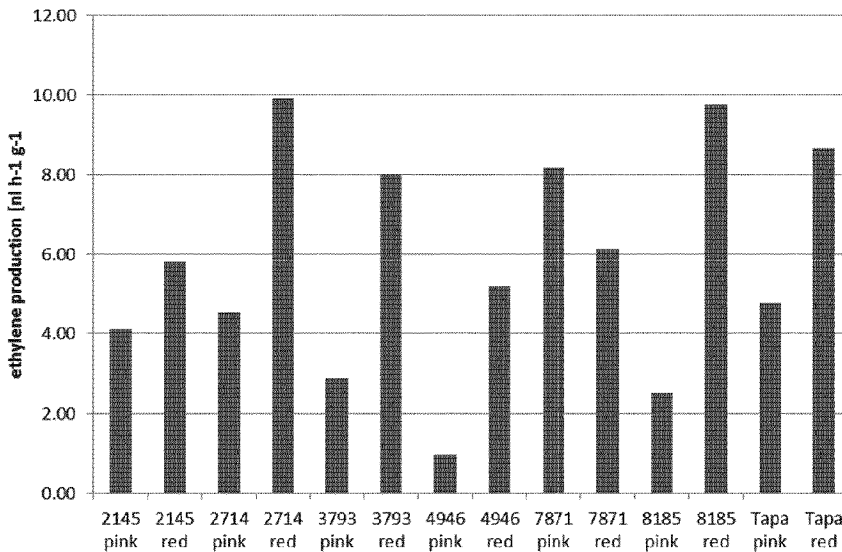




(86) Date de dépôt PCT/PCT Filing Date: 2013/11/20  
 (87) Date publication PCT/PCT Publication Date: 2014/05/30  
 (45) Date de délivrance/Issue Date: 2022/05/03  
 (85) Entrée phase nationale/National Entry: 2015/05/15  
 (86) N° demande PCT/PCT Application No.: EP 2013/074309  
 (87) N° publication PCT/PCT Publication No.: 2014/079896  
 (30) Priorité/Priority: 2012/11/21 (EP12193592.8)

(51) Cl.Int./Int.Cl. *A01H 6/82* (2018.01),  
*A01H 1/00* (2006.01), *A01H 1/06* (2006.01),  
*A01H 5/00* (2018.01), *A01H 5/08* (2018.01),  
*A01H 5/10* (2018.01), *A23L 19/00* (2016.01),  
*C12N 15/60* (2006.01), *C12N 5/04* (2006.01),  
*C12N 9/88* (2006.01), *C12N 15/82* (2006.01)  
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(54) Titre : PLANTES DE SOLANUM LYCOPERSICUM PRESENTANT DES ALTERATIONS NON-TRANSGENIQUES  
 DANS LE GENE ACS2  
 (54) Title: SOLANUM LYCOPERSICUM PLANTS HAVING NON-TRANSGENIC ALTERATIONS IN THE ACS2 GENE



(57) **Abrégé/Abstract:**

The present invention relates to cultivated plant of the species *Solanum lycopersicum* comprising a *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein having loss-of-function *acs2* protein or reduced function compared to wild type *Acs2* protein.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau(10) International Publication Number  
**WO 2014/079896 A1**(43) International Publication Date  
30 May 2014 (30.05.2014)

## (51) International Patent Classification:

A01H 5/08 (2006.01) C12N 15/82 (2006.01)  
C12N 9/88 (2006.01)

## (21) International Application Number:

PCT/EP2013/074309

## (22) International Filing Date:

20 November 2013 (20.11.2013)

## (25) Filing Language:

English

## (26) Publication Language:

English

## (30) Priority Data:

12193592.8 21 November 2012 (21.11.2012) EP

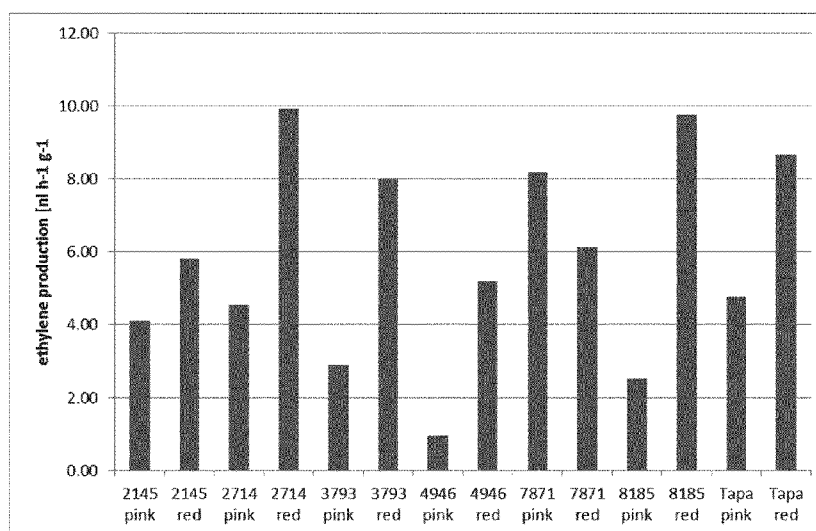
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GmbH, Creative Campus Monheim, Alfred-Nobel-Str. 10,  
40789 Monheim (DE).(81) Designated States (unless otherwise indicated, for every  
kind of national protection available): AE, AG, AL, AM,  
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,  
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,  
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,  
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,  
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,  
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,  
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,  
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,  
ZW.(84) Designated States (unless otherwise indicated, for every  
kind of regional protection available): ARIPO (BW, GH,  
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,  
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,  
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,  
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,  
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,  
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,  
KM, ML, MR, NE, SN, TD, TG).

## Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments (Rule 48.2(h))
- with (an) indication(s) in relation to deposited biological  
material furnished under Rule 13bis separately from the  
description (Rules 13bis.4(d)(i) and 48.2(a)(viii))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: SOLANUM LYCOPERSICUM PLANTS HAVING NON-TRANSGENIC ALTERATIONS IN THE ACS2 GENE

Figure 1

(57) Abstract: The present invention relates to cultivated plant of the species *Solanum lycopersicum* comprising a *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein having loss-of-function *acs2* protein or reduced function compared to wild type *Acs2* protein.

WO 2014/079896 A1

**Solanum lycopersicum plants having non-transgenic alterations in the ACS2 gene**

FIELD OF THE INVENTION

[0001] This invention relates to the field of plant biotechnology and plant breeding. Provided are  
5 *Solanum lycopersicum* plants comprising an *acs2* allele having one or more mutations, said mutations  
resulting in production of a mutant *acs2* protein having loss-of-function *acs2* protein or reduced activity  
compared to wild type *Acs2* protein. The invention provides plants the fruits of which show a lower  
ethylene production and/or slower fruit ripening and/or a longer shelf life compared to *Solanum*  
*lycopersicum* being homozygous for the wild type *Acs2* allele. In addition, the invention provides tomato  
10 fruit, seeds, pollen, plant parts, and progeny of the *Solanum lycopersicum* plants of the invention. Food  
and food products comprising or consisting of fruits of the plants of the invention are provided too.

[0002] The invention further provides an endogenous *acs2* gene and *acs2* protein encoded by said  
gene, having at least one human-induced non-transgenic mutation.

[0003] In another embodiment methods for making tomato plants comprising one or more mutant  
15 *acs2* alleles in their genome are provided herein.

BACKGROUND OF THE INVENTION

[0004] Breeding of *Solanum lycopersicum* aims at the production of commercial varieties  
optimally adapted to growing and storage conditions. A challenge breeders are facing is finding an  
improved balance between fruit firmness post-harvest and consumer desires in terms of taste, texture  
20 and colour. These consumer desires relate strongly to fruit ripening. Fruit ripening is a complex  
developmental process responsible for the transformation of the seed-containing organ into a tissue  
attractive to seed dispersers and agricultural consumers. The changes associated with fruit ripening, in  
particular post-harvest softening, limit the shelf life of fresh tomatoes.

[0005] For tomato fruit growth and development, a number of consecutive phases can be  
25 discerned: floral development, pollination, then early fruit development takes place which is  
characterised by a high frequency of cell division and the fruit is rapidly increasing in size mainly due to  
cell expansion. At the end of the third phase the fruit reaches the mature green stage. During the fourth  
phase, fruit ripening takes place which is characterised by a change in colour and flavour as well as fruit  
firmness and texture.

[0006] The build-up of the characteristic red colour of the tomato fruit is caused by the  
30 accumulation of lycopene and carotene. In general, different colouration phases are distinguished:  
mature green, breaker, pink and red. At the breaker stage, the typical red pigmentation initiates. Red ripe

- 2 -

stage or red ripe harvested fruit stage is the stage where the fruit has reached its mature colour on the major part of the fruit.

[0007] In addition to the colour changes, during fruit ripening enzymatic activity leads to degradation of the middle lamellar region of the cell walls which leads to cell loosening which is manifested as softening and loss of texture of the fruit. Softening of the fruit is often measured as external resistance to compression which can be quantified for example by a penetrometer.

[0008] Modification of single genes known to be involved in ripening has not yet resulted in a fruit with normal ripening but minimal tissue softening.

[0009] Ripening and senescence in climacteric fruits such as tomatoes are promoted by ethylene. Ethylene is autocatalytic for its own biosynthesis through increases in 1-Aminocyclopropae-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO). ACS is also referred to as 1-aminocyclopropane-1-carboxylate synthase; Le-ACS; or S-adenosyl-L-methionine methylthioadenosine-lyase. An increase in the amount of ACS and ACO thus leads to an increased conversion of L-methionine into ethylene. At least eight ACS genes (LEACS1A, LEACS1B, and LEACS2-7) have been identified in tomato (Alexander et. al., Journal of Experimental Botany, Vol 53, No 377, pp 2039-2055, 2002) and each ACS has a different expression pattern.

[0010] ACC synthase (ACS) is an enzyme that catalyzes the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-Adenosyl methionine. ACC is then converted into ethylene catalyzed by ACO. The biosynthesis of ethylene is for example described by Stearns and Glick (Biotechnology Advances 2003, vol 21 pp 193-210), which is enclosed by reference.

[0011] ACS belongs to the  $\alpha$ -family of pyridoxal-5'-phosphate (PLP) dependent enzymes and shares a modest level of similarity with other members of this family like aspartate amino-transferase (AATase and tyrosine aminotransferase (TATase). The structure of ACS from various sources has been described by Capitani *et al.* In a sequence alignment of eight ACS proteins (*Malus domestica*, *Phaseolus aureus*, *Solanum tuberosum*, *Pelargonium hortorum*, *Nicotiana tabacum*, *Cucumis melo*, *Lycopersicon esculentum*, and *Brassica oleracea*) they describe conserved regions which are indicated in Figure 1 in this Capitani publication. (Capitani *et al.*, Journal of Molecular Biology, 1999, vol 294, pp 745-756).

[0012] Two systems have been proposed to operate in climacteric plants regulating ethylene production. The first is functional during normal vegetative growth (system 1); it is auto inhibitory and responsible for production of basal ethylene levels that are detected in all tissues including those in non-climacteric plants. System 1 continues during fruit development until a competence to fruit ripening is attained. Then a transition period is reached wherein LEACS1A and LEACS4 are activated resulting in an increased level of ethylene. This increased ethylene level induces the expression of LEACS2 which starts system 2 which is active during the ripening of climacteric fruit. In system 2, ethylene production

- 3 -

is auto catalytic. This complexity of the ethylene regulation has been studied using antisense inhibition of LEACS2 in transgenic plants (Barry et al., Plant Physiology vol 123, pp 979-986, 2000).

[0013] WO2005/016504 discloses "stay green" plants, i.e. a plant phenotype whereby leaf senescence is delayed compared to a standard reference. It discloses plants with disrupted ACS2, ACS6, 5 ACS7 genes which disruption inhibits the expression or activity of said ACS.

[0014] Yokotani *et al* describe transgenic tomatoes with all known LeEIL genes (Ethylene Insensitive Like genes) suppressed to study the regulatory mechanisms of ethylene biosynthesis (Yokotani *et al*, Journal of Experimental Botany, vol 60, pp 3433-3442, 2009).

[0015] ACS2 mutants are known for instance from Vijee Mohan et al. They disclose (Vijee Mohan 10 et al. Sol 2010, page 67 [http://www.sol2010.org/sol2010/files/file/SOL2010\\_Abstracts.pdf](http://www.sol2010.org/sol2010/files/file/SOL2010_Abstracts.pdf)) EcoTILLING in tomato wherein nucleotide sequence diversity is used as a measure of the genetic variation that is present in a species, especially Single Nucleotide Polymorphisms (SNPs) are used to represent the most common variations across a genome. EcoTILLING is used as a high throughput, low cost technique for rapid discovery of polymorphisms in natural populations by heteroduplex analysis 15 using a mismatch-specific endonuclease. A collection of tomato accessions was obtained from different sources like NBPGR (India), IIVR (India) and TGRC (California, USA) and were analyzed for the frequency of naturally occurring SNPs, among others in ACS2. A number of SNPs were detected for different genes but with a varying frequency. The morphological features during different developmental stages and chemotypic observations indicated wide variations among the accessions. 20 Correlations between these variations with the location of the SNPs is suggested to be useful in finding promising alleles for crop development.

[0016] Studies on transgenic tomato fruit (ACS2 mutants) in which ethylene production was suppressed (Oeller et al, 1991 Science, vol 254, pp 437-439); (Picton et al. 1993 The Plant Journal vol 3 pp 469-481) showed delayed fruit ripening and revealed a critical role of ethylene in fruit ripening.

[0017] WO 92/04456 discloses control of plant development characteristics effected by ethylene in 25 tomato and zucchini, among others by recombinant production of ACS and by using antisense technology or mutated ACS genes.

[0018] Despite the above, no useful alleles for tomato fruit ripening improvement have been found so far.

[0019] There is thus a need for cultivated tomato plants with a modified ethylene production 30 having a delayed ripening and/or longer shelf-life of the tomato fruits compared to wild type tomato plants.

## SUMMARY OF THE INVENTION

[0020] It is, thus, an object of the invention to generate and identify cultivated plants of the species *Solanum lycopersicum* having fruits that have delayed ripening and/or a longer shelf-life of the fruits.

5 [0021] The invention thus relates to a cultivated plant of the species *Solanum lycopersicum* comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein, wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y of SEQ ID NO: 1 or in a wild type *Acs2* protein having at least 85% amino  
10 acid sequence identity to SEQ ID NO: 1, i.e. in a variant of SEQ ID NO: 1 (as defined below).

[0022] In one embodiment the invention relates to a plant of the invention wherein said mutation results in production of a mutant *acs2* protein having loss-of-function *acs2* protein or reduced function compared to wild type *Acs2* protein, wherein said wild type *acs2* protein is a protein having at least 85% amino acid sequence identity to SEQ ID NO: 1 and wherein said  
15 mutant *acs2* protein comprises sufficient function to result in ripening of the tomato fruits to the red stage when the mutant allele is present in heterozygous or homozygous form.

[0022a] The invention as claimed relates to:

- a cell of a cultivated plant of the species *Solanum lycopersicum* comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein  
20 having loss-of-function or reduced function compared to a wild type *Acs2* protein, wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y in said wild type *Acs2* protein, and wherein said wild type *Acs2* protein is a protein having at least 85% amino acid sequence identity to the full length of SEQ ID NO: 1;

- a cell of a cultivated plant of the species *Solanum lycopersicum* comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein having loss-of-function or reduced function compared to a wild type *Acs2* protein, wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y in said wild type *Acs2* protein, wherein said wild type *Acs2* protein is a protein having at least 85% amino acid sequence identity to the full length of SEQ ID NO: 1; and wherein the plant additionally comprises an *acs4* allele having one or more mutations, said mutations in said *acs4* allele resulting in production of a mutant *acs4* protein having loss-of-function or reduced function compared to wild type *Acs4* protein and having at least 85% amino acid sequence identity to the *Acs4* amino acid sequence provided in GenBank Accession number M63490.1 and wherein said mutant *acs4* protein has one or more amino acids changed selected from the group consisting of A248V, S279N, L321F, V250E and T316I or wherein said mutant *acs4* protein misses all amino acids 204 to 476 of the *Acs4* amino acid sequence provided in GenBank Accession number M63490.1; and

- a cell of a tomato fruit comprising a mutant *acs2* protein having loss-of-function or reduced function compared to a wild type *Acs2* protein, wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y in said wild type *Acs2* protein, and wherein said wild type *Acs2* is a protein having at least 85% amino acid sequence identity to the full length of SEQ ID NO: 1.

#### GENERAL DEFINITIONS

[0023] The term "nucleic acid sequence" (or nucleic acid molecule) refers to a DNA or RNA molecule in single or double stranded form, particularly a DNA encoding a protein or protein fragment according to the invention. An "isolated nucleic acid sequence" refers to a nucleic acid sequence which is no longer in the natural environment from which it was isolated, e.g. the nucleic acid sequence in a bacterial host cell or in the plant nuclear or plastid genome.

[0024] The terms "protein" or "polypeptide" are used interchangeably and refer to molecules consisting of a chain of amino acids, without reference to a specific mode of action, size, 3-dimensional structure or origin. A "fragment" or "portion" of *Acs2* protein may thus still be referred to as a "protein". An "isolated protein" is used to refer to a protein which is no longer  
5 in its natural environment, for example in vitro or in a recombinant bacterial or plant host cell.

[0025] The term "gene" means a DNA sequence comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an m RNA, hpRNA or an RNAi molecule) in a cell, operably linked to suitable regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked sequences, such as a promoter, a 5' leader sequence  
10 comprising e.g. sequences involved in translation initiation, a (protein) coding region (cDNA or genomic DNA) and a 3' non-translated sequence comprising e.g. transcription termination sites. A gene may be an endogenous gene (in the species of origin) or a chimeric gene (e.g. a transgene or cis-gene).

- 5 -

[0026] “Expression of a gene” refers to the process wherein a DNA region, which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA, which is biologically active, i.e. which is capable of being translated into a biologically active protein or peptide (or active peptide fragment) or which is active itself (e.g. in posttranscriptional gene silencing or RNAi).  
5 The coding sequence may be in sense-orientation and encodes a desired, biologically active protein or peptide, or an active peptide fragment.

[0027] An “active protein” or “functional protein” is a protein which has protein activity as measurable *in vitro*, e.g. by an *in vitro* activity assay, and/or *in vivo*, e.g. by the phenotype conferred by the protein. A “wild type” protein is a fully functional protein comprising at least 85% amino acid  
10 sequence identity to SEQ ID NO: 1 (also referred to as variant of SEQ ID NO:1). Likewise, the wild type *Acs2* allele is the allele encoding said wild type protein or variant. A “mutant protein” is herein a protein comprising one or more mutations in the nucleic acid sequence encoding the protein, whereby the mutation results in (the mutant nucleic acid molecule encoding) a “reduced-function” or “loss-of-function” protein, as e.g. measurable *in vivo*, e.g. by the phenotype conferred by the mutant allele.

[0028] A “reduced function *acs2* protein” or “reduced activity *acs2* protein” refers to a mutant *acs2*  
15 protein which has a reduced catalytic activity in synthesizing ACC from S-Adenosyl methionine, leading to reduced ethylene synthesis compared to wild-type *Acs2* protein. Said reduced catalytic activity of the *acs2* protein affects the ripening behaviour of the fruits comprising such reduced function *acs2* protein when the allele encoding the mutant protein is present in homozygous or heterozygous form  
20 in the tomato plant, i.e. delayed ripening and/or longer shelf-life of the fruits. Such a reduced function *acs2* protein can be obtained by the transcription and translation of a “partial knockout mutant *acs2* allele” which is, for example, a wild-type *Acs2* allele, which comprises one or more mutations in its nucleic acid sequence. In one aspect, such a partial knockout mutant *acs2* allele is a wild-type *Acs2* allele, which comprises one or more mutations that preferably result in the production of an *acs2* protein  
25 wherein at least one conserved and/or functional amino acid is substituted for another amino acid, such that the biological activity is significantly reduced but not completely abolished. However, other mutations, such as one or more non-sense, missense, splice-site or frameshift mutations in the tomato *Acs2* allele may also result in reduced function *acs2* protein and such reduced function proteins may have one or more amino acids replaced, inserted or deleted, relative to the wild type ACS2 protein. Such  
30 partial knockout mutant *acs2* allele may also encode a dominant negative *acs2* protein, which is capable of adversely affecting the biological activity of other *Acs2* proteins within the same cell. Such a dominant negative *acs2* protein can be an *Acs2* protein that is still capable of interacting with the same elements as the wild-type *Acs2* protein, but that blocks some aspect of its function. Examples of dominant negative *Acs2* proteins are *acs2* proteins that lack, or have modifications in specific amino  
35 acid residues critical for activation and/or dimerization, but still contain their binding domain, such that not only their own biological activity is reduced or abolished, but that they further reduce the total *acs2* activity in the cell by competing with wild type and/or partial knockout *acs2* proteins present in the cell

- 6 -

for binding sites. Mutant alleles can be either "natural mutant" alleles, which are mutant alleles found in nature (e.g. produced spontaneously without human application of mutagens) or "induced mutant" alleles, which are induced by human intervention, e.g. by mutagenesis.

[0029] A "loss-of-function Acs2 protein" refers to a mutant Acs2 protein which is has essentially  
5 no catalytic activity in synthesising ACC from S-Adenosyl methionine compared to wild-type Acs2  
protein, leading to reduced ethylene synthesis compared to wild type Acs2 protein. Said lack of catalytic  
activity synthesis affects the ripening behaviour of the fruits comprising such loss-of-function acs2  
protein when the allele encoding the mutant protein is present in homozygous or heterozygous form in  
10 the tomato plant. Fruits of tomato plants homozygous for such a "loss-of-function acs2 protein" may still  
produce ethylene catalysed by other proteins (e.g. other Acs proteins like Acs1A or Acs4). As a  
consequence, fruits of tomato plants homozygous for such a "loss-of-function acs2 protein" may still  
ripen, but ripening may be delayed and/or shelf life may be longer.

[0030] A "mutation" in a nucleic acid molecule coding for a protein is a change of one or more  
nucleotides compared to the wild type sequence, e.g. by replacement, deletion or insertion of one or  
15 more nucleotides. A "point mutation" is the replacement of a single nucleotide, or the insertion or  
deletion of a single nucleotide.

[0031] A "nonsense" mutation is a (point) mutation in a nucleic acid sequence encoding a protein,  
whereby a codon is changed into a stop codon. This results in a premature stop codon being present in  
the mRNA and in a truncated protein. A truncated protein may have reduced function or loss of  
20 function.

[0032] A "missense" or non-synonymous mutation is a (point) mutation in a nucleic acid sequence  
encoding a protein, whereby a codon is changed to code for a different amino acid. The resulting protein  
may have reduced function or loss of function.

[0033] A "splice-site" mutation is a mutation in a nucleic acid sequence encoding a protein,  
25 whereby RNA splicing of the pre-mRNA is changed, resulting in an mRNA having a different  
nucleotide sequence and a protein having a different amino acid sequence than the wild type. The  
resulting protein may have reduced function or loss of function.

[0034] A "frame-shift" mutation is a mutation in a nucleic acid sequence encoding a protein by  
which the reading frame of the mRNA is changed, resulting in a different amino acid sequence. The  
30 resulting protein may have reduced function or loss of function.

[0035] A mutation in a regulatory sequence, e.g. in a promoter of a gene, is a change of one or  
more nucleotides compared to the wild type sequence, e.g. by replacement, deletion or insertion of one  
or more nucleotides, leading for example to reduced or no mRNA transcript of the gene being made.

- 7 -

[0036] “Silencing” refers to a down-regulation or complete inhibition of gene expression of the target gene or gene family.

[0037] A “target gene” in gene silencing approaches is the gene or gene family (or one or more specific alleles of the gene) of which the endogenous gene expression is down-regulated or completely inhibited (silenced) when a chimeric silencing gene (or ‘chimeric RNAi gene’) is expressed and for example produces a silencing RNA transcript (e.g. a dsRNA or hairpin RNA capable of silencing the endogenous target gene expression). In mutagenesis approaches, a target gene is the endogenous gene which is to be mutated, leading to a change in (reduction or loss of) gene expression or a change in (reduction or loss of) function of the encoded protein.

10 [0038] As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter, or rather a transcription regulatory sequence, is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein encoding regions, contiguous and in reading frame so as to produce a “chimeric protein”. A “chimeric protein” or “hybrid protein” is a protein composed of various protein “domains” (or motifs) which is not found as such in nature but which is joined to form a functional protein, which displays the functionality of the joined domains. A chimeric protein may also be a fusion protein of two or more proteins occurring in nature.

20 [0039] The term “food” is any substance consumed to provide nutritional support for the body. It is usually of plant or animal origin, and contains essential nutrients, such as carbohydrates, fats, proteins, vitamins, or minerals. The substance is ingested by an organism and assimilated by the organism's cells in an effort to produce energy, maintain life, or stimulate growth. The term food includes both substance consumed to provide nutritional support for the human and animal body.

25 [0040] The term “shelf life” or “post-harvest shelf life” designates the (average) length of time that a fruit is given before it is considered unsuitable for sale or consumption (‘bad’ or ‘bad stage’). Shelf life is the period of time that products can be stored, during which the defined quality of a specified proportion of the goods remains acceptable under expected conditions of distribution, storage and display. Shelf life is influenced by several factors: exposure to light and heat, transmission of gases (including humidity), mechanical stresses, and contamination by things such as micro-organisms. Product quality is often mathematically modelled around the fruit firmness/softness parameter. Shelf-life can be defined as the (average) time it takes for fruits of a plant line to start to become bad and unsuitable for sale or consumption, starting for example from the first fruit of a plant entering breaker stage or turning stage or from the first fruit becoming fully red or from harvest. In one embodiment the mutants according to the invention have a shelf life that is significantly longer than the shelf life of wild type plants, for example the number of days from the first fruit being in breaker stage (or turning stage,

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- 8 -

pink stage, red stage or from harvest) up to the first fruit starting to become 'bad' and unsuitable for sale or consumption is significantly longer, e.g. at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, days longer than fruits of control plants (such as wild type *Acs2/Acs2* plants), when plants are grown under the same conditions and fruits are treated the same way and kept under the same conditions. Thus, to determine the number of days required from a certain stage (e.g. from breaker stage or a later stage) to 'bad' stage, the day when the first fruit of the wild type control plant (grown under the same conditions as the mutant plants and being at the same developmental stage) enters a certain stage (e.g. breaker stage or a later stage) can, for example, be taken as the starting point (day 1) from when on periodically (at certain time intervals, e.g. after 1, 2, 3, 4, 5 or 6 days) the fruits are observed until the day that the first fruit has passed the fully ripe stage and becomes 'bad' (as determinable visually and/or through assessing fruit softness).

[0041] In this application the words "improved", "increased", "longer" and "extended" as used in conjunction with the word "shelf-life" are interchangeable and all mean that the fruits of a tomato plant according to the invention have on average, a longer shelf-life than the control fruits (*Acs2/Acs2* fruits) such as Pusa Sheetal, Tapa, or TPAADASU.

[0042] "Delayed ripening" means that the fruits of a tomato plant or plant line (e.g. a mutant) according to the invention require on average significantly more days to reach the red stage from the mature green, breaker, turning stage, and/or pink stages of tomato fruit ripening compared to wild type control fruits of plants homozygous for the wild type *Acs2* allele (*Acs2/Acs2*). Delayed ripening can be measured on the plant and/or after harvest as days required for a certain percentage of fruits (e.g. 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and/or 100% of fruits) to reach the red stage. A plant is said to have a delayed ripening phenotype if it takes at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 days longer for 10%, 20%, 30% 40%, 50%, 60%, 70%, 80%, 90% and/or 100% of fruits to reach the red stage than it takes for the wild type control fruits to develop the same percentage of red fruits. It is understood that each combination of above-cited number of days (i.e. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15) with each % of fruits to reach the red stage (i.e. 10%, 20%, 30% 40%, 50%, 60%, 70%, 80%, 90% and/or 100%) is enclosed herein, both for the delayed ripening to be measured on the plant and after harvest. For example if it takes at least 2 days longer for 10%, 20%, 30% 40%, 50%, 60%, 70%, 80%, 90% and/or 100% of fruits to reach the red stage than it takes for the wild type control fruits to develop the same percentage of red fruits. Another example of how delayed ripening can be measured on the plant and/or after harvest is it takes at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 days longer for 100% of fruits to reach the red stage than it takes for the wild type control fruits to develop the same percentage of red fruits. The day when the first fruit of the wild type control plant (grown under the same conditions as the mutant plants and being at the same developmental stage) enters a certain stage (e.g. breaker stage) can, for example, be taken as the starting point (day 1) from when on periodically (at certain time intervals (e.g. after 1, 2, 3, 4, 5 or 6 days) the number of fruits that are in

- 9 -

breaker stage and the number of fruit that are in red stage are counted, both for the mutant plant line and control plants (see Examples).

[0043] In this application the word senescence means biological aging i.e. the change in the biology of an organism as it ages after its maturity.

5 [0044] As used herein, "reduced ethylene production" refers herein to statistically significant reduced amounts of ethylene being produced by tomato fruits according to the invention (compared to wild type Acs2/Acs2 fruits) during fruit ripening, e.g. at the pink stage and/or at the light red stage and/or at the red stage, as described in the Examples, and as measurable by real time ethylene  
10 measurements. In one embodiment, ethylene levels are significantly reduced throughout fruit ripening from pink stage through to red stage.

[0045] It is understood that comparisons between different plant lines involves growing a number of plants of a line (e.g. at least 5 plants, preferably at least 10 plants per line) under the same conditions as the plants of one or more control plant lines (preferably wild type plants) and the determination of statistically significant differences between the plant lines when grown under the same environmental  
15 conditions.

[0046] "Delay of breaker stage" refers to the mutants according to the invention requiring significantly more days than wild type controls for the first fruits and/or for all fruits to have entered breaker stage, e.g. at least 1 more day, preferably at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 more days than the wild type control, when grown under the same conditions.

20 [0047] The "ripening stage" of a tomato fruit can be divided as follows: (1) Mature green stage: surface is completely green; the shade of green may vary from light to dark. (2) Breaker stage: there is a definite break in color from green to tannish-yellow, pink or red on not more than 10% of the surface; (3) Turning stage: 10% to 30% of the surface is not green; in the aggregate, shows a definite change from green to tannish-yellow, pink, red, or a combination thereof. (4) Pink stage: 30% to 60% of the  
25 surface is not green; in the aggregate, shows pink or red color. (5) Light red stage: 60% to 90% of the surface is not green; in the aggregate, shows pinkish-red or red. (6) Red stage: More than 90% of the surface is not green; in the aggregate, shows red color.

[0048] "Sequence identity" and "sequence similarity" can be determined by alignment of two peptide or two nucleotide sequences using global or local alignment algorithms. Sequences may then be  
30 referred to as "substantially identical" or "essentially similar" when they are optimally aligned by for example the programs GAP or BESTFIT or the Emboss program "Needle" (using default parameters, see below) share at least a certain minimal percentage of sequence identity (as defined further below). These programs use the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length, maximizing the number of matches and minimises the number of gaps. Generally, the  
35 default parameters are used, with a gap creation penalty = 10 and gap extension penalty = 0.5 (both for

- 10 -

nucleotide and protein alignments). For nucleotides the default scoring matrix used is DNAFULL and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 10915-10919). Sequence alignments and scores for percentage sequence identity may for example be determined using computer programs, such as EMBOSS (http://www.ebi.ac.uk/Tools/psa/emboss\_needle/). Alternatively sequence similarity or identity may be determined by searching against databases such as FASTA, BLAST, etc., but hits should be retrieved and aligned pairwise to compare sequence identity. Two proteins or two protein domains, or two nucleic acid sequences have "substantial sequence identity" if the percentage sequence identity is at least 85%, 90%, 95%, 98%, 99% or more (e.g. at least 99.1, 99.2 99.3 99.4, 99.5, 99.6, 99.7, 99.8, 99.9 or more (as determined by Emboss "needle" using default parameters, i.e. gap creation penalty = 10, gap extension penalty = 0.5, using scoring matrix DNAFULL for nucleic acids an Blosum62 for proteins).

[0049] In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one". It is further understood that, when referring to "sequences" herein, generally the actual physical molecules with a certain sequence of subunits (e.g. amino acids) are referred to.

[0050] As used herein, the term "plant" includes the whole plant or any parts or derivatives thereof, such as plant organs (e.g., harvested or non-harvested fruits, flowers, leaves, etc.), plant cells, plant protoplasts, plant cell or tissue cultures from which whole plants can be regenerated, regenerable or non-regenerable plant cells, plant calli, plant cell clumps, and plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, ovaries, fruits (e.g., harvested tissues or organs, such as harvested tomatoes or parts thereof), flowers, leaves, seeds, tubers, clonally propagated plants, roots, stems, cotyledons, hypocotyls, root tips and the like. Also any developmental stage is included, such as seedlings, immature and mature, etc.

[0051] A "plant line" or "breeding line" refers to a plant and its progeny. As used herein, the term "inbred line" refers to a plant line which has been repeatedly selfed.

[0052] "Plant variety" is a group of plants within the same botanical taxon of the lowest grade known, which (irrespective of whether the conditions for the recognition of plant breeder's rights are fulfilled or not) can be defined on the basis of the expression of characteristics that result from a certain genotype or a combination of genotypes, can be distinguished from any other group of plants by the expression of at least one of those characteristics, and can be regarded as an entity, because it can be multiplied without any change. Therefore, the term "plant variety" cannot be used to denote a group of plants, even if they are of the same kind, if they are all characterized by the presence of 1 locus or gene

- 11 -

(or a series of phenotypical characteristics due to this single locus or gene), but which can otherwise differ from one another enormously as regards the other loci or genes.

[0053] “F1, F2, etc.” refers to the consecutive related generations following a cross between two parent plants or parent lines. The plants grown from the seeds produced by crossing two plants or lines is called the F1 generation. Selfing the F1 plants results in the F2 generation, etc. “F1 hybrid” plant (or F1 seed) is the generation obtained from crossing two inbred parent lines. An “M1 population” is a plurality of mutagenized seeds / plants of a certain plant line or cultivar. “M2, M3, M4, etc.” refers to the consecutive generations obtained following selfing of a first mutagenized seed / plant (M1).

[0054] The term “allele(s)” means any of one or more alternative forms of a gene at a particular locus, all of which alleles relate to one trait or characteristic at a specific locus. In a diploid cell of an organism, alleles of a given gene are located at a specific location, or locus (loci plural) on a chromosome. One allele is present on each chromosome of the pair of homologous chromosomes. A diploid plant species may comprise a large number of different alleles at a particular locus. These may be identical alleles of the gene (homozygous) or two different alleles (heterozygous).

[0055] The term “locus” (loci plural) means a specific place or places or a site on a chromosome where for example a gene or genetic marker is found. The ACS2 locus is thus the location in the genome where the ACS2 gene is found.

[0056] “Wild type allele” (WT or Wt) refers herein to a version of a gene encoding a fully functional protein (wild type protein). Such a sequence encoding a fully functional Acs2 protein is for example the wild type Acs2 cDNA (mRNA) sequence depicted in SEQ ID NO: 9, based on GenBank NM001247249.1 Tomato 1-aminocyclopropane-1-carboxylate synthase mRNA, complete cds [http://www.ncbi.nlm.nih.gov/nuccore/NM\\_001247249.1](http://www.ncbi.nlm.nih.gov/nuccore/NM_001247249.1) or <http://www.ncbi.nlm.nih.gov/nuccore/AY326958.1>, or the wild type Acs2 genomic sequence depicted in SEQ ID NO: 17. The protein sequence encoded by this wild type Acs2 mRNA is depicted in SEQ ID NO: 1. It consists of 485 amino acids. Other fully functional Acs2 protein encoding alleles (i.e. alleles which confer ripening and ethylene production to the same extent as the protein of SEQ ID NO 1) may exist in other *Solanum lycopersicum* plants and may comprise substantial sequence identity with SEQ ID NO: 1, i.e. at least about 85%, 90%, 95%, 98%, 99%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7% sequence identity with SEQ ID NO: 1. Such fully functional wild type Acs2 proteins are herein referred to as “variants” of SEQ ID NO: 1. Likewise the nucleotide sequences encoding such fully functional Acs2 proteins are referred to as variants of SEQ ID NO: 9 and SEQ ID NO: 17.

[0057] The following mutant *acs2* alleles are exemplary of the reduced ethylene production and/or delayed-ripening and/or extended shelf-life conferring *acs2* mutations identified according to the present invention. It is noted that nucleotide sequences referred to herein (SEQ ID NO: 9-16) are cDNA, i.e. coding DNA sequences, encoding the proteins of SEQ ID NO: 1-8. Obviously, when reference is made

- 12 -

to these cDNA nucleotide sequences, it is understood that the cDNA is the coding region of the corresponding *Solanum lycopersicum* genomic *acs2* sequence, which, however, additionally contains introns and therefore the nucleotides have different numbering. Thus, when reference is made to a tomato plant comprising an *acs2* sequence according to e.g. any one of SEQ ID NO: 9-16, it is, therefore, understood that the tomato plant comprising the genomic *acs2* sequence which comprises the coding DNA (cDNA), from which the mRNA of SEQ ID NO: 8-14 is transcribed (and which is in turn translated into protein). The mRNA has the same nucleotide sequence as the cDNA, except that Thymine (T) is Uracil (U) in the mRNA. Further, when reference is made to a tomato plant comprising a nucleotide sequence encoding a protein according to the invention (i.e. a mutant protein of SEQ ID No: 2, 3, 4, 5, 6, 7, or 8), this encompasses different nucleotide sequences, due to the degeneracy of the genetic code. In one embodiment the plant comprises the genomic *Acs2* sequence depicted in SEQ ID NO:17 or a genomic *Acs2* sequence substantially identical thereto (e.g. having at least about 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7% sequence identity with SEQ ID NO: 17), but with one or more mutations in said sequence, especially in the exons of said genomic sequence (exon 1 ranges from nucleotide 1 to 171; exon 2 ranges from nucleotide 270 to 399, exon 3 ranges from nucleotide 485 to 644 and exon 4 ranges from nucleotide 1526 to 2523; counting A in the ATG of the START CODON as nucleotide position 1), causing reduced function or loss of function of the encoded mutant *acs2* protein.

[0058] One exemplary mutant *acs2* allele (mutant 783) conferring reduced ethylene production and/or delayed ripening and/or extended shelf-life, identified according to the present invention, comprises a mutation resulting in an alanine (Ala or A) to threonine (Thr or T) substitution at amino acid 103 (A103T) in the encoded protein. The protein sequence of mutant 783 is depicted in SEQ ID NO: 2. The amino acid substitution is due to a G to A mutation at nucleotide 307 of SEQ ID NO: 9 counting A in the ATG of the START CODON as nucleotide position 1. The mutant cDNA of mutant 783 is depicted in SEQ ID NO: 10.

[0059] Yet another exemplary mutant *acs2* allele (mutant 2145) conferring reduced ethylene production and/or delayed ripening and/or extended shelf-life, identified according to the present invention, comprises a mutation resulting in a glycine (Gly or G) to arginine (Arg or R) substitution at amino acid 112 (G112R) in the encoded protein. The protein sequence of mutant 2145 is depicted in SEQ ID NO: 3. The amino acid substitution is due to a change from G to A at nucleotide 334 as shown in SEQ ID NO: 9 counting A in the ATG of the START CODON as nucleotide position 1. The mutant cDNA of mutant 2145 is depicted in SEQ ID NO: 11.

[0060] Another exemplary mutant *acs2* allele (mutant 2714) conferring reduced ethylene production and/or delayed ripening and/or extended shelf-life, identified according to the present invention, comprises a mutation resulting in a change from a proline (Pro or P) to leucine (Leu or L) at amino acid 118 (P118L) in the encoded protein (SEQ ID NO: 4). The protein sequence of mutant 2714

- 13 -

is depicted in SEQ ID NO: 4. The amino acid substitution is due to a C to T mutation at nucleotide 353 (C353T) of SEQ ID NO: 9, counting A in the ATG of the START CODON as nucleotide position 1. The mutant cDNA 2714 is depicted in SEQ ID NO: 12.

5 [0061] Still another exemplary mutant *acs2* allele (mutant 3793) conferring reduced ethylene production and /or delayed ripening and/or extended shelf-life, identified according to the present invention, comprises a mutation resulting in a change from an alanine (Ala or A) to valine (Val or V) at amino acid 101 (A101V) in the encoded protein (SEQ ID NO: 5). The amino acid substitution is due to a C to T mutation at nucleotide 302 (C302T) of SEQ ID NO: 9, counting A in the ATG of the START CODON as nucleotide position 1. The mutant cDNA is depicted in SEQ ID NO: 13.

10 [0062] Another exemplary mutant *acs2* allele (mutant 4946) conferring reduced ethylene production and /or delayed ripening and/or extended shelf-life, identified according to the present invention, comprises a mutation resulting in a change from alanine (Ala or A) to threonine (Thr or T) at amino acid 101 (A101T) in the encoded protein (SEQ ID NO: 6). The amino acid substitution is due to a change from G to A at nucleotide 301 (G301A) of SEQ ID NO: 9, counting A in the ATG of the  
15 START CODON as nucleotide position 1. The mutant cDNA is depicted in SEQ ID NO: 14.

[0063] Yet another exemplary mutant *acs2* allele (mutant 7871) conferring reduced ethylene production and /or delayed ripening and/or extended shelf-life, identified according to the present invention, comprises a mutation resulting in a change from a cysteine (Cys or C) to tyrosine (Tyr or Y) at amino acid 265 (C265Y) in the encoded protein (SEQ ID NO: 7). The amino acid substitution is due  
20 to a G to A mutation at nucleotide 794 (G794A) of SEQ ID NO: 9, counting A in the ATG of the START CODON as nucleotide position 1. The mutant cDNA is depicted in SEQ ID NO: 15.

[0064] Another exemplary mutant *acs2* allele (mutant 8185) conferring reduced ethylene production and /or delayed ripening and/or extended shelf-life, identified according to the present invention, comprises a mutation resulting in a change from valine (Val or V) to glutamic acid (Glu or E)  
25 at amino acid 147 (V147E) in the encoded protein (SEQ ID NO: 8). The amino acid substitution is due to a change from T to A at nucleotide 440 (T440A) of SEQ ID NO: 9, counting A in the ATG of the START CODON as nucleotide position 1. The mutant cDNA is depicted in SEQ ID NO: 16.

[0065] "Mutant allele" refers herein to an allele comprising one or more mutations in the coding sequence (mRNA, cDNA or genomic sequence) compared to the wild type allele. Such mutation(s) (e.g.  
30 insertion, inversion, deletion and/or replacement of one or more nucleotide(s)) may lead to the encoded protein having reduced *in vitro* and/or *in vivo* functionality (reduced function) or no *in vitro* and/or *in vivo* functionality (loss-of-function), e.g. due to the protein e.g. being truncated or having an amino acid sequence wherein one or more amino acids are deleted, inserted or replaced. Such changes may lead to the protein having a different 3D conformation, being targeted to a different sub-cellular compartment,  
35 having a modified catalytic domain, having a modified binding activity to nucleic acids or proteins, etc.

- 14 -

[0066] “Wild type plant” and “wild type fruits” or “normal ripening” plants/fruits refers herein to a tomato plant comprising two copies of a wild type (WT or Wt) *Acs2* allele (*Acs2/Acs2*) encoding a fully functional *Acs2* protein (e.g. in contrast to “mutant plants”, comprising a mutant *acs2* allele). Such plants are for example suitable controls in phenotypic assays. Preferably wild type and/or mutant plants are “cultivated tomato plants”. For example the cultivar MoneyMaker is a wild type plant, as is cultivar Ailsa Craig, cultivar Tapa and many others.

[0067] “Tomato plants” or “cultivated tomato plants” are plants of the *Solanum lycopersicum*, i.e. varieties, breeding lines or cultivars of the species *Solanum lycopersicum*, cultivated by humans and having good agronomic characteristics; preferably such plants are not “wild plants”, i.e. plants which generally have much poorer yields and poorer agronomic characteristics than cultivated plants and e.g. grow naturally in wild populations. “Wild plants” include for example ecotypes, PI (Plant Introduction) lines, landraces or wild accessions or wild relatives of a species. The so-called heirloom varieties or cultivars, i.e. open pollinated varieties or cultivars commonly grown during earlier periods in human history and often adapted to specific geographic regions, are in one aspect of the invention encompassed herein as cultivated tomato plants.

[0068] Wild relatives of tomato include *S. arcanum*, *S. chmielewskii*, *S. neorickii* (= *L. parviflorum*), *S. cheesmaniae*, *S. galapagense*, *S. pimpinellifolium*, *S. chilense*, *S. corneliomulleri*, *S. habrochaites* (= *L. hirsutum*), *S. huaylasense*, *S. sisymbriifolium*, *S. peruvianum*, *S. hirsutum* or *S. pennellii*.

[0069] “Average” refers herein to the arithmetic mean.

#### BRIEF DESCRIPTION OF THE SEQUENCE LISTING

- [0070] SEQ ID NO: 1 shows the *Solanum lycopersicum* wild type, fully functional, ACS2 protein sequence as derived from the mRNA based on NCBI Reference Sequence: NM\_001247249.1; [http://www.ncbi.nlm.nih.gov/nuccore/NM\\_001247249](http://www.ncbi.nlm.nih.gov/nuccore/NM_001247249).
- [0071] SEQ ID NO: 2 shows the *Solanum lycopersicum* mutant 783 *acs2* protein sequence.
- [0072] SEQ ID NO: 3 shows the *Solanum lycopersicum* mutant 2145 *acs2* protein sequence.
- [0073] SEQ ID NO: 4 shows the *Solanum lycopersicum* mutant 2714 *acs2* protein sequence.
- [0074] SEQ ID NO: 5 shows the *Solanum lycopersicum* mutant 3793 *acs2* protein sequence.
- [0075] SEQ ID NO: 6 shows the *Solanum lycopersicum* mutant 4946 *acs2* protein sequence.
- [0076] SEQ ID NO: 7 shows the *Solanum lycopersicum* mutant 7871 *acs2* protein sequence.
- [0077] SEQ ID NO: 8 shows the *Solanum lycopersicum* mutant 8185 *acs2* protein sequence.
- [0078] SEQ ID NO: 9 shows the *Solanum lycopersicum* wild type *Acs2* cDNA based on NCBI Reference Sequence: NM\_001247249.1 ([http://www.ncbi.nlm.nih.gov/nuccore/NM\\_001247249](http://www.ncbi.nlm.nih.gov/nuccore/NM_001247249)) for *Solanum lycopersicum* ripening-related ACC synthase 2 (ACS2), mRNA.

- 15 -

- [0079] SEQ ID NO: 10 shows the *Solanum lycopersicum* mutant 783 *acs2* cDNA.
- [0080] SEQ ID NO: 11 shows the *Solanum lycopersicum* mutant 2145 *acs2* cDNA.
- [0081] SEQ ID NO: 12 shows the *Solanum lycopersicum* mutant 2714 *acs2* cDNA.
- [0082] SEQ ID NO: 13 shows the *Solanum lycopersicum* mutant 3793 *acs2* cDNA.
- 5 [0083] SEQ ID NO: 14 shows the *Solanum lycopersicum* mutant 4946 *acs2* cDNA.
- [0084] SEQ ID NO: 15 shows the *Solanum lycopersicum* mutant 7871 *acs2* cDNA.
- [0085] SEQ ID NO: 16 shows the *Solanum lycopersicum* mutant 8185 *acs2* cDNA.
- [0086] SEQ ID NO: 17 shows the *Solanum lycopersicum* wild type *Acs2* genomic DNA as  
 10 obtained from the solgenomics network (<http://solgenomics.net> sequence region sl2.40ch01:78217541-78213542). The position of the exons as described herein, is derived from this sequence.
- [0087] SEQ ID NO: 18 shows amino acids 58 to and including 154 of the *Solanum lycopersicum* mutant 783 *acs2* protein sequence as shown in SEQ ID NO: 2.
- [0088] SEQ ID NO: 19 shows amino acids 58 to and including 154 of the *Solanum lycopersicum* mutant 2145 *acs2* protein sequence as shown in SEQ ID NO: 3.
- 15 [0089] SEQ ID NO: 20 shows amino acids 58 to and including 154 of the *Solanum lycopersicum* mutant 2714 *acs2* protein sequence as shown in SEQ ID NO: 4.
- [0090] SEQ ID NO: 21 shows amino acids 58 to and including 154 of the *Solanum lycopersicum* mutant 3793 *acs2* protein sequence as shown in SEQ ID NO: 5.
- [0091] SEQ ID NO: 22 shows amino acids 58 to and including 154 of the *Solanum*  
 20 *lycopersicum* mutant 4946 *acs2* protein sequence as shown in SEQ ID NO: 6.
- [0092] SEQ ID NO: 23 shows amino acids 184 to and including 297 of the *Solanum lycopersicum* mutant 7871 *acs2* protein sequence as shown in SEQ ID NO: 7.
- [0093] SEQ ID NO: 24 shows amino acids 58 to and including 154 of the *Solanum lycopersicum* mutant 8185 *acs2* protein sequence as shown in SEQ ID NO: 8.
- 25

#### BRIEF DESCRIPTION OF THE FIGURES

[0094] Figure 1: Ethylene-release measured in  $\text{nl} / (\text{h} \cdot \text{g})$ , also written as  $\text{nl} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ , from tomato fruits of various *acs2* mutants at Pink stage and Red stage. Tapa is a type (*Acs2/Acs2*) plant.

[0095] Figure 2: In this graph the percentage of fruits in red stage is shown, determined at various  
 30 days after the wild type control fruits started entering breaker stage [at day 1, the first fruit of Wild type was in breaker stage]. Fruits of the mutant plants (indicated by the mutant number) were homozygous for a specific *acs2* mutation (*acs2/acs2*).

[0096] Figure 3: Alignment of SEQ ID NO: 1 – 8. The mutations are depicted in bold and underlined.

- 16 -

[0097] Figure 4: Average firmness necessary to decrease the fruit diameter 1 mm per cm fruit (10%) of mutant 8185 (8185 Ho) and wild type (wt; i.e. Tapa) in Newton [N]. The same fruits were repeatedly measured at 0 (red stage, day of harvest) and 7, 14, and 21 days after harvest.

[0098] Figure 5: Alignment of amino acid sequence of wild type Acs4 amino acid sequence with six acs4 mutants: mutant 2477, mutant 4043, mutant 4222, mutant 4303, mutant 4691, and mutant 5251. Acs4 small and large domains are also depicted (light gray), as are the mutations (in bold and underlined).

#### DETAILED DESCRIPTION OF THE INVENTION

[0099] The present invention discloses a cultivated plant of the species *Solanum lycopersicum* comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein having loss-of-function and / or reduced function compared to wild type Acs2 protein.

[0100] In one aspect the invention relates to a cultivated plant of the species *Solanum lycopersicum*, and/or parts thereof (e.g. fruits), comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein having loss-of-function or reduced function compared to wild type Acs2 protein wherein said mutation or mutations result in reduced ethylene production and /or delayed fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* plants which are homozygous for the wild type fully functional Acs2 allele (*Acs2/Acs2*) (encoding a functional Acs2 protein of SEQ ID NO: 1 or a functional variant).

[0101] A *S. lycopersicum* plant encoding the protein of SEQ ID NO: 1 is for example described by Kamiyoshihara Y, *et al.* in Plant J. 2010 Vol 64(1) pp 140-50; Turnover of LeACS2, a wound-inducible 1-aminocyclopropane-1-carboxylic acid synthase in tomato, is regulated by phosphorylation/dephosphorylation.

[0102] In one aspect the invention relates to a cultivated plant of the species *Solanum lycopersicum* and/or parts thereof (e.g. fruit or seed) comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein, wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y in the wild type Acs2 protein of SEQ ID NO: 1 or in a functional variant thereof. Which functional variant is a wild type Acs2 protein having at least 85% sequence identity to SEQ ID NO: 1; and optionally wherein said mutation results in production of a mutant *acs2* protein having loss-of-function or reduced function compared to wild type Acs2 protein.

[0103] In another aspect the invention relates to a plant of the invention wherein said mutant *acs2* protein comprises amino acids 58 to and including 154 and / or amino acids 184 to and including 297 or SEQ ID NO: 1 and wherein said mutant *acs2* protein has one or more amino acids changed selected

- 17 -

from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y of SEQ ID NO: 1.

[0104] In yet another aspect the invention relates to a cultivated plant of the species *Solanum lycopersicum* and/or parts thereof (e.g. fruit or seed) comprising an *acs2* allele having one or more mutations, wherein said mutant *acs2* protein encoded by said allele, has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y of SEQ ID NO:1 or of a wild type variant of SEQ ID NO: 1 having at least 85% amino acid sequence identity to SEQ ID NO:1.

[0105] In a further aspect the invention relates to a mutant *acs2* protein having one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y of SEQ ID NO:1 or of a wild type variant of SEQ ID NO: 1 having at least 85% amino acid sequence identity to SEQ ID NO:1. Preferably said mutant *acs2* protein is a *Solanum lycopersicum acs2* protein.

[0106] In another aspect the invention relates to a plant of the invention wherein said mutation or mutations result in reduced ethylene production and /or delayed fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* plants which are homozygous for the wild type fully functional *Acs2* allele (*Acs2/Acs2*) (encoding a functional *Acs2* protein of SEQ ID NO: 1 or a functional variant of SEQ ID NO: 1). In another aspect, the mutation or mutations in the plant of the invention result in reduced ethylene production compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele encoding the protein of SEQ ID NO:1 or of a wild type variant of SEQ ID NO: 1 having at least 85% amino acid sequence identity to SEQ ID NO:1.

[0107] In another aspect, the mutation or mutations in the plant of the invention result in delayed fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele.

[0108] In yet another aspect, the invention relates to a cultivated plant of the species *Solanum lycopersicum* and/or parts thereof (e.g. fruits) comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein, wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, C265Y of SEQ ID NO: 1 or variants of SEQ ID NO: 1 and wherein said mutation(s) result(s) in production of a mutant *acs2* protein having loss-of-function or reduced function compared to wild type *Acs2* protein (or wild type variant) whereby the fruits of the plant do ripen to the red stage (preferably slower than plants homozygous for the wild type allele, encoding a fully functional *Acs2* protein (or wild type variant)).

[0109] In one aspect the plant is homozygous for the allele encoding the mutant *acs2* protein.

- 18 -

[0110] In still another embodiment such mutant *acs2* allele is derived from and/or generated in a cultivated tomato (e.g. a breeding line, variety or heirloom variety) or a wild relative of tomato. Such a human-induced mutation may, for example, be induced using targeted mutagenesis as described in EP1963505. Mutant *acs2* alleles generated in wild relatives of tomato are then easily transferred into cultivated tomato by breeding.

[0111] In yet another aspect, the invention relates to a plant of the invention having reduced ethylene production and/or delayed ripening and/or longer shelf-life than wild type (*Acs2/Acs2*) plants, due to said plants comprising an endogenous *acs2* allele encoding a loss-of-function *acs2* protein or reduced-function *acs2* protein having substantial sequence identity to SEQ. ID NO: 2 or to SEQ. ID NO: 3, or to SEQ. ID NO: 4, or to SEQ. ID NO: 5 or to SEQ. ID NO: 6, or to SEQ. ID NO: 7 or to SEQ. ID NO: 8.

[0112] In a specific aspect, the invention relates to cultivated tomato plants comprising an *acs2* allele as found in, and is derivable from or obtainable from (or derived from or obtained from) seed deposited under accession number NCIMB 42032, NCIMB 42033, NCIMB 42035, NCIMB 42036, NCIMB 42040, NCIMB 42042, or NCIMB 42043 in one or two copies, i.e. in homozygous or heterozygous form. In heterozygous form, the other allele may be a wild type *Acs2* allele or another mutant *acs2* allele, such as from any one of the other mutants provided herein, or any other mutant *acs2* allele encoding for a loss-of-function *acs2* protein or reduced-function *acs2* protein as described herein. In heterozygous form, the other allele may, thus, be a reduced function or a loss-of-function *acs2* allele.

[0113] In still another aspect, the invention relates to an endogenous *acs2* allele, or to a loss-of-function *acs2* protein or a reduced-function *acs2* protein encoded by it, said protein comprising an amino acid sequence having substantial sequence identity to SEQ. ID NO: 18 or to SEQ. ID NO: 19 or to SEQ. ID NO: 20 or to SEQ. ID NO: 21 or to SEQ. ID NO: 22 or to SEQ. ID NO: 23 or to SEQ. ID NO: 24.

[0114] In another aspect the invention relates to an endogenous *acs2* allele, or to a loss-of-function *acs2* protein or a reduced-function *acs2* protein encoded by it, said protein having substantial sequence identity to SEQ. ID NO: 2 or to SEQ. ID NO: 3, or to SEQ. ID NO: 4, or to SEQ. ID NO: 5 or to SEQ. ID NO: 6, or to SEQ. ID NO: 7, or to SEQ. ID NO: 8 as found in and/or as derivable from or obtainable from (and as derived from or obtained from) seed deposited under accession number NCIMB 42032, NCIMB 42033, NCIMB 42035, NCIMB 42036, NCIMB 42040, NCIMB 42042, or NCIMB 42043, respectively.

[0115] In yet another aspect, the invention relates to a tomato plant or plant part of the invention comprising an endogenous *acs2* allele encoding an *acs2* protein having 100% sequence identity to SEQ. ID NO: 2, or to SEQ. ID NO: 3, or to SEQ. ID NO: 4, or to SEQ. ID NO: 5, or to SEQ. ID NO: 6, or to SEQ. ID NO: 7 or to SEQ. ID NO: 8.

- 19 -

[0116] In still another aspect the invention relates to a tomato plant or plant part comprising an endogenous *acs2* allele encoding an *acs2* protein wherein said protein comprises a part having substantial sequence identity to SEQ. ID NO: 18 or to SEQ. ID NO: 19 or to SEQ. ID NO: 20 or to SEQ. ID NO: 21 or to SEQ. ID NO: 22 or to SEQ. ID NO: 23 or to SEQ. ID NO: 24; preferably wherein said protein comprises a part having 100% sequence identity to SEQ. ID NO: 18 or to SEQ. ID NO: 19 or to SEQ. ID NO: 20 or to SEQ. ID NO: 21 or to SEQ. ID NO: 22 or to SEQ. ID NO: 23 or to SEQ. ID NO: 24.

[0117] The invention further relates to tomato seeds, plants and plant parts comprising an endogenous *acs2* gene encoding a cDNA (mRNA) having substantial sequence identity to SEQ. ID NO: 9 and having at least one non-transgenic mutation within said endogenous *acs2* gene, wherein the mutation results in an amino acid substitution selected from the group consisting of Ala101, Ala103, Gly112, Pro118, and Val147 and Cys265 of the wild type *Acs2* protein, e.g. wherein the mutation is selected from the group consisting of Ala101Thr, Ala101Val, Ala103Thr, Gly112Arg, Pro118Leu, Val147Glu, and Cys265Tyr.

[0118] In another aspect the invention relates to tomato seeds, plants and plant parts of the invention wherein said at least one non-transgenic mutation results in the production of a mutant *acs2* protein having loss-of-function *acs2* protein or reduced activity compared to wild type *Acs2* protein. Preferably, said mutation results in reduced ethylene production and/or slower fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being homozygous for the functional wild type *Acs2* allele, encoding the protein of SEQ ID NO: 1 or a functional variant thereof. The mutation described anywhere herein may be human-induced or it may be a natural mutation. The plant is preferably a cultivated tomato plant. In another embodiment, said mutation is selected from the group consisting of G307A, G334A, C353T, C302T, G301A, G794A, and T440A of SEQ ID NO: 9.

[0119] In another aspect the invention relates to tomato seeds, plants and plant parts comprising an endogenous mutant *acs2* gene wherein said non-transgenic mutation creates an amino acid change in the *acs2* protein encoded by and produced by transcription and translation of the *acs2* gene, wherein said amino acid change is selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y of SEQ ID NO: 1 or of a functional variant of SEQ ID NO: 1 having at least 85% amino acid sequence identity to SEQ ID NO: 1.

[0120] In yet another aspect the invention relates to *acs2* protein having substantial sequence identity to SEQ ID NO: 2. In still another aspect the invention relates to *acs2* protein having substantial sequence identity to SEQ ID NO: 3. In a further aspect the invention relates to *acs2* protein having substantial sequence identity to SEQ ID NO: 4. In yet another aspect the invention relates to *acs2* protein having substantial sequence identity to SEQ ID NO: 5. In still another aspect the invention relates to *acs2* protein having substantial sequence identity to SEQ ID NO: 6. In a further aspect the invention relates to *acs2* protein having substantial sequence identity to SEQ ID NO: 7. In yet another aspect the

- 20 -

invention relates to *acs2* protein having substantial sequence identity to SEQ ID NO: 8. The invention also relates to tomato seeds, plants and plant parts comprising a nucleotide sequence encoding these proteins.

5 [0121] In still another aspect, the invention relates to tomato fruit, seeds, pollen, plant parts, and/or progeny of a plant of the invention. Preferably, the invention relates to fruit or seeds of the plant of the invention. More preferably, the invention relates to tomato fruit having delayed ripening and/or an increased post-harvest shelf life caused by a non-transgenic mutation in at least one *acs2* allele, as described elsewhere herein.

10 [0122] In still another aspect, the invention relates to tomato fruit, seeds, pollen, plant parts, and/or progeny of a plant of the invention comprising an *acs2* protein having one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y in a wild type *Acs2* protein, said protein having at least 85% amino acid sequence identity to SEQ ID NO: 1. In another embodiment, the invention relates to fruit or seeds of such plant of the invention. In still another embodiment, the invention relates to tomato fruit having delayed ripening and/or an increased  
15 post-harvest shelf life caused by a non-transgenic mutation in at least one *acs2* allele, as described elsewhere herein.

[0123] In one aspect the tomato plants according to the invention have a delay of breaker stage, meaning that the mutants according to the invention require significantly more days e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more days than wild type *Acs2/Acs2* controls, such as TAPA, TPAADASU, or Pusa  
20 Sheetal, for the first fruits and/or for all fruits to have entered breaker stage.

[0124] In another aspect fruits of the tomato plants of the invention require more days to go from breaker stage to red stage , e.g. fruits of the plants of the invention require 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 or more days than wild type *Acs2/Acs2* controls to go from breaker stage to red stage.

25 [0125] In another aspect the invention relates to a fruit of a plant of the invention having a the shelf life that is at least 2 days longer than the shelf life of a tomato fruit being homozygous for the wild type *Acs2* allele. In still another aspect the invention relates to a fruit according to a plant of the invention having a reduced ethylene production that is at least 10 % reduced, or at least 15% reduced or at least 20 % reduced compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele. In another aspect the invention relates to a fruit according to a plant of the invention having a reduced  
30 ethylene production that is at least 10 % reduced, or at least 15% reduced or at least 20 % reduced compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele, when measured at the pink or red stage.

[0126] In a particular aspect the tomato plants according to the invention have a shelf life that is significantly longer than the shelf life of wild type plants, for example the number of days from the first  
35 fruit being in breaker stage (or turning stage, pink stage, red stage or from harvest) up to the first fruit

- 21 -

starting to become 'bad' and unsuitable for sale or consumption is significantly longer, e.g. at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, days longer than fruits of control plants (such as wild type *Acs2/Acs2* plants), when plants are grown under the same conditions and fruits are treated the same way and kept under the same conditions.

5 [0127] A delayed ripening and/or extended shelf-life can have the advantage that more time is available for transport of picked fruits e.g. to retailers and supermarkets and/or that the consumer can keep the fruits longer. Tomatoes can be harvested at mature green stage or at breaker stage, or thereafter. When harvested before breaker stage, ethylene exposure is needed, while harvest around breaker stage  
10 Figure 2, delayed-ripening mutants according to the invention produce less ethylene at pink stage and red stage than wild type fruits, but sufficient ethylene to ripen to the red stage. In one aspect of the invention tomato plants are provided comprising a mutant *acs2* allele encoding a loss-of-function *acs2* protein or reduced function *acs2* protein, wherein the fruits of said plants produce significantly less ethylene than wild type (*Acs2/Acs2*) plants. "Significantly less ethylene" refers to the fruit producing  
15 equal to or less than 75%, equal to or less than 70%, equal to or less than 65%, equal to or less than 60%, equal to or less than 55%, equal to or less than 50%, equal to or less than 45%, equal to or less than 40%, equal to or less than 35%, equal to or less than 30%, equal to or less than 25% equal to or less than 20%, or equal to or less than 15% of the ethylene produced by homozygous *Acs2/Acs2* fruits at the pink or red stage. Thus, the ethylene produced at the pink stage is in one aspect below about 3.5 nl/(h · g), such as equal to or below about 3 nl/(h · g) or equal to or below about 2.5 nl/(h · g) or equal to or below about 2.0 nl/(h · g) or equal to or below about 1.5 nl/(h · g) or equal to or below about 1.0 nl/(h · g) or equal to or below about 0.5 nl/(h · g). The ethylene produced at the red stage is in one aspect below  
20 about 6 nl/(h · g), such as equal to or below about 5.5 nl/(h · g) or equal to or below about 5.0 nl/(h · g), or equal to or below 4.5 nl/(h · g), or equal to or below about 3.5 nl/(h · g), or equal to or below about 3  
25 nl/(h · g) or equal to or below about 2.5 nl/(h · g) or equal to or below about 2.0 nl/(h · g) or equal to or below about 1.5 nl/(h · g) or equal to or below about 1.0 nl/(h · g) or equal to or below about 0.5 nl/(h · g).

[0128] In another aspect, the invention relates to tomato fruit of a plant of the invention having a longer ripening period and/or an increased post-harvest shelf life caused by a non-transgenic mutation in  
30 at least one *acs2* allele wherein the longer ripening period and/or the longer post-harvest shelf life is at least 110% of the ripening period and/or of the post-harvest shelf life of a tomato fruit being homozygous for the wild type *Acs2* allele. Preferably, the ripening period and/or post-harvest shelf life is at least 115%, more preferably at least 120%, even more preferably at least 125% of the ripening period and/or post-harvest shelf life of a tomato fruit being homozygous for the wild type *Acs2* allele. In  
35 another aspect, the ripening period and/or post-harvest shelf life is at least 135%, more preferably at least 150%, even more preferably at least 165% of the ripening period and/or post-harvest shelf life of a tomato fruit being homozygous for the wild type *Acs2* allele. In yet another aspect, the ripening period

- 22 -

and/or post-harvest shelf life is at least 180%, more preferably at least 200% even more preferably at least 250 % of the ripening period and/or post-harvest shelf life of a tomato fruit being homozygous for the wild type *Acs2* allele.

5 [0129] In yet another aspect, the invention relates to a tomato plant comprising an *acs2* allele encoding a mutant *acs2* protein said allele being derived or derivable, or obtained or obtainable from a plant of the invention representative seed of which having been deposited under Accession Number NCIMB 42032, NCIMB 42033, NCIMB 42035, NCIMB 42036, NCIMB 42040, NCIMB 42042, or NCIMB 42043.

10 [0130] In still another aspect of the invention tomato plants are provided that have the same or similar delayed ripening and/or increased shelf life as tomato plants of the invention, of which representative seeds were deposited by Nunhems B.V. and accepted for deposit on 21 August 2012 at the NCIMB Ltd. (Ferguson Building, Craibstone Estate, Bucksburn Aberdeen, Scotland AB21 9YA, UK) according to the Budapest Treaty, under the Expert Solution (EPC 2000, Rule 32(1)). Seeds were given the following deposit numbers: NCIMB 42032 (mutant 783), NCIMB 42033 (mutant 2145),  
15 NCIMB 42035 (mutant 2714), NCIMB 42036 mutant (3793), NCIMB 42040 (mutant 4946), NCIMB 42042 (mutant 7871), or NCIMB 42043 (mutant 8185).

[0131] According to a further aspect the invention provides a cell culture or tissue culture of the tomato plant of the invention. The cell culture or tissue culture comprises regenerable cells. Such cells can be derived from leaves, pollen, embryos, cotyledon, hypocotyls, meristematic cells, roots, root tips,  
20 anthers, flowers, seeds and stems.

[0132] Seeds from which plants according to the invention can be grown are also provided, as well as packages or containers containing such seeds. Also a vegetative propagation of plants according to the invention are an aspect encompassed herein. Likewise harvested fruits and fruit parts, either for fresh consumption or for processing or in processed form are encompassed. Fruits may be graded, sized  
25 and/or packaged. Fruits may be sliced or diced or further processed.

[0133] In another aspect the invention relates to one or more cells of a plant of the invention.

[0134] The invention also relates to food and/ or food products comprising or consisting of the fruit, or part of a fruit, of a tomato plant of the invention. As used herein, food refers to nutrients consumed by human or animal species. Examples are sandwiches, salads, sauces, ketchup and the like.

30 [0135] A method of producing a tomato plant of the invention comprising the steps of:  
a. obtaining plant material from a tomato plant;  
b. treating said plant material with a mutagen to create mutagenized plant material;

- 23 -

- c. analyzing said mutagenized plant material to identify a plant having at least one mutation in at least one *acs2* allele having substantial sequence identity to SEQ ID NO: 1 or in a functional variant thereof.

[0136] The method may further comprise analyzing the ripening period and/or shelf life of tomato  
5 fruits of the selected plant or progeny of the plant and selecting a plant of which the fruit have delayed ripening and/or extended shelf-life.

In one aspect the mutation is selected from a mutation resulting in an amino acid substitution selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, C265Y of SEQ ID NO: 1 or of a part thereof. In a further aspect, the mutation is selected from a mutation causing a change in the  
10 cDNA selected from the group consisting of G307A, G334A, C353T, C302T, G301A, G794A, and T440A of SEQ ID NO: 9. In this method, the plant material of step a) is preferably selected from the group consisting of seeds, pollen, plant cells, or plant tissue of a tomato plant line or cultivar. Plant seeds being more preferred. In another aspect, the mutagen used in this method is ethyl methanesulfonate. In step b) and step c) the mutagenized plant material is preferably a mutant population, such as a tomato  
15 TILLING population.

Thus, in one aspect a method for producing a tomato plant comprising delayed fruit ripening and/or longer fruit shelf-life is provided comprising the steps of:

- a) providing a tomato TILLING population,  
b) screening said TILLING population for mutants in the *acs2* gene, and  
20 c) selecting from the mutant plants of b) those plants (or progeny of those plants) of which the fruits have a reduced ethylene production and/or a delayed ripening and/or longer shelf life than wild type (*Acs2/Acs2*) fruits.

[0137] Mutant plants (M1) are preferably selfed one or more times to generate for example M2 populations or preferably M3 or M4 populations for phenotyping. In M2 populations the mutant allele is  
25 present in a ratio of 1 (homozygous for mutant allele) : 2 (heterozygous for mutant allele): 1 (homozygous for wild type allele).

[0138] In yet a further aspect the invention relates to a method for producing a hybrid *Solanum lycopersicum* plant, said method comprising:

- (a) obtaining a first *Solanum lycopersicum* plant of the current invention or from a seed from  
30 which a plant of the invention can be grown; and  
(b) crossing said first *Solanum lycopersicum* plant with a second *Solanum lycopersicum* plant to obtain hybrid seeds,

wherein said hybrid *Solanum lycopersicum* plant comprises an *acs2* allele having one or more mutations wherein said mutations result in production of a mutant *acs2* protein having one or more amino acids

- 24 -

changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y of SEQ ID NO: 1 or of a variant of SEQ ID NO: 1.

[0139] Plants and plant parts (e.g. fruits, cells, etc.) of the invention can be homozygous or heterozygous for the mutant *acs2* allele.

5 [0140] Preferably the plants according to the invention, which comprise one or more mutant *acs2* alleles, and which produce a mutant *acs2* protein having loss-of-function *acs2* protein or reduced activity compared to wild type *Acs2* protein, do not produce fewer fruits than the wild type plants. Thus, fruit number per plant is preferably not reduced.

[0141] Other putative ACS2 genes/proteins can be identified *in silico*, e.g. by identifying nucleic acid or protein sequences in existing nucleic acid or protein database (e.g. GENBANK, SWISSPROT, TrEMBL) and using standard sequence analysis software, such as sequence similarity search tools  
10 (BLASTN, BLASTP, BLASTX, TBLAST, FASTA, etc.).

[0142] In one embodiment loss-of-function *acs2* protein or reduced-function mutant *acs2* proteins (including variants or orthologs, such as *acs2* proteins of wild tomato relatives) are provided and plants and plant parts comprising one or more *acs2* alleles in their genome, which encode loss-of-function *acs2* protein or reduced-function mutants, whereby the reduced-function confers reduced ethylene production and /or slower fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being  
15 homozygous for the wild type *Acs2* allele.

[0143] Any type of mutation may lead to a reduction in function of the encoded *Acs2* protein, e.g. insertion, deletion and/or replacement of one or more nucleotides in the genomic DNA which comprises the cDNA (SEQ ID NO: 9, or variants thereof). In a preferred embodiment an *acs2* nucleic acid sequence, encoding a loss-of-function *acs2* protein or reduced-function *acs2* protein due to one or more mutation(s), is provided, said *acs2* protein causing reduced ethylene production and/or conferring slower fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being homozygous for the  
20 wild type *Acs2* allele.  
25

[0144] The *in vivo* loss-of-function *acs2* protein or reduced-function of such proteins can be tested as described herein, by determining the effect this mutant allele has on ethylene production and/or ripening period and/or shelf life period. Plants comprising a nucleic acid sequence encoding such mutant loss-of-function *acs2* protein or reduced-function proteins and having a reduced ethylene production and/or slower fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being  
30 homozygous for the wild type *Acs2* allele can for example be generated using e.g. mutagenesis and identified by TILLING or identified using EcoTILLING, as known in the art. Also transgenic methods can be used to test *in vivo* functionality of a mutant *acs2* allele encoding a mutant *acs2* protein. A mutant allele can be operably linked to a plant promoter and the chimeric gene can be introduced into a tomato  
35 plant by transformation. Regenerated plants (or progeny, e.g. obtained by selfing), can be tested for

- 25 -

ethylene production and/or fruit ripening period and/or shelf life. For example a tomato plant comprising a non-functional *acs2* allele can be transformed to test the functionality of the transgenic *acs2* allele.

[0145] TILLING (Targeting Induced Local Lesions IN Genomes) is a general reverse genetics technique that uses traditional chemical mutagenesis methods to create libraries of mutagenized individuals that are later subjected to high throughput screens for the discovery of mutations. TILLING combines chemical mutagenesis with mutation screens of pooled PCR products, resulting in the isolation of missense and non-sense mutant alleles of the targeted genes. Thus, TILLING uses traditional chemical mutagenesis (e.g. EMS or MNU mutagenesis) or other mutagenesis methods (e.g. radiation such as UV) followed by high-throughput screening for mutations in specific target genes, such as *Acs2* according to the invention. S1 nucleases, such as CEL1 or ENDO1, are used to cleave heteroduplexes of mutant and wildtype target DNA and detection of cleavage products using e.g. electrophoresis such as a LI-COR gel analyzer system, see e.g. Henikoff *et al.* Plant Physiology 2004, 135: 630-636. TILLING has been applied in many plant species, such as tomato. (see [http://tilling.ucdavis.edu/index.php/Tomato\\_Tilling](http://tilling.ucdavis.edu/index.php/Tomato_Tilling) ), rice (Till *et al.* 2007, BMC Plant Biol 7: 19), Arabidopsis (Till *et al.* 2006, Methods Mol Biol 323: 127-35), Brassica, maize (Till *et al.* 2004, BMC Plant Biol 4: 12), etc. Also EcoTILLING, whereby mutants in natural populations are detected, has been widely used, see Till *et al.* 2006 (Nat Protoc 1: 2465-77) and Comai *et al.* 2004 (Plant J 37: 778-86).

[0146] In one embodiment of the invention (cDNA or genomic) nucleic acid sequences encoding such mutant *acs2* proteins comprise one or more non-sense and/or missense mutations, e.g. transitions (replacement of purine with another purine (A ↔ G) or pyrimidine with another pyrimidine (C ↔ T)) or transversions (replacement of purine with pyrimidine, or *vice versa* (C/T ↔ A/G)). In one embodiment the non-sense and/or missense mutation(s) is/are in the nucleotide sequence encoding any of the *Acs2* exons, or an essentially similar domain of a variant *Acs2* protein, i.e. in a domain comprising at least 80%, 90%, 95%, 98%, 99% amino acid sequence identity to amino acids of SEQ ID NO: 1 or to a variant thereof.

[0147] In one embodiment an *acs2* nucleotide sequence comprising one or more non-sense and/or missense mutations in one of the exon- encoding sequence are provided, as well as a plant comprising such a mutant allele resulting in reduced ethylene production and/or delayed fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele.

[0148] In a specific embodiment of the invention tomato plants and plant parts (fruits, seeds, etc.) comprising a mutant loss-of-function or reduced-function *acs2* allele are provided.

[0149] Also provided are nucleic acid sequences (genomic DNA, cDNA, RNA) encoding loss-of-function *acs2* protein or reduced-function *acs2* proteins, such as for example *acs2* depicted in SEQ ID NO: 2, 3, 4, 5, 6, 7, or 8; or variants thereof as defined above (including any chimeric or hybrid proteins or mutated proteins or truncated proteins). Due to the degeneracy of the genetic code various nucleic

- 26 -

acid sequences may encode the same amino acid sequence. The nucleic acid sequences provided include naturally occurring, artificial or synthetic nucleic acid sequences. A nucleic acid sequence encoding Acs2 is provided for in SEQ ID NO: 9 (wild type cDNA), NCBI Reference Sequence: NM\_001247249.1 [http://www.ncbi.nlm.nih.gov/nuccore/NM\\_001247249](http://www.ncbi.nlm.nih.gov/nuccore/NM_001247249).

5 [0150] It is understood that when sequences are depicted as DNA sequences while RNA is referred to, the actual base sequence of the RNA molecule is identical with the difference that thymine (T) is replaced by uracil (U). When referring herein to nucleotide sequences (e.g DNA or RNA) italics are used, e.g. *acs2* allele, while when referring to proteins, no italics are used, e.g. *acs2* protein. Mutants are in small letters (e.g *acs2* allele or *acs2* protein), while wild type / functional forms start with a capital letter  
10 (*Acs2* allele or *Acs2* protein).

[0151] Also provided are nucleic acid sequences (genomic DNA, cDNA, RNA) encoding mutant *acs2* proteins, i.e. loss-of-function *acs2* protein or reduced function *acs2* proteins, as described above, and plants and plant parts comprising such mutant sequences. For example, *acs2* nucleic acid sequences comprising one or more non-sense and/or missense mutations in the wild type *Acs2* coding sequence,  
15 rendering the encoded protein having a loss-of-function or reduced function *in vivo*. Also sequences with other mutations are provided, such as splice-site mutants, i.e. mutations in the genomic *acs2* sequence leading to aberrant splicing of the pre-mRNA, and/or frame-shift mutations, and/or insertions (e.g. transposon insertions) and/or deletions of one or more nucleic acids.

[0152] It is clear that many methods can be used to identify, synthesise or isolate variants or  
20 fragments of *acs2* nucleic acid sequences, such as nucleic acid hybridization, PCR technology, *in silico* analysis and nucleic acid synthesis, and the like. Variants of SEQ ID NO: 9, may either encode wild type, functional *Acs2* proteins, or they may encode loss-of-function *acs2* protein or reduced-function mutant alleles of any of these, as for example generated e.g. by mutagenesis and/or identified by methods such as TILLING or EcoTILLING, or other methods.

25 [0153] A plant of the invention can be used in a conventional plant breeding scheme to produce more plants with the same characteristics or to introduce the mutated *acs2* allele into other plant lines or varieties of the same or related plant species.

[0154] Also transgenic plants can be made using the mutant *acs2* nucleotide sequences of the invention using known plant transformation and regeneration techniques in the art. An "elite event" can  
30 be selected, which is a transformation event having the chimeric gene (comprising a promoter operably linked to a nucleotide sequence encoding a loss-of-function *acs2* protein or reduced-function *acs2* protein) inserted in a particular location in the genome, which results in good expression of the desired phenotype.

[0155] The plants of the invention as described above are homozygous for the mutant *acs2* allele,  
35 or heterozygous. To generate plants comprising the mutant allele in homozygous form, selfing can be

- 27 -

used. The mutant *acs2* alleles according to the invention can be transferred to any other tomato plant by traditional breeding techniques, such as crossing, selfing, backcrossing, etc. Thus any type of tomato having delayed ripening and/or longer shelf life due to the presence of at least one mutant *acs2* allele according to the invention can be generated. Any *S. lycopersicum* may be generated and/or identified  
5 having at least one mutant *acs2* allele in its genome and producing a *acs2* protein having loss-of-function *acs2* protein or reduced activity compared to wild type *Acs2* protein. The tomato plant may, thus, be any cultivated tomato, any commercial variety, any breeding line or other, it may be determinate or indeterminate, open pollinated or hybrid, producing fruits of any colour, shape and size. The mutant allele generated and/or identified in a particular tomato plant, or in a sexually compatible relative of  
10 tomato, may be easily transferred into any other tomato plant by breeding (crossing with a plant comprising the mutant allele and then selecting progeny comprising the mutant allele).

[0156] The presence or absence of a mutant *acs2* allele according to the invention in any tomato plant or plant part and/or the inheritance of the allele to progeny plants can be determined phenotypically and/or using molecular tools (e.g. detecting the presence or absence of the *acs2*  
15 nucleotide sequence or *acs2* protein using direct or indirect methods).

[0157] The mutant allele is in one embodiment generated or identified in a cultivated plant, but may also be generated and/or identified in a wild plant or non-cultivated plant and then transferred into an cultivated plant using e.g. crossing and selection (optionally using interspecific crosses with e.g. embryo rescue to transfer the mutant allele). Thus, a mutant *acs2* allele may be generated (human  
20 induced mutation using mutagenesis techniques to mutagenize the target *acs2* gene or variant thereof) and/or identified (spontaneous or natural allelic variation) in *Solanum lycopersicum* or in other *Solanum* species include for example wild relatives of tomato, such as *S. cheesmanii*, *S. chilense*, *S. habrochaites* (*L. hirsutum*), *S. chmielewskii*, *S. lycopersicum* x *S. peruvianum*, *S. glandulosum*, *S. hirsutum*, *S. minutum*, *S. parviflorum*, *S. pennellii*, *S. peruvianum*, *S. peruvianum* var. *humifusum* and *S.*  
25 *pimpinellifolium*, and then transferred into a cultivated *Solanum* plant, e.g. *Solanum lycopersicum* by traditional breeding techniques. The term “traditional breeding techniques” encompasses herein crossing, selfing, selection, double haploid production, embryo rescue, protoplast fusion, transfer via bridge species, etc. as known to the breeder, i.e. methods other than genetic modification by which alleles can be transferred.

[0158] In another embodiment, the plant comprising the mutant *acs2* allele (e.g. tomato) is crossed  
30 with another plant of the same species or of a closely related species, to generate a hybrid plant (hybrid seed) comprising the mutant *acs2* allele. Such a hybrid plant is also an embodiment of the invention.

[0159] In one embodiment F1 hybrid tomato seeds (i.e. seeds from which F1 hybrid tomato plants can be grown) are provided, comprising at least one *acs2* allele according to the invention. F1 hybrid  
35 seeds are seeds harvested from a cross between two inbred tomato parent plants. Such an F1 hybrid may comprise one or two mutant *acs2* alleles according to the invention. Such an F1 hybrid comprising two

- 28 -

mutant *acs2* alleles according to the invention may comprise two copies of the same *acs2* allele or two different *acs2* alleles according to the invention. Thus, in one embodiment a plant according to the invention is used as a parent plant to produce an F1 hybrid, the fruit of which have reduced ethylene production and/or delayed ripening and/or longer shelf-life than wild type *Acs2/Acs2* plants.

5 [0160] Also a method for transferring a mutant *acs2* allele to another plant is provided, comprising providing a plant comprising a mutant *acs2* allele in its genome, whereby the plant comprising the mutant allele produce fruits that show reduced ethylene production and/or slower fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele (as described above), crossing said plant with another plant and obtaining the seeds of said cross. Optionally  
10 plants obtained from these seeds may be further selfed and/or crossed and progeny selected comprising the mutant allele and producing fruits with delayed ripening and/or longer shelf-life and / or reduced ethylene production due to the presence of the mutant allele compared to plants comprising the wild type *Acs2* allele.

[0161] As mentioned, it is understood that other mutagenesis and/or selection methods may  
15 equally be used to generate mutant plants according to the invention. Seeds may for example be radiated or chemically treated to generate mutant populations. Also direct gene sequencing of *acs2* may be used to screen mutagenized plant populations for mutant alleles. For example KeyPoint screening is a sequence based method which can be used to identify plants comprising mutant *acs2* alleles (Rigola *et al.* PloS One, March 2009, Vol 4(3):e4761).

20 [0162] Thus, non-transgenic mutant tomato plants which produce lower levels of wild type *Acs2* protein in fruits are provided, or which completely lack wild type *Acs2* protein in fruits, and which produce loss-of-function *acs2* protein or reduced-function *acs2* protein in fruits due to one or more mutations in one or more endogenous *acs2* alleles, are provided. These mutants may be generated by mutagenesis methods, such as TILLING or variants thereof, or they may be identified by EcoTILLING  
25 or by any other method. *Acs2* alleles encoding loss-of-function *acs2* protein or reduced-functional *acs2* protein may be isolated and sequenced or may be transferred to other plants by traditional breeding methods.

[0163] Any part of the plant, or of the progeny thereof, is provided, including harvested fruit, harvested tissues or organs, seeds, pollen, flowers, ovaries, etc. comprising a mutant *acs2* allele  
30 according to the invention in the genome. Also plant cell cultures or plant tissue cultures comprising in their genome a mutant *acs2* allele are provided. Preferably, the plant cell cultures or plant tissue cultures can be regenerated into whole plants comprising a mutant *acs2* allele in its genome. Also double haploid plants (and seeds from which double haploid plants can be grown), generated by chromosome doubling of haploid cells comprising an *acs2* mutant allele, and hybrid plants (and seeds from which hybrid plants  
35 can be grown) comprising a mutant *acs2* allele in their genome are encompassed herein, whereby the

- 29 -

double haploid plants and hybrid plants produce delayed ripening and/or longer shelf life fruits according to the invention.

[0164] The invention further relates to an endogenous *acs2* protein having at least one human-induced non-transgenic mutation selected from A101T, A101V, A103T, G112R, P118L, V147E and C265Y of SEQ ID NO: 1 or an endogenous *acs2* allele encoding such protein.

[0165] In another aspect the invention relates to a cultivated plant or plant part (e.g. seed) of the species *Solanum lycopersicum* of the invention comprising in addition to the one or more mutations in the *acs2* allele as described herein, an *acs4* allele having one or more mutations, said mutations in said *acs4* allele resulting in production of a mutant *acs4* protein having loss-of-function or reduced function compared to *Solanum lycopersicum* wild type *Acs4* protein, having a protein sequence as shown in Figure 5 and/or as derived from the mRNA based on Genbank Accession number AAA34131.1 (encoded by the cDNA of GenBank Accession number M63490.1)..

[0166] A "reduced function *acs4* protein" or "reduced activity *acs4* protein" refers to a mutant *acs4* protein which has a reduced catalytic activity in synthesizing ACC from S-Adenosyl methionine, leading to reduced ethylene synthesis compared to wild-type *Acs4* protein. Said reduced catalytic activity of the *acs4* protein affects the ripening behaviour of the fruits comprising such reduced function *acs4* protein when the allele encoding the mutant protein is present in homozygous or heterozygous form in the tomato plant, i.e. delayed ripening and/or longer shelf-life of the fruits. Such a reduced function *acs4* protein can be obtained by the transcription and translation of a "partial knockout mutant *acs4* allele" which is, for example, a wild-type *Acs4* allele, which comprises one or more mutations in its nucleic acid sequence. In one aspect, such a partial knockout mutant *acs4* allele is a wild-type *Acs4* allele, which comprises one or more mutations that preferably result in the production of an *acs4* protein wherein at least one conserved and/or functional amino acid is substituted for another amino acid, such that the biological activity is significantly reduced but not completely abolished. However, other mutations, such as one or more non-sense, missense, splice-site or frameshift mutations in the tomato *Acs4* allele may also result in reduced function *acs4* protein and such reduced function proteins may have one or more amino acids replaced, inserted or deleted, relative to the wild type *ACS4* protein. Such partial knockout mutant *acs4* allele may also encode a dominant negative *acs4* protein, which is capable of adversely affecting the biological activity of other *Acs4* proteins within the same cell. Such a dominant negative *acs4* protein can be an *acs4* protein that is still capable of interacting with the same elements as the wild-type *Acs4* protein, but that blocks some aspect of its function. Examples of dominant negative *acs4* proteins are *acs4* proteins that lack, or have modifications in specific amino acid residues critical for activation, but still contain their binding domain, such that not only their own biological activity is reduced or abolished, but that they further reduce the total *acs4* activity in the cell by competing with wild type and/or partial knockout *acs4* proteins present in the cell for binding sites. Mutant alleles can be either "natural mutant" alleles, which are mutant alleles found in nature (e.g.

- 30 -

produced spontaneously without human application of mutagens) or "induced mutant" alleles, which are induced by human intervention, e.g. by mutagenesis.

[0167] A "loss-of-function *acs4* protein" refers to a mutant *acs4* protein which has essentially no catalytic activity in synthesising ACC from S-Adenosyl methionine compared to wild-type *Acs4* protein, leading to reduced ethylene synthesis compared to wild type *Acs4* protein. Said lack of catalytic activity synthesis affects the ripening behaviour of the fruits comprising such loss-of-function *acs4* protein when the allele encoding the mutant protein is present in homozygous or heterozygous form in the tomato plant. Fruits of tomato plants homozygous for such a "loss-of-function *acs4* protein" may still produce ethylene catalysed by other proteins (e.g. other *Acs* proteins like *Acs1A*). As a consequence, fruits of tomato plants homozygous for such a "loss-of-function *acs4* protein" may still ripen, but ripening may be delayed and/or shelf life may be longer.

[0168] In one aspect said mutant *acs4* allele is the allele as found in, and as obtained from and/or obtainable from and/or derived from and/or derivable from seed of mutant 2477 and/or mutant 4043 and/or mutant 4222, and/or mutant 4303 and/or mutant 4691 and/or mutant 5251. These *acs4* mutants have been described in detail in EP application number 12186606.5. Preferably, said mutations in said *acs2* and/or *acs4* alleles results in reduced ethylene production and/or slower fruit ripening and/or a longer shelf life of tomato fruits compared to *Solanum lycopersicum* being homozygous for the functional wild type *Acs2* and *Acs4* allele (e.g. Pusa Sheetal, Tapa, or TPAADASU) or variants thereof. Such plants may be obtained via breeding methods known in the art by crossing a plant having the desired *acs2* mutation with a plant having the desired *acs4* mutation. Such plants or plant parts can be homozygous or heterozygous for the *acs2* mutation or for the *acs4* mutation or for both the *acs2* and *acs4* mutation. Thus, the plant may genetically be *acs2/Acs2 acs4/Acs4* or *acs2/acs2 acs4/Acs4* or *acs2/Acs2 acs4/acs4* or *acs2/acs2 acs4/acs4*.

[0169] Preferably, the mutant plants also have good other agronomic characteristics, i.e. they do not have reduced fruit numbers and/or reduced fruit quality compared to wild type plants. In a preferred embodiment the plant is a tomato plant and the fruit is a tomato fruit, such as a processing tomato, fresh market tomato of any shape or size or colour. Thus, also harvested products of plants or plant parts comprising one or two mutant *acs2* alleles are provided. This includes downstream processed products, such as tomato paste, ketchup, tomato juice, cut tomato fruit, canned fruit, dried fruit, peeled fruit, etc. The products can be identified by comprising the mutant allele in their genomic DNA.

#### Seed Deposits

[0170] A representative sample of seeds of seven (7) tomato TILLING mutants (*acs2* mutants) according to Example 1, were deposited by Nunhems B.V. and accepted for deposit on 21 August 2012 at the NCIMB Ltd. (Ferguson Building, Craibstone Estate, Bucksburn Aberdeen, Scotland AB21 9YA, UK) according to the Budapest Treaty, under the Expert Solution (EPC 2000, Rule 32(1)). Seeds were

- 31 -

given the following deposit numbers: NCIMB 42032 (mutant 783), NCIMB 42033 (mutant 2145), NCIMB 42035 (mutant 2714), NCIMB 42036 mutant (3793), NCIMB 42040 (mutant 4946), NCIMB 42042 (mutant 7871), or NCIMB 42043 (mutant 8185).

[0171] A representative sample of seeds of five tomato TILLING mutants (acs4 mutants) according to Example 1, were deposited by Nunhems B.V. and accepted for deposit on 21 August 2012 at the NCIMB Ltd. (Ferguson Building, Craibstone Estate, Bucksburn Aberdeen, Scotland AB21 9YA, UK) according to the Budapest Treaty, under the Expert Solution (EPC 2000, Rule 32(1)). Seeds were given the following deposit numbers: NCIMB 42034 (mutant 2477), NCIMB 42037 (mutant 4043), NCIMB 42038 (mutant 4222), NCIMB 42039 (mutant 4691), NCIMB 42041 (mutant 5251). These acs4 mutants have been described in European Patent Application number 12186606.5.

[0172] The Applicant requests that samples of the biological material and any material derived therefrom be only released to a designated Expert in accordance with Rule 32(1) EPC or related legislation of countries or treaties having similar rules and regulation, until the mention of the grant of the patent, or for 20 years from the date of filing if the application is refused, abandoned, withdrawn or deemed to be withdrawn.

[0173] Access to the deposit will be available during the pendency of this application to persons determined by the Director of the U.S. Patent Office to be entitled thereto upon request. Subject to 37 C.F.R. § 1.808(b), all restrictions imposed by the depositor on the availability to the public of the deposited material will be irrevocably removed upon the granting of the patent. The deposit will be maintained for a period of 30 years, or 5 years after the most recent request, or for the enforceable life of the patent whichever is longer, and will be replaced if it ever becomes nonviable during that period. Applicant does not waive any rights granted under this patent on this application or under the Plant Variety Protection Act (7 USC 2321 et seq.).

## EXAMPLES

### General methods

[0174] PCR amplification products were directly sequenced by a service company (BaseClear, The Netherlands, <http://www.baseclear.com/>) using the same primers as were used for the amplification. The  
5 obtained sequences were aligned using a computer program (CLC Bio Main Work Bench, Denmark, [www.clcbio.com](http://www.clcbio.com)) to identify the nucleotide changes.

### Materials

[0175] Water used for analyses and mutagenesis is tap water filtered in an Milli-Q water Integral system, Milli-Q type Reference A+ supplied with a Q-gard T2 Cartridge and a Quantum TEX Cartridge.  
10 Water resistance is  $\geq 18$  M $\Omega$ .

[0176] Ethyl Methanesulfonate (EMS) (pure) was obtained from Sigma, product number M0880.

### Measurement of tomato ripening and/or shelf-life time or periods

[0177] Tomato ripening and/or shelf life time or periods can be measured by various methods known in the art like for example making periodically visual assessments of fruits and/or measurement  
15 of fruit firmness or softening, measurement of lycopene contents in the tomato fruits, ethylene production by the fruits, colour of the fruits or any alternative method or combination of methods. Fruit firmness can for example be measured by evaluating resistance to deformation in units of for example 0.1 mm as measured with a penetrometer fitted with a suitable probe (e.g. a probe of 3 mm) (Mutschler *et al*, 1992, Hortscience 27 pp 352-355) (Martinez *et al* 1995 Acta Horticulturae 412 pp 463-469).  
20 Alternative methods exist in the art, such as use of a texturometer (Bui *et al*. 2010; International Journal of Food Properties, Volume 13, Issue 4 pp 830 846).

[0178] Fruit colour can be classified by the U.S. standards for grades of fresh tomato (U.S. Dept of Agriculture, 1973, US standards for grades of fresh tomatoes, U.S. Dept Agr. Agr. Mktg. Serv., Washington D.C.), measuring the colour with a chromometer (Mutschler *et al*, 1992, Hortscience 27 pp  
25 352-355) or by comparing the colour to a colour chart like the Royal Horticultural Society (RHS) Color Chart ([www.rhs.org.uk](http://www.rhs.org.uk)).

[0179] Lycopene content can be determined according to the reduced volumes of organic solvents method of Fish *et al*. A quantitative assay for lycopene that utilizes reduced volumes of organic solvents. Fish *et al*. *J. Food Compos. Anal.* **2002**, 15, 309–317. This method can be used to determine lycopene  
30 content measured directly on intact tomato fruit while simultaneously estimating the basic physicochemical characteristics: color, firmness, soluble solids, acidity, and pH (Clement *et al*, *J. Agric. Food Chem.* **2008**, 56, 9813–9818).

[0180] Ethylene release can be measured by placing the fruit in a closed space, e.g. in a 0.5 l glass holder. One ml of holder atmosphere can be extracted after one hour and amount of ethylene gas  
35 produced can be quantified using a gas chromatograph (e.g. a Hewlett-Packard 5890) equipped with a

- 33 -

suitable detection unit, e.g. a flame ionisation detector, and a suitable column (e.g. a 3 m stainless steel column with an inner diameter of 3.5 mm containing activated alumina of 80/100 mesh). Ethylene production can be expressed as the amount in nl of ethylene given off per gram of fruit per hour (nl g<sup>-1</sup> h<sup>-1</sup>) (Martinez et al 1995 Acta Horticulturae 412 pp 463-469).

- 5 [0181] Alternatively, ethylene production can be measured as described further below, using real-time measurements with a laser-based ethylene detector (ETD-300, Sensor Sense B.V., Nijmegen, the Netherlands) in combination with a gas handling system (Cristescu et al., 2008 Laser-based systems for trace gas detection in life sciences. Appl Phys B 2008; 92 pp 343-9).

#### EXAMPLE 1

##### 10 Mutagenesis

[0182] A highly homozygous inbred line used in commercial processing tomato breeding was used for mutagenesis treatment with the following protocol. After seed imbibition on damp Whatman® paper for 24h, ~20,000 seeds, divided in 8 batches of 2500 respectively, were soaked in 100 ml of ultrapure water and ethyl methanesulfonate (EMS) at a concentration of 1% in conical flasks. The flasks were  
15 gently shaken for 16h at room temperature. Finally, EMS was rinsed out under flowing water. Following EMS treatment, seeds were directly sown in the greenhouse. Out of the 60% of the seeds that germinated, 10600 plantlets were transplanted in the field. From these 10600 plantlets, 1790 were either sterile or died before producing fruit. For each remaining M1 mutant plant one fruits was harvested and its seeds isolated. The obtained population, named M2 population, is composed of 8810 seeds lots each  
20 representing one M2 family. Of these, 585 families were excluded from the population due to low seed set.

[0183] DNA was extracted from a pool of 10 seeds originating from each M2 seed lot. Per mutant line, 10 seeds were pooled in a Micronic® deepwell tube; <http://www.micronic.com> from a 96 deep-well plate, 2 stainless balls were added to each tube. The tubes and seeds were frozen in liquid nitrogen for 1  
25 minute and seeds were immediately ground to a fine powder in a Deepwell shaker (Vaskon 96 grinder, Belgium; <http://www.vaskon.com>) for 2 minutes at 16,8 Hz (80% of the maximum speed). 300 µl Agowa® Lysis buffer P from the AGOWA® Plant DNA Isolation Kit <http://www.agowa.de> was added to the sample plate and the powder was suspended in solution by shaking 1 minute at 16,8 Hz in the Deepwell shaker. Plates were centrifuged for 10 minutes at 4000 rpm. 75 µl of the supernatant was  
30 pipetted out to a 96 Kingfisher plate using a Janus MDT® (Perkin Elmer, USA; <http://www.perkinelmer.com>) platform (96 head). The following steps were performed using a Perkin Elmer Janus® liquid handler robot and a 96 Kingfisher® (Thermo labsystems, Finland; <http://www.thermo.com>). The supernatant containing the DNA was diluted with binding buffer (150 µl) and magnetic beads (20 µl). Once DNA was bound to the beads, two successive washing steps were  
35 carried out (Wash buffer 1: Agowa wash buffer 1 1/3, ethanol 1/3, isopropanol 1/3; Wash buffer 2: 70% ethanol, 30% Agowa wash buffer 2) and finally eluted in elution buffer (100 µl MQ, 0,025 µl Tween).

- 34 -

[0184] Grinding ten *S. lycopersicum* seeds produced enough DNA to saturate the magnetic beads, thus highly homogenous and comparable DNA concentrations of all samples were obtained. Comparing with lambda DNA references, a concentration of 30 ng/ $\mu$ l for each sample was estimated. Two times diluted DNA was 4 fold flat pooled. 2  $\mu$ l pooled DNA was used in multiplex PCRs for mutation  
5 detection analysis.

[0185] Primers used to amplify gene fragments for HRM were designed using a computer program (Primer3, <http://primer3.sourceforge.net/>). The length of the amplification product was limited between 200 and 400 base pairs. Quality of the primers was determined by a test PCR reaction that should yield a single product.

10 [0186] Polymerase Chain Reaction (PCR) to amplify gene fragments. 10ng of genomic DNA was mixed with 4 $\mu$ l reaction buffer (5x Reaction Buffer), 2 $\mu$ l 10xLC dye (LCGreen+ dye, Idaho Technology Inc., UT, USA), 5pmole of forward and reverse primers each, 4nmole dNTPs (Life Technologies, NY, USA) and 1 unit DNA polymerase (Hot Start II DNA Polymerase) in a total volume of 10 $\mu$ l. Reaction conditions were: 30s 98°C, then 40 cycles of 10s. 98°C, 15s 60°C, 25s of 72°C and  
15 finally 60s at 72°C.

[0187] High Resolution Melt curve analysis (HRM) has been proven to be sensitive and high-throughput methods in human and plant genetics. HRM is a non-enzymatic screening technique. During the PCR amplification dye (LCGreen+ dye, Idaho Technology Inc., UT, USA) molecules intercalate between each annealed base pair of the double stranded DNA molecule. When captured in the molecule,  
20 the dye emits fluorescence at 510 nm after excitation at 470 nm. A camera in a fluorescence detector (LightScanner, Idaho Technology Inc., UT, USA) records the fluorescence intensity while the DNA sample is progressively heated. At a temperature dependent on the sequence specific stability of the DNA helices, the double stranded PCR product starts to melt, releasing the dye. The release of dye results in decreased fluorescence that is recorded as a melting curve by the fluorescence detector. Pools  
25 containing a mutation form hetero duplexes in the post-PCR fragment mix. These are identified as differential melting temperature curves in comparison to homo duplexes.

[0188] The presence of the particular mutation in individual plants was confirmed repeating the HRM analysis on DNA from the individual M2 seed lots of the identified corresponding DNA pool. When the presence of the mutation, based on the HRM profile, was confirmed in one of the four  
30 individual M2 family DNA samples, the PCR fragments were sequenced to identify the mutation in the gene.

[0189] Once the mutation was known the effect of such an mutation was predicted using a computer program CODDLe (for Choosing codons to Optimize Discovery of Deleterious Lesions, <http://www.proweb.org/coddle/>) that identifies the region(s) of a user-selected gene and of its coding

- 35 -

sequence where the anticipated point mutations are most likely to result in deleterious effects on the gene's function.

[0190] Seeds from M2 families that contain mutations with predicted effect on protein activity were sown for phenotypic analysis of the plants.

5 [0191] Homozygous mutants were selected or obtained after selfing and subsequent selection. The effect of the mutation on the corresponding protein and phenotype of the plant was determined.

[0192] Seeds containing the different identified mutations were germinated and plants were grown in pots with soil the greenhouse with 16/8 light dark regime and 18°C night and 22-25°C day temperature. For each genotype 5 plants were raised. The second, third and fourth inflorescence were used for the analysis. The inflorescences were pruned leaving six flowers per inflorescence that were allowed to set fruit by self-pollination. The dates of fruit set of the first and sixth flower was recorded as was the date of breaker and red stage of the first and sixth fruit. At breaker of the sixth fruit the truss was harvested and stored in an open box in the greenhouse. Condition of the fruits was recorded during the whole ripening period.

15 [0193] At later stages fruit condition was determined based on visual assessment of the fruits and the date when the oldest fruit became 'bad' was recorded and further fruit deterioration was recorded (indicated by further fruit softness assessed by pinching the fruits, and visual assessment of dehydration/water loss, breaking of the skin and fungal growth).

[0194] The following mutants were identified: mutant 783, mutant 2145, mutant 2714, mutant 20 3793, mutant 4946, mutant 7871, and mutant 8185, and seeds were deposited at the NCIMB under the Accession numbers given above.

[0195] The mutations in the nucleotide sequence compared to the cDNA of wild type *Acs2* as depicted in SEQ ID NO 9, and its effect on the protein sequence of each mutant has been described above.

25 [0196] Plants comprising mutations in the target sequence, such as the above mutant plants or plants derived therefrom (e.g. by selfing or crossing) and comprising the mutant *acs2* allele, show a normal vegetative growth of all plant parts when compared to wild-type plants except for the ripening of the tomato fruits. The plants comprising mutations in the target sequence were screened phenotypically for their fruit ripening, ethylene production and shelf live.

30 EXAMPLE 2

Ripening behaviour of the *acs2* mutants

- 36 -

[0197] Seeds containing the different mutations were germinated and plants were grown in pots with soil the greenhouse with 16/8 light dark regime and 18 °C night and 22-25°C day temperature. For each genotype 5 plants were raised. The second, third and fourth inflorescence were used for the analysis. The inflorescences were pruned, leaving six flowers per inflorescence that were allowed to set fruit by self-pollination. The dates of fruit set of the first and sixth flower was recorded as was the date of breaker and red stage of the first and sixth fruit. At red stage of the 4<sup>th</sup> fruit the truss was harvested and stored in an open box in the greenhouse. Condition of the fruits was recorded during the whole ripening period by making pictures from each truss. After harvest pictures were made per box containing all trusses from one genotype.

10 [0198] At later stages fruit condition was determined based on visual assessment of the fruits and the date when the oldest fruit became 'bad' was recorded and further fruit deterioration was recorded (indicated by further fruit softness assessed by pinching the fruits, and visual assessment of dehydration/water loss, breaking of the skin and fungal growth).

15 [0199] The ripening behaviour of the fruits is shown in Figure 2. The day on which the first fruit of the wild type plant came into breaker stage was taken as day 1. The days thereafter were numbered as consecutive days. Mutants show a delay in ripening, i.e. fruits of the mutants require more days to become red and / or become "bad". Especially mutant 3793 and 2714 show a significant delay of several days. Mutant 3793 shows that it takes more time for this mutant to go from first fruit in breaker stage to 100 % fruit in red stage.

20 [0200] A characteristic of fruits of the plants of the invention is that breaker stage starts later (e.g. mutant 783, 2145, 2714, 3793). Post-harvest characteristics are shown below. The day on which the first fruit of the wild type plant came into breaker stage was taken as day 1. The days thereafter were numbered as consecutive days.

	First fruit in Breaker stage	All Fruits in Breaker stage	First Fruit in red stage	All fruits in red stage	First fruits in "bad" stage
Wt	1	25	4	29	42
783 Ho	8	18	14	22	49
2145 Ho	4	22	8	22	>51
2714 Ho	4	25	23	29	>51
3793 Ho	4	35	8	39	>51

25 As can be seen, mutant fruits enter breaker stage later. Equally, mutant fruits come into the red stage later and the date when the first fruits of a mutant line are in "bad" stage is also significantly later than for the wild type.

- 37 -

EXAMPLE 3

## Ethylene Release

[0201] Ethylene released by tomato fruits was measured in real-time with a laser-based ethylene detector (ETD-300, Sensor Sense B.V., Nijmegen, the Netherlands) in combination with a gas handling system (Cristescu et al., Laser-based systems for trace gas detection in life sciences. Appl Phys B 2008; 5 92 pp 343-9). Six glass cuvettes (100 mL volume) were used per experiment, one as a reference without plant material. Air was sampled from the lab and passed through a platinum based catalyzer (Sensor Sense B.V., Nijmegen, the Netherlands) to remove traces of ethylene or other hydrocarbons. Between the sample and the detector scrubbers with KOH and CaCl<sub>2</sub> were placed to reduce the CO<sub>2</sub> 10 concentration (to less than 1 ppm) and decrease the water content in the gas flow, respectively.

[0202] Comparison of the ethylene released from fruits of mutant 2145, 2714, 3793, 4946, 7871, and 8185 with wild type (Tapa) at pink stage and red stage (as shown in Figure 1) revealed that the ethylene production of all mutants had reduced compared to wild type (Tapa) in at least one of these stages. Tapa is a highly homozygous inbred parental line used in commercial processing tomato 15 (TPAADASU in Gady et al, 2012 Molecular Breeding 29 pp 801-812) and is homozygous for the wild type *Acs2* allele (*Acs2/Acs2*). Mutant 2145, 3793, and 4946 produced less ethylene compared to Tapa in both stages whereas mutant 2714 and 8185 only in pink, and 7871 only in red stage.

[0203] At pink stage mutant 2145 produced about 14% less ethylene than wild type, mutant 2714 produced about 5% less ethylene than wild type, mutants 3793, and 8185 produced between about 39 20 and about 47% less ethylene than wild type. Mutant 4946 produced about 80% less ethylene at pink stage compared to wild-type: < about 1.0 nl/(h · g) versus about 4.8 nl/(h · g) for the wild type. While in red stage mutants 2714 and 8185 produce about 14 and 12%, respectively more ethylene than wild type. Mutant 3793 produced at red stage about 8% less ethylene than wild type, mutants 7871, 2145, and 4946 produced about 29, 33, 40%, less ethylene than wild type, respectively. Wherein nl/(h · g) means nano 25 liter per hour per gram of fruit.

EXAMPLE 4

## Tomato Fruit firmness / compression test

[0204] Seeds of mutant 8185 lines were sown and 6 plants were grown from February to September in 5L pots under standard greenhouse conditions. Per plant three clusters of tomatoes were 30 selected and labeled. From each fruit cluster the 3rd and 4th tomato were selected for fruit development and softening analysis during the ripening process. In total six tomatoes per plant were used for the analysis. Date of the breaker, yellow/pink, and full red stage were noted for each tomato. Stages were defined based on United States Standards for Grade of Fresh Tomatoes (USDA; 1997, US department of Agriculture, Agricultural Marketing, Service, Washington, DC. Tomato ripening stage was determined

- 38 -

by the tomato color (RHS color chart). Mature green, 144B; Breaker, N144D; Orange, N163C/D; Red, 44A/B; Overripe (bad) N34A and 46A).

[0205] Tomatoes that entered the full red stage (day 0) were labeled and, either picked from the plant for analysis, or left on the plant to be analyzed at later time point. In the latter case fruits that remained on the plant were picked at day 3, 7, 10, 14 or 18 after full red for analysis. Thus 6 fruits were picked at each time point. Harvested tomatoes were stored at 22°C after measurements. Fruit firmness was measured with a texturometer (Compressor/load frame Instron, <http://www.instron.us>, System ID: 3342L2018; Force Transducer model 2519-104) controlled by a computer running Bluehill 3 program (Instron).

10 [0206] Firmness of tomatoes was measured according a method developed by Sirisomboon and Tanaka (Panmanas Sirisomboon, Munehiro Tanaka, Takayuki Kojima 2012 Evaluation of tomato textural mechanical properties. J Food Engineering 111, 618-624) adjusted to our preferences. The fruit was compressed between two steel plates (the upper one is the load cell plateau) moving with 1 mm per second generating an incremental force until 4 Newton was reached. This force was empirically determined as a high enough to generate quantifiable fruit compression without damaging the fruit tissue, also allowing repetitive measurements. Immediately thereafter the pressure was released to 0.1N. Then pressure was increased again until 4N was measured. The average deformation ( $D_{av}$ ) during the force increment from 0.1N to 4N was calculated from the two measurements ( $D_{av}/3.9$  (mm/N)). As fruits differ in size a deformation was calculated relative to the fruit diameter ( $D_{rel}=D_{av}/\text{Fruit diameter}$  (mm/(N·cm)). The firmness of a fruit was expressed as the force necessary to decrease the fruit diameter 1 mm per cm fruit (10%) (Firmness=1/ $D_{rel}$  (N)).

[0207] Day =0 equals to the day at which the fruits were harvested and measured for the first time. So, the same fruits were measured 4 times to obtain data points at 7, 7, 14 and 21 days. Fruit firmness measurement shows that mutant 8185 has a higher fruit firmness especially at about 7, 14, and / or 21 days where fruits of mutant 8185 require about 1 N more to decrease the fruit diameter 1mm per cm fruit (10%).

CLAIMS:

1. A cell of a cultivated plant of the species *Solanum lycopersicum* comprising an *acs2* allele having one or more mutations, said mutations resulting in production of a mutant *acs2* protein having loss-of-function or reduced function compared to a wild type *Acs2* protein,  
5 wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y in said wild type *Acs2* protein, and wherein said wild type *Acs2* protein is a protein having at least 85% amino acid sequence identity to the full length of SEQ ID NO: 1.
2. The cell of a cultivated plant according to claim 1, wherein said mutation or  
10 mutations result in reduced ethylene production and/or delayed fruit ripening and/or a longer shelf life compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele encoding said wild type *Acs2* protein.
3. The cell of a cultivated plant according to claim 1 or 2, wherein said mutation or  
15 mutations result in the tomato fruits requiring at least 1 more day to reach the red stage compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele encoding said wild type *Acs2* protein.
4. The cell of a cultivated plant according to any one of claims 1 to 3, wherein said  
20 mutation or mutations result in the tomato fruits requiring at least 1 more day to reach the bad stage compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele encoding said wild type *Acs2* protein, wherein a fruit is considered to reach the bad stage when said fruit is considered unsuitable for sale or consumption.
5. The cell of a cultivated plant according to any one of claims 1 to 4, wherein said  
25 mutation or mutations result in the tomato fruits of said plant having at least a 10 % reduced ethylene production compared to *Solanum lycopersicum* being homozygous for the wild type *Acs2* allele encoding said wild type *Acs2* protein.
6. The cell of a cultivated plant according to any one of claims 1 to 5, wherein the  
plant is an F1 hybrid plant.

7. The cell of a cultivated plant according to any one of claims 1 to 6, wherein the *acs2* allele having one or more mutations, is in homozygous form.

8. The cell of a cultivated plant according to any one of claims 1 to 7, wherein the plant additionally comprises an *acs4* allele having one or more mutations, said mutations in said  
5 *acs4* allele resulting in production of a mutant *acs4* protein having loss-of-function or reduced function compared to wild type *Acs4* protein and having at least 85% amino acid sequence identity to the *Acs4* amino acid sequence provided in GenBank Accession number M63490.1, and wherein said mutant *acs4* protein has one or more amino acids changed selected from the group consisting of A248V, S279N, L321F, V250E and T316I, or wherein said mutant *acs4*  
10 protein misses all amino acids 204 to 476 of the *Acs4* amino acid sequence provided in GenBank Accession number M63490.1.

9. The cell of a cultivated plant according to any one of claims 1 to 8, which is a seed cell.

10. A cell of a tomato fruit comprising a mutant *acs2* protein having loss-of-function  
15 or reduced function compared to a wild type *Acs2* protein, wherein said mutant *acs2* protein has one or more amino acids changed selected from the group consisting of A101T, A101V, A103T, G112R, P118L, V147E, and C265Y in said wild type *Acs2* protein, and wherein said wild type *Acs2* is a protein having at least 85% amino acid sequence identity to the full length of SEQ ID NO: 1.

20 11. The cell of a tomato fruit of claim 10, wherein the tomato fruit has reduced ethylene production and/or delayed ripening and/or an increased shelf life compared to fruits from *Solanum lycopersicum* plants being homozygous for the wild type *Acs2* allele encoding the wild type *Acs2* protein.

25 12. The cell of a tomato fruit according to claim 11, wherein the shelf life of the tomato fruit is at least 2 days longer than the shelf life of a tomato fruit being homozygous for the wild type *Acs2* allele.

13. The cell of a tomato fruit according to claim 10, wherein the tomato fruit has reduced ethylene production that is at least 10% reduced compared to fruits from *Solanum lycopersicum* plants being homozygous for the wild type *Acs2* allele encoding the wild type *Acs2* protein.

5 14. The cell of a cultivated plant according to claim 5 or the cell of a tomato fruit according to claim 13, wherein the reduced ethylene production is determined in the pink and/or red stage of the fruit.

1/7

Figure 1

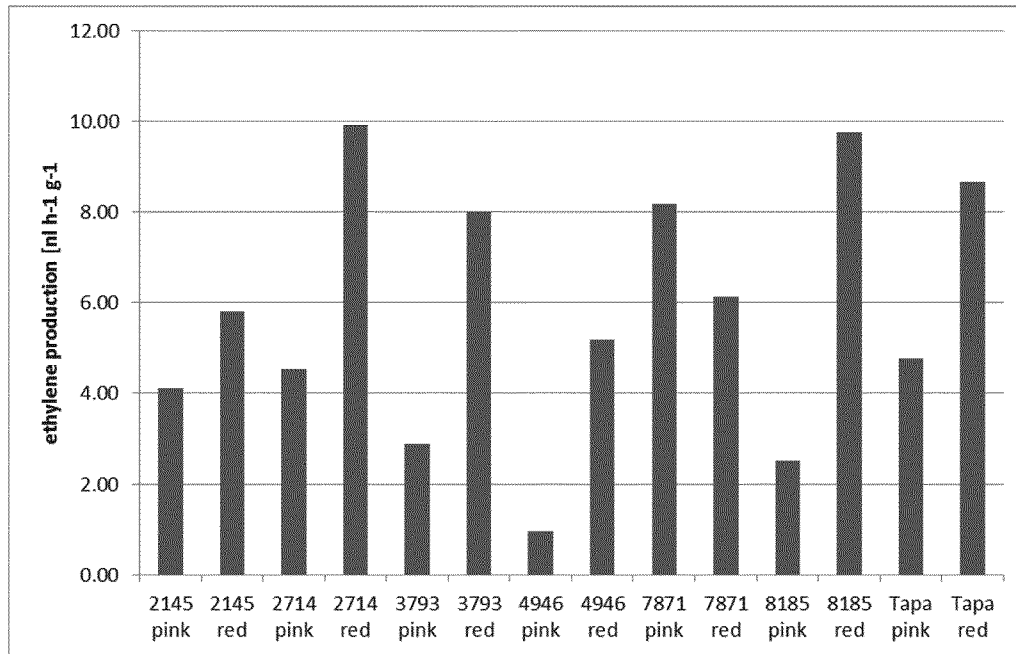


Figure 2

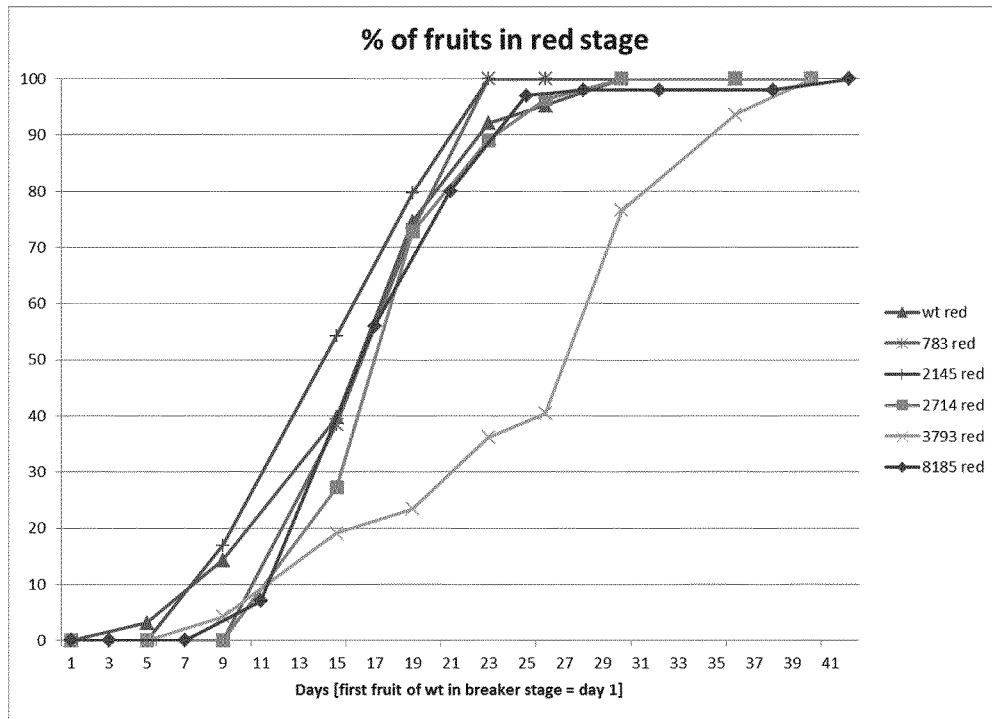


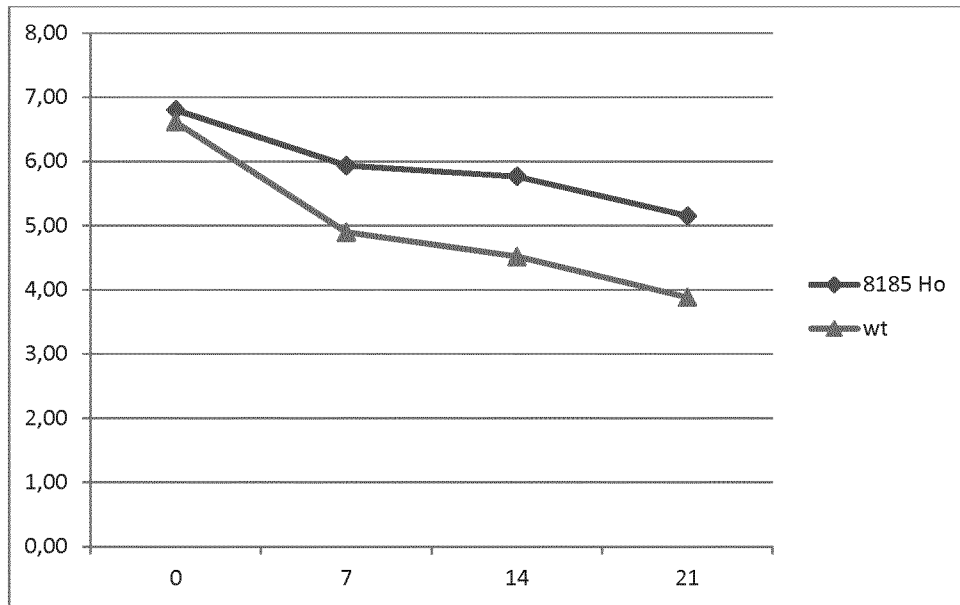
Figure 3

	Le-ACS2	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
	LeACS2_783_prot	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
5	LeACS2_2145_prot	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
	LeACS2_2714_prot	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
	LeACS2_3793_prot	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
	LeACS2_4946_prot	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
	LeACS2_7871_prot	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
10	LeACS2_8185_prot	MGFEIAKTNS	ILSKLATNEE	HGENSPYFDG	WKAYSDPPFH	PLKNPNGVIQ
	Le-ACS2	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
	LeACS2_783_prot	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
15	LeACS2_2145_prot	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
	LeACS2_2714_prot	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
	LeACS2_3793_prot	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
	LeACS2_4946_prot	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
	LeACS2_7871_prot	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
20	LeACS2_8185_prot	MGLAENQLCL	DLIEDWIKRN	PKGSICSEGI	KSFKAIANFQ	DYHGLPEFRK
	Le-ACS2	AIAKFMEKTR	GGRVRFDPER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
25	LeACS2_783_prot	AIAKFMEKTR	GGRVRFDPER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
	LeACS2_2145_prot	AIAKFMEKTR	GGRVRFDPER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
	LeACS2_2714_prot	AIAKFMEKTR	GGRVRFDLER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
	LeACS2_3793_prot	VIKAFMEKTR	GGRVRFDPER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
	LeACS2_4946_prot	TIKAFMEKTR	GGRVRFDPER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
30	LeACS2_7871_prot	AIKAFMEKTR	GGRVRFDPER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
	LeACS2_8185_prot	AIKAFMEKTR	GGRVRFDPER	VVMAGGATGA	NETIIFCLAD	PGDAFLVPSP
	Le-ACS2	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
35	LeACS2_783_prot	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
	LeACS2_2145_prot	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
	LeACS2_2714_prot	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
	LeACS2_3793_prot	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
40	LeACS2_4946_prot	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
	LeACS2_7871_prot	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
	LeACS2_8185_prot	YYPAFNRDLR	WRTGVQLIPI	HCESSNNFKI	TSKAVKEAYE	NAQKSNIKVK
	Le-ACS2	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
45	LeACS2_783_prot	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
	LeACS2_2145_prot	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
	LeACS2_2714_prot	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
50	LeACS2_3793_prot	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
	LeACS2_4946_prot	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
	LeACS2_7871_prot	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
	LeACS2_8185_prot	GLILTNPSNP	LGTTLDKDTL	KSVLSFTNQH	NIHLVCDEIY	AATVFDTQPQF
	Le-ACS2	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
55	LeACS2_783_prot	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
	LeACS2_2145_prot	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
60	LeACS2_2714_prot	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
	LeACS2_3793_prot	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
	LeACS2_4946_prot	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
	LeACS2_7871_prot	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
	LeACS2_8185_prot	VSAEILDEQ	EMTYCNKDLV	HIVYSLSKDM	GLPGFRVGI	YSFNDDVVNC
65	Le-ACS2	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL
	LeACS2_783_prot	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL

	LeACS2_2145_prot	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL
	LeACS2_2714_prot	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL
	LeACS2_3793_prot	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL
5	LeACS2_4946_prot	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL
	LeACS2_7871_prot	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL
	LeACS2_8185_prot	ARKMSSFGLV	STQTQYFLAA	MLSDEKFVDN	FLRESAMRLG	KRHKHFTNGL
10	Le-ACS2	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
	LeACS2_783_prot	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
	LeACS2_2145_prot	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
	LeACS2_2714_prot	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
	LeACS2_3793_prot	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
15	LeACS2_4946_prot	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
	LeACS2_7871_prot	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
	LeACS2_8185_prot	EVVGIKCLKN	NAGLFCWMDL	RPLLRESTFD	SEMSLWRVII	NDVKLNVSPG
20	Le-ACS2	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
	LeACS2_783_prot	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
	LeACS2_2145_prot	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
	LeACS2_2714_prot	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
25	LeACS2_3793_prot	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
	LeACS2_4946_prot	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
	LeACS2_7871_prot	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
	LeACS2_8185_prot	SSFECQEPGW	FRVCFANMDD	GTVDIALARI	RRFVGVEKSG	DKSSSMEQKQ
30	Le-ACS2	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	
	LeACS2_783_prot	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	
	LeACS2_2145_prot	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	
35	LeACS2_2714_prot	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	
	LeACS2_3793_prot	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	
	LeACS2_4946_prot	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	
	LeACS2_7871_prot	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	
40	LeACS2_8185_prot	QWKKNLRLS	FSKRMIDESV	LSPLSSPIPP	SPLVR	

5/7

Figure 4



5

6/7

Figure 5

				<b>Small domain</b>	
	ACS4_WT_ID1	MDLETSEISN	YKSSVVLSKL	ASNEQHGENS	PYFDGWKAYD NDPFHLVNNL 50
	ACS4_2477_ID2	MDLETSEISN	YKSSVVLSKL	ASNEQHGENS	PYFDGWKAYD NDPFHLVNNL
5	ACS4_4043_ID3	MDLETSEISN	YKSSVVLSKL	ASNEQHGENS	PYFDGWKAYD NDPFHLVNNL
	ACS4_4222_ID4	MDLETSEISN	YKSSVVLSKL	ASNEQHGENS	PYFDGWKAYD NDPFHLVNNL
	ACS4_4303_ID5	MDLETSEISN	YKSSVVLSKL	ASNEQHGENS	PYFDGWKAYD NDPFHLVNNL
	ACS4_4691_ID6	MDLETSEISN	YKSSVVLSKL	ASNEQHGENS	PYFDGWKAYD NDPFHLVNNL
10	ACS4_5251_ID7	MDLETSEISN	YKSSVVLSKL	ASNEQHGENS	PYFDGWKAYD NDPFHLVNNL
				<b>Large domain</b>	
	ACS4_WT_ID1	NGVIQMLAE	NQLSVDLIEE	WIKRNPKASI	CTNDGIESFR RIANFQDYHG 100
	ACS4_2477_ID2	NGVIQMLAE	NQLSVDLIEE	WIKRNPKASI	CTNDGIESFR RIANFQDYHG
	ACS4_4043_ID3	NGVIQMLAE	NQLSVDLIEE	WIKRNPKASI	CTNDGIESFR RIANFQDYHG
15	ACS4_4222_ID4	NGVIQMLAE	NQLSVDLIEE	WIKRNPKASI	CTNDGIESFR RIANFQDYHG
	ACS4_4303_ID5	NGVIQMLAE	NQLSVDLIEE	WIKRNPKASI	CTNDGIESFR RIANFQDYHG
	ACS4_4691_ID6	NGVIQMLAE	NQLSVDLIEE	WIKRNPKASI	CTNDGIESFR RIANFQDYHG
20	ACS4_5251_ID7	NGVIQMLAE	NQLSVDLIEE	WIKRNPKASI	CTNDGIESFR RIANFQDYHG
	ACS4_WT_ID1	LPEFTNAIAK	FMEKTRGGKV	KFDAKRVMMA	GGATGANETL ILCLADPGDA 150
	ACS4_2477_ID2	LPEFTNAIAK	FMEKTRGGKV	KFDAKRVMMA	GGATGANETL ILCLADPGDA
	ACS4_4043_ID3	LPEFTNAIAK	FMEKTRGGKV	KFDAKRVMMA	GGATGANETL ILCLADPGDA
25	ACS4_4222_ID4	LPEFTNAIAK	FMEKTRGGKV	KFDAKRVMMA	GGATGANETL ILCLADPGDA
	ACS4_4303_ID5	LPEFTNAIAK	FMEKTRGGKV	KFDAKRVMMA	GGATGANETL ILCLADPGDA
	ACS4_4691_ID6	LPEFTNAIAK	FMEKTRGGKV	KFDAKRVMMA	GGATGANETL ILCLADPGDA
30	ACS4_5251_ID7	LPEFTNAIAK	FMEKTRGGKV	KFDAKRVMMA	GGATGANETL ILCLADPGDA
	ACS4_WT_ID1	FLVPTPYYPG	FNRDLRWRSG	VQLLPISCKS	CNNFKITIEA IEEAYEKGQQ 200
	ACS4_2477_ID2	FLVPTPYYPG	FNRDLRWRSG	VQLLPISCKS	CNNFKITIEA IEEAYEKGQQ
	ACS4_4043_ID3	FLVPTPYYPG	FNRDLRWRSG	VQLLPISCKS	CNNFKITIEA IEEAYEKGQQ
35	ACS4_4222_ID4	FLVPTPYYPG	FNRDLRWRSG	VQLLPISCKS	CNNFKITIEA IEEAYEKGQQ
	ACS4_4303_ID5	FLVPTPYYPG	FNRDLRWRSG	VQLLPISCKS	CNNFKITIEA IEEAYEKGQQ
	ACS4_4691_ID6	FLVPTPYYPG	FNRDLRWRSG	VQLLPISCKS	CNNFKITIEA IEEAYEKGQQ
40	ACS4_5251_ID7	FLVPTPYYPG	FNRDLRWRSG	VQLLPISCKS	CNNFKITIEA IEEAYEKGQQ
	ACS4_WT_ID1	ANVKIKGLIL	TNPCNPLGTI	LDRDTLKKIS	TFTNEHNIHL VCDEIYAATV 250
	ACS4_2477_ID2	ANVKIKGLIL	TNPCNPLGTI	LDRDTLKKIS	TFTNEHNIHL VCDEIYAATV
	ACS4_4043_ID3	ANVKIKGLIL	TNPCNPLGTI	LDRDTLKKIS	TFTNEHNIHL VCDEIYAATV
	ACS4_4222_ID4	ANV*			
	ACS4_4303_ID5	ANVKIKGLIL	TNPCNPLGTI	LDRDTLKKIS	TFTNEHNIHL VCDEIYAATV
45	ACS4_4691_ID6	ANVKIKGLIL	TNPCNPLGTI	LDRDTLKKIS	TFTNEHNIHL VCDEIYAATE
	ACS4_5251_ID7	ANVKIKGLIL	TNPCNPLGTI	LDRDTLKKIS	TFTNEHNIHL VCDEIYAATV
	ACS4_WT_ID1	FNSPKFVSI	EIINEDNCIN	KDLVHIVSSL	SKDLGFPGFR VGIVYSFNDD 300
	ACS4_2477_ID2	FNSPKFVSI	EIINEDNCIN	KDLVHIVSNL	SKDLGFPGFR VGIVYSFNDD
50	ACS4_4043_ID3	FNSPKFVSI	EIINEDNCIN	KDLVHIVSSL	SKDLGFPGFR VGIVYSFNDD
	ACS4_4222_ID4	FNSPKFVSI	EIINEDNCIN	KDLVHIVSSL	SKDLGFPGFR VGIVYSFNDD
	ACS4_4303_ID5	FNSPKFVSI	EIINEDNCIN	KDLVHIVSSL	SKDLGFPGFR VGIVYSFNDD
	ACS4_4691_ID6	FNSPKFVSI	EIINEDNCIN	KDLVHIVSSL	SKDLGFPGFR VGIVYSFNDD
55	ACS4_5251_ID7	FNSPKFVSI	EIINEDNCIN	KDLVHIVSSL	SKDLGFPGFR VGIVYSFNDD
					<b>Small domain</b>
	ACS4_WT_ID1	VVNCARKMSS	FGLVSTQTQH	LLAFMLSDDDE	FVEEFLIESA KRLRERYEKF 350
	ACS4_2477_ID2	VVNCARKMSS	FGLVSTQTQH	LLAFMLSDDDE	FVEEFLIESA KRLRERYEKF
	ACS4_4043_ID3	VVNCARKMSS	FGLVSTQTQH	LLAFMLSDDDE	FVEEFLIESA KRLRERYEKF
60	ACS4_4222_ID4	VVNCARKMSS	FGLVSTQTQH	LLAFMLSDDDE	FVEEFLIESA KRLRERYEKF
	ACS4_4303_ID5	VVNCARKMSS	FGLVSTQTQH	FLAFMLSDDDE	FVEEFLIESA KRLRERYEKF
	ACS4_4691_ID6	VVNCARKMSS	FGLVSTQTQH	LLAFMLSDDDE	FVEEFLIESA KRLRERYEKF
65	ACS4_5251_ID7	VVNCARKMSS	FGLVSIQTQH	LLAFMLSDDDE	FVEEFLIESA KRLRERYEKF
	ACS4_WT_ID1	TRGLEEIGIK	CLESNAGVYC	WMDLRSLLKE	ATLDAEMSLW KLIINEVKLN 400
	ACS4_2477_ID2	TRGLEEIGIK	CLESNAGVYC	WMDLRSLLKE	ATLDAEMSLW KLIINEVKLN

	ACS4_4043_ID3	TRGLEEIGIK	CLESNAGVYC	WMDLRSLLKE	ATLDAEMSLW	KLIINEVKLN	
	ACS4_4222_ID4						
	ACS4_4303_ID5	TRGLEEIGIK	CLESNAGVYC	WMDLRSLLKE	ATLDAEMSLW	KLIINEVKLN	
5	ACS4_4691_ID6	TRGLEEIGIK	CLESNAGVYC	WMDLRSLLKE	ATLDAEMSLW	KLIINEVKLN	
	ACS4_5251_ID7	TRGLEEIGIK	CLESNAGVYC	WMDLRSLLKE	ATLDAEMSLW	KLIINEVKLN	
	ACS4_WT_ID1	VSPGSSFNC	EVGWFRVCFA	NIDDQTMEIA	LARIRMFMDA	YNNVNKNGVM	450
10	ACS4_2477_ID2	VSPGSSFNC	EVGWFRVCFA	NIDDQTMEIA	LARIRMFMDA	YNNVNKNGVM	
	ACS4_4043_ID3	VSPGSSFNC	EVGWFRVCFA	NIDDQTMEIA	LARIRMFMDA	YNNVNKNGVM	
	ACS4_4222_ID4						
	ACS4_4303_ID5	VSPGSSFNC	EVGWFRVCFA	NIDDQTMEIA	LARIRMFMDA	YNNVNKNGVM	
	ACS4_4691_ID6	VSPGSSFNC	EVGWFRVCFA	NIDDQTMEIA	LARIRMFMDA	YNNVNKNGVM	
15	ACS4_5251_ID7	VSPGSSFNC	EVGWFRVCFA	NIDDQTMEIA	LARIRMFMDA	YNNVNKNGVM	
	ACS4_WT_ID1	KNKHNGRGT	YDLTPQMGST	MKMLLA			
	ACS4_2477_ID2	KNKHNGRGT	YDLTPQMGST	MKMLLA			
20	ACS4_4043_ID3	KNKHNGRGT	YDLTPQMGST	MKMLLA			
	ACS4_4222_ID4						
	ACS4_4303_ID5	KNKHNGRGT	YDLTPQMGST	MKMLLA			
	ACS4_4691_ID6	KNKHNGRGT	YDLTPQMGST	MKMLLA			
	ACS4_5251_ID7	KNKHNGRGT	YDLTPQMGST	MKMLLA			
25							

