotype.

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10. A fruit produced by the plant of claim 1.

11. A method for producing a transgenic fruit-bearing plant, where fruit produced by said plant has a modified ripening phenotype, comprising

introducing into progenitor cells of the plant (i) a DNA sequence encoding a product effective to reduce ethylene biosynthesis in fruit from the plant, and (ii) a promoter whose expression is induced during fruit ripening or by ethylene synthesis by said fruit, where said DNA sequence is heterologous to said promoter and said DNA sequence is operably linked to said promoter to enable expression of said product, and

growing the transformed progenitor cells to produce a transgenic plant bearing fruit, wherein fruit produced by said plant has an initial burst of ethylene production, followed by a reduction in the level of ethylene synthesis by said fruit, resulting in fruit having a modified ripening phenotype.



* C L M S *

12. A method of claim 11, where said introducing includes transforming progenitor cells of the plant with a selectable vector containing said DNA sequence and said promoter.

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13. A method of claim 11, wherein said heterologous DNA sequence encodes S-adenosylmethionine hydrolase.

14. A method of claim 11, wherein said DNA sequence encodes a product selected from the group consisting of aminocyclopropane-1-carboxylic acid (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC oxidase cosuppression molecule, and ACC synthase cosuppression molecule.

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15. A method of claim 11, wherein the promoter is obtained from a gene homologous to a tomato E4 or E8 gene.

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16. A method of claim 15, wherein the promoter is from a tomato E4 or E8 gene.

17. A method of claim 15, wherein the promoter is from a raspberry E4 gene.

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18. A method of claim 11, wherein the promoter is obtained from a gene selected from the group consisting of avocado cellulase gene and tomato polygalacturonase gene.

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19. A method of claim 11, wherein the promoter is obtained from a gene homologous to a gene selected from the group consisting of avocado cellulase gene and tomato polygalacturonase gene.

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20. A method of claim 11, wherein the promoter is isolated by the steps of:

- (i) selecting a probe DNA molecule containing a sequence homologous to a region of tomato E4 gene DNA,
- 5 (ii) contacting the probe with a plurality of target DNA molecules derived from the genome of a selected fruit-bearing plant under conditions favoring specific hybridization between the probe molecule and a target molecule homologous to the probe molecule,
- 10 (iii) identifying a target molecule having a DNA sequence homologous to tomato E4 gene, and
- (iv) isolating promoter sequences associated with the target molecule.

15 21. A method of claim 11, wherein the promoter is isolated by the steps of:

- (i) selecting a probe DNA molecule containing a sequence homologous to a region of tomato E8 gene DNA,
- (ii) contacting the probe with a plurality of target
- 20 DNA molecules derived from the genome of a selected fruit-bearing plant under conditions favoring specific hybridization between the probe molecule and a target molecule homologous to the probe molecule,
- (iii) identifying a target molecule having a DNA
- 25 sequence homologous to tomato E4 gene, and
- (iv) isolating promoter sequences associated with the target molecule.

22. An expression vector for use in transforming

30 plant cells, comprising

- (i) a DNA sequence encoding a product that is effective to reduce ethylene biosynthesis in fruit from a plant, and
- (ii) a promoter whose expression is induced during
- 35 fruit ripening or by ethylene synthesis by said fruit,



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where said DNA sequence is heterologous to said promoter and said DNA sequence is operably linked to said promoter to enable expression of said product.

5 23. A plant cell expression vector of claim 22, wherein said DNA sequence encodes S-adenosylmethionine hydrolase.

 24. A plant cell expression vector of claim 22,
10 wherein said DNA sequence encodes a product selected from the group consisting of aminocyclopropane-1-carboxylic acid (ACC) deaminase, ACC oxidase antisense molecule, ACC synthase antisense molecule, ACC oxidase cosuppression molecule, and ACC synthase cosuppression molecule.

15 25. A plant cell expression vector of claim 22, wherein the promoter is obtained from a gene homologous to a tomato E4 or E8 gene.

20 26. A plant cell expression vector of claim 25, wherein the promoter is from a tomato E4 or E8 gene.

 27. A plant cell expression vector of claim 25,
25 wherein the promoter is from a raspberry E4 gene.

 28. A plant cell expression vector of claim 22,
wherein the promoter is obtained from a gene selected from the group consisting of avocado cellulase gene and tomato polygalacturonase gene.

30 29. A plant cell expression vector of claim 22, wherein the promoter is obtained from a gene homologous to a gene selected from the group consisting of avocado cellulase gene and tomato polygalacturonase gene.

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30. A kit for use in plant transformation,
comprising the vector of claim 22.

31. A kit of claim 28, where the plant is a fruit
5 bearing plant.

32. A chimeric gene capable of expressing a
polypeptide in a plant, comprising
(i) a DNA sequence encoding a product that is
10 effective to reduce ethylene biosynthesis in fruit from
the plant, and (ii) a promoter whose expression is
induced during fruit ripening or by ethylene synthesis by
said fruit, where said DNA sequence is heterologous to
said promoter and said DNA sequence is operably linked to
15 said promoter to enable expression of said product.

33. A plant cell containing the chimeric gene of
claim 32.

20 34. A plant transformation vector containing the
chimeric gene of claim 32.

