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(71) Applicant(s)
**Keygene N.V.**

(72) Inventor(s)
**Op Den Camp, Rik Hubertus Martinus;Van Dijk, Peter Johannes;Gallard, Anthony**

(74) Agent / Attorney
**FPA Patent Attorneys Pty Ltd, Level 43 101 Collins Street, Melbourne, VIC, 3000, AU**

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(71) Applicant: **KEYGENE N.V.** [NL/NL]; P.O. Box 216, 6700 AE Wageningen (NL).

(72) Inventors: **OP DEN CAMP, Rik Hubertus Martinus**; c/o P.O. Box 216, 6700 AE Wageningen (NL). **VAN DIJK, Peter Johannes**; c/o P.O. Box 216, 6700 AE Wageningen (NL). **GALLARD, Anthony**; c/o P.O. Box 216, 6700 AE Wageningen (NL).

(74) Agent: **KINGMA, R.L.**; P.O. Box 3241, 2280 GE Rijswijk (NL).

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(54) Title: METHOD FOR THE PRODUCTION OF HAPLOID AND SUBSEQUENT DOUBLED HAPLOID PLANTS

(57) Abstract: It was found that plants comprising modified CENPC protein comprising one or more active mutations which affect the functioning of CENPC protein yet allow plants expressing said modified CENPC protein to be viable, are able to induce haploid offspring after a cross to or with a wild type plant comprising a endogenous CENPC protein. The invention relates to generation of haploid and doubled haploid plants.

Title: Method for the production of haploid and subsequent doubled haploid plants

Field of the invention

The disclosure relates to the field of agriculture. In particular, the disclosure relates to the production of haploid and subsequent doubled haploid plants.

Background of the invention

Reference to any prior art in the specification is not an acknowledgement or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be combined with any other piece of prior art by a skilled person in the art.

A high degree of heterozygosity in breeding material can make plant breeding and selection for beneficial traits a very time consuming process. Extensive population screening, even with the latest molecular breeding tools, is both laborious and costly.

The creation of haploid plants followed by chemical or spontaneous genome doubling is an efficient way to solve the problem of high heterozygosity. Such doubled haploids bypass at least 7 generations of selfing otherwise needed to reduce the heterozygosity to an acceptable level. Haploid plants can be produced in some crops by microspore culture. However, this is costly and time-consuming. More importantly, in many crops microspore culture methods do not work. In some crop species, (doubled) haploid plants can be obtained by parthenogenesis of the egg cell or by elimination of one of the parental genomes. However, these methods are also restricted to a few selected crops and the production rates of doubled haploid plants are low.

WO2011/044132 discloses methods of producing haploid plants. One of the methods employed is inactivating or knocking out CenH3 protein. This was done by adding an N-terminal GFP to the CenH3 protein, thereby creating GFP-CenH3. This is also called a "tailswap". The tailswap was sufficient to induce uni-parental genome elimination upon a cross to a plant without such modified N-terminal part of the CenH3 protein. The uni-parental genome elimination resulted in the production of a haploid plant. So far this process has only been demonstrated in the model plant *Arabidopsis thaliana* and not in crop plants. Additionally, when another artificial construct, which consisted of a different trans-genetically modified N-terminal part of the CenH3 protein, was introduced in a plant with a genetic background lacking the endogenous CenH3, it appeared that this did not resulted in uni-parental genome elimination and subsequent production of a haploid plant (WO 2014/110274). Therefore it remains elusive which modifications of the CenH3 protein are sufficient for uni-parental genome elimination.

Thus, there remains a need in the art for methods that allow efficient generation of haploid plants which can subsequently be doubled, to produce doubled haploid plants. With doubled haploid production systems, homozygosity is achieved in one generation.

Summary of the invention

The present inventors have now found that plants with modified CENPC protein, due to unique single nucleotide polymorphisms, are both able to induce haploid offspring after a cross to or with a wild type plant lacking these particular unique single nucleotide polymorphisms. One polymorphism comprised four nucleotide changes in a single plant, resulting in; (1) a G to T nucleotide modification of a splice acceptor site of the CENPC genomic DNA sequence which leads to the insertion of an H amino acid in the CENPC protein, (2) a D to K amino acid modification in the CENPC protein and (3) a N to Y amino acid modification in the CENPC protein. The other unique single nucleotide polymorphism resulted in a M to V amino acid modification in the CENPC protein. Reciprocal crosses with control plants, never yielded any haploid offspring.

The invention relates to a CENPC protein of plant origin comprising one or more active mutations. The one or more active mutations may be present in a protein comprising the amino acid sequence of any of SEQ ID NO: 2, 3, 4 or 15-19. The one or more active mutations may be made in a protein comprising the amino acid sequence of any of SEQ ID NO:2, 3, 4 or 15-19, or a variant thereof having at least 70%, more preferably at least 80%, even more preferably at least 90%, yet even more preferably at least 95%, most preferably at least 98% or 99% sequence identity to the amino acid sequence of any of SEQ ID NO:2, 3, 4 or 15-19.

In an embodiment, the one or more active mutations are present in a doubled haploid inducer domain represented by the amino acid sequences as depicted in any of SEQ ID NO: 5 or 6.

Said one or more active mutations may be chosen from the group consisting of a mutation between amino acid residues at position 552 and 553, a mutation of an amino acid residue at position 554, a mutation of an amino acid residue at position 555 and a mutation of an amino acid residue at position 556, or any combination thereof, in the amino acid sequence of any of SEQ ID NO: 3 or 4; or of a mutation between amino acid residues at position 555 and 556, a mutation of an amino acid residue at position 557, a mutation of an amino acid residue at position 558 and a mutation of an amino acid residue at position 559, or any combination thereof, in the amino acid sequence of SEQ ID NO: 2.

In an embodiment, the amino acid that is mutated at position 554 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is aspartic acid and/or the amino acid that is changed at position 555 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 558 in the amino acid sequence of SEQ ID NO: 2, is asparagine and/or the amino acid that is changed at position 556 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 559 in the amino acid sequence of SEQ ID NO: 2, is methionine.

In an embodiment, the amino acid at position 554 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is changed into a positively charged amino acid residue, preferably into lysine, and/or the amino acid at position 555 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 558 in the amino acid sequence of SEQ ID NO: 2, is changed into a tyrosine and/or the amino acid at position 556 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is changed into valine, and/or wherein a positively charged residue, preferably histidine, is inserted between amino acid residues 552 and 553 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or between amino acid residues 555 and 556 in the amino acid sequence of SEQ ID NO: 2.

In an embodiment, a histidine is inserted between amino acid residues 552 and 553 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or between amino acid residues 555 and 556 in the amino acid sequence of SEQ ID NO: 2, and/or an aspartic acid at position 554 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is changed into a lysine and/or an asparagine at position 555 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 558 in the amino acid sequence of SEQ ID NO: 2, is changed into a tyrosine.

In an embodiment, said protein comprises the amino acid sequence of SEQ ID NO:8 or of SEQ ID NO:10.

Said protein may be encoded by a polynucleotide comprising the nucleic acid sequence of any of SEQ ID NO:7 or 12, or said protein may be encoded by a polynucleotide comprising the nucleic acid sequence of any of SEQ ID NO:9 or 13.

In an embodiment, said protein is encoded by a CENPC protein-encoding polynucleotide having an active mutation, which, when present in a plant in the absence of its endogenous CENPC protein-encoding polynucleotide and/or endogenous CENPC protein, allows said plant to be viable, and allows generation of some haploid progeny, or progeny with aberrant ploidy, when said plant is crossed with a wild-type plant.

Preferably, at least 0.1, 0.5, 1 or 5% of the progeny generated is haploid or has an aberrant ploidy.

Said protein may be derived from an endogenous CENPC protein by introducing mutations in the polynucleotide encoding said endogenous CENPC protein using targeted nucleotide exchange or by applying an endonuclease.

In an embodiment, the one or more active mutations are not present in a protein domain comprising the amino acid sequence depicted in SEQ ID NO:1.

In a further aspect, the present invention pertains to a polynucleotide encoding the CENPC protein as taught herein.

The invention also relates to a polynucleotide comprising the nucleic acid sequence of any of SEQ ID NO:11 or 14, or a variant thereof having at least 70%, more preferably at least 80%, even more preferably at least 90%, yet even more preferably at least 95%, most preferably at least 98% or 99% sequence identity to the amino acid sequence of any of SEQ ID NO:11 or 14, but in which one or more nucleotides at positions 2449-2450 and/or 2454-2462 of the nucleic acid sequence of SEQ ID NO:11 or at positions 1660-1668 in SEQ ID NO:14 are modified such that the nucleic acid encodes a CENPC protein in which the amino acid sequence of SEQ ID NO:3 has an altered residue at position 554 and/or at position 555 and/or at position 556, and/or has an insertion of an amino acid residue, such as a histidine between the amino acid residues 552 and 553 of the amino acid sequence of SEQ ID NO:3.

Additionally, a polynucleotide comprising the nucleic acid sequence of any of SEQ ID NO:7 or 12 is taught herein.

Also, a polynucleotide comprising the nucleic acid sequence of any of SEQ ID NO: 9 or 13 is taught herein.

The polynucleotide taught herein may be isolated.

The invention is further concerned with a chimeric gene comprising the polynucleotide as taught herein, a vector comprising the polynucleotide or the chimeric gene as taught herein, and a host cell comprising a polynucleotide as taught herein, a chimeric gene as taught herein, or a vector as taught herein. The host cell may be a plant cell, preferably a tomato plant cell.

The invention also provides a plant comprising a polynucleotide as taught herein, a chimeric gene as taught herein, or a vector as taught herein.

In an embodiment, the endogenous CENPC protein is not expressed in said plant.

The plant may be a *Solanum* plant, preferably a *Solanum lycopersicum* plant.

The invention further relates to a method for making a plant as taught herein, said method comprising the steps of:

a) modifying an endogenous plant CENPC protein-encoding polynucleotide within a plant cell to obtain an plant CENPC protein-encoding polynucleotide having an active mutation;

b) selecting a plant cell comprising the plant CENPC protein-encoding polynucleotide having an active mutation; and

c) optionally, regenerating a plant from said plant cell.

Also, the invention relates to a method for making a plant as taught herein, said method comprising the steps of:

a) transforming a plant cell with a polynucleotide as taught herein, a chimeric gene as taught herein, or a vector as taught herein;

b) selecting a plant cell comprising said polynucleotide, chimeric gene or vector; and

c) optionally, regenerating a plant from said plant cell.

Said method mat further comprise the step of modifying said plant cell to prevent expression of endogenous CENPC protein.

An endogenous plant CENPC protein-encoding polynucleotide within said plant cell may be modified to prevent expression of endogenous CENPC protein.

The invention further pertains to a method of generating a haploid plant, or a plant with aberrant ploidy, said method comprising the steps of:

a) crossing a plant expressing an endogenous plant CENPC protein to the plant as taught herein, wherein the plant as taught herein does not express an endogenous CENPC protein at least in its reproductive parts and/or during embryonic development;

b) harvesting seed;

c) growing at least one seedling, plantlet or plant from said seed; and

d) selecting a haploid seedling, plantlet or plant; a seedling, plantlet or plant with aberrant ploidy; or a doubled haploid seedling, plantlet or plant.

The disclosure further teaches a method of generating a doubled haploid plant, said method comprising the steps of converting the haploid plant obtained in step d) into a doubled haploid plant.

The conversion may be performed by treatment with colchicine.

In an embodiment, said plant expressing an endogenous plant CENPC protein is an F1 plant.

The plant expressing an endogenous CENPC protein may be a pollen parent of the cross, or may be an ovule parent of the cross.

In an embodiment, the cross is performed at a temperature in the range of about 24 to about 30°C.

In an embodiment, the methods taught herein do not comprise sexually crossing the whole genomes of said plants.

The polynucleotide taught herein may be used for producing a haploid inducer line.

A *Solanum lycopersicum* plant comprising the polynucleotide as taught herein, a chimeric gene as taught herein, or a vector as taught herein, is also provided.

Additionally, a *Solanum lycopersicum* plant comprising a polynucleotide encoding a protein comprising the amino acid sequence of any SEQ ID NO:8 or 10 is provided.

Further, a *Solanum lycopersicum* plant comprising a polynucleotide comprising the nucleic acid sequence of any of SEQ ID NO:7, 12, 9, or 13 is taught herein.

The invention further provides a *Solanum lycopersicum* plant comprising a polynucleotide that encodes a CENPC protein as taught herein.

A *Solanum lycopersicum* plant comprising one or more active mutations in the polypeptide comprising the amino acid sequence of SEQ ID NO:4, is also provided.

The *Solanum lycopersicum* plants as taught herein may be used for producing a haploid *Solanum lycopersicum* plant, and/or for producing a doubled haploid *Solanum lycopersicum* plant.

In an embodiment, the *Solanum lycopersicum* plant as taught herein does not express an endogenous CENPC protein at least in its reproductive parts and/or during embryonic development.

In a final aspect, the present invention is concerned with a method of generating a haploid or doubled haploid plant, said method comprising identifying a plant expressing an endogenous CENPC protein and a plant as taught herein, wherein the plant as taught herein does not express an endogenous CENPC protein.

In an embodiment, said method does not comprise sexually crossing the whole genomes of said plants.

Definitions

The abbreviation "CENPC" or "CENP-C" denotes Centromere Protein C. Centromere Protein C is characterized by a CENPC motif, which is a protein sequence defining a CENPC protein from any organism. The CENPC motif is highly conserved among animals, yeast and plants (Talbert et al 2004 Journal of biology 3(4),18). The consensus CENPC motif from human; mouse; cow; chicken; Caenorhabditis elegans; budding yeast; Schizosaccharomyces pombe; Physcomitrella patens; maize; rice; Arabidopsis thaliana; black cottonwood, soybean and tomato is provided as SEQ ID NO:1.

A "mutation" is a permanent change of the nucleotide sequence of the genome of an organism, virus, or extrachromosomal DNA or other genetic elements. Mutations result from damage to DNA which is not repaired or to RNA genomes (typically caused by radiation or chemical mutagens), errors in the process of replication, or from the insertion or deletion of segments of DNA by mobile genetic elements. Mutations may or may not produce discernible changes in the observable characteristics (phenotype) of an organism. A mutation can result in several different types of change in sequences. Mutations in genes can either have no effect, alter the product of a gene, or prevent the gene from functioning properly or completely. Mutations can also occur in nongenic regions.

An "CENPC-encoding polynucleotide having an active mutation" refers to a non-endogenous, mutated CENPC-encoding polynucleotide that encodes a CENPC protein having an active mutation, which, when present in a plant in the absence of its endogenous

CENPC-encoding polynucleotide and/or endogenous CENPC protein, allows said plant to be viable, and allows generation of some haploid progeny, or progeny with aberrant ploidy, when said plant is crossed with a wild-type plant, preferably a wild-type plant of the same species. The plant comprising a CENPC-encoding polynucleotide having an active mutation may be referred to as a "modified plant". The percentage of haploid progeny or progeny with aberrant ploidy that is generated upon crossing with a wild-type plant can, for instance, be at least 0.1, 0.5, 1, 5, 10, 20% or more. A mutation that causes a transition from the endogenous CENPC-encoding polynucleotide to a CENPC-encoding polynucleotide having an active mutation is herein referred to as an "active mutation". An active mutation in a CENPC protein context may result, among other things, in reduced centromere loading, a less functional CENPC protein and/or a reduced functionality in the separation of chromosomes during cell division. An active mutation may be introduced into the CENPC-encoding polynucleotide by any of several methods well-known to the skilled person, for example, by random mutagenesis, such as induced by treatment of seeds or plant cells with chemicals or radiation, targeted mutagenesis, the application of endonucleases, by generation of partial or complete protein domain deletions, or by fusion with heterologous sequences. Alternatively, several of these technologies may be used to introduce one or more active mutations.

A "CENPC protein having an active mutation" is encoded by a CENPC-encoding polynucleotide having an active mutation. The endogenous CENPC-encoding polynucleotide encodes the endogenous CENPC protein.

A plant may be made to lack the endogenous CENPC-encoding polynucleotide by knocking out or inactivating said endogenous CENPC-encoding polynucleotide. Alternatively, said endogenous CENPC-encoding polynucleotide may be modified to encode an inactive or non-functional CENPC protein.

The modified plant comprising the CENPC-encoding polynucleotide having an active mutation taught herein may be crossed to a wild-type plant either as a pollen parent or as an ovule parent. In an embodiment, a CENPC protein having an active mutation may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20 or more amino acid changes relative to the endogenous CENPC protein. In an embodiment, a CENPC-encoding polynucleotide having an active mutation has 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 99.5% sequence identity to the endogenous CENPC-encoding polynucleotide, preferably over the full length.

The skilled person would readily be able to ascertain whether or not a modified plant as taught herein comprises an active mutation. For example, the skilled person may make use of predictive tools such as SIFT (Kumar P, Henikoff S, Ng PC. (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc; 4(7):1073-81. doi:10.1038/nprot.2009.86) to propose such active mutation. The active mutation may then be made in a plant, and expression of endogenous CENPC protein in said

plant should be knocked out. The plant may be considered to comprise an active mutation when the percentage of haploid progeny or progeny with aberrant ploidy that is generated upon crossing with a wild-type plant is at least 0.1, 0.5, 1, 5, 10, 20% or more.

Crossing a plant that lacks an endogenous CENPC-encoding polynucleotide or that lacks expression of endogenous CENPC protein and that expresses a CENPC protein having an active mutation either as a pollen or as an ovule parent with a plant that expresses an endogenous CENPC protein results in a certain percentage (for instance at least 0.1, 0.5, 1, 5, 10, 20% or more) of progeny that is haploid or shows aberrant ploidy. Such a plant comprises only chromosomes of the parent that expresses the endogenous CENPC protein, and no chromosomes of the plant expressing the CENPC protein having an active mutation.

Two plants that are crossed have to be sexually compatible. They may be of the same genus or of the same species.

The term "endogenous" as used in the context of the present invention in combination with protein or gene means that said protein or gene originates from the plant in which it is still contained. Often an endogenous gene will be present in its normal genetic context in the plant.

The term "haploid inducer line" used in the context of the present disclosure refers to a plant line which differs in at least one single nucleotide polymorphism from the non-inducer line. When an haploid inducer line is crossed, either used as female or as pollen donor, it results in uni-parental genome elimination of the haploid inducer line's genome.

The term "uni-parental genome elimination" as used herein refers to the effect of losing all the genetic information, meaning all chromosomes, of one parent after a cross irrespective of the direction of the cross. This occurs in such way that the offspring of such cross will only contain chromosomes of the non-eliminated parental genome. The genome which is eliminated always has the origin in the haploid inducer parent.

A "doubled haploid" is a genotype formed when haploid cells undergo chromosome doubling. It may be produced by induced or spontaneous chromosome doubling from haploid cells. For diploid plants, the haploid cells are monoploid, and the term "doubled monoploid" may also be used for the doubled haploids.

The "Solanaceae CENPC DH-inducer domain protein sequence" defines a specific region of the CENPC protein from a species belonging to the Solanaceae plant family that is highly conserved among Solanaceae species. It represents the consensus protein sequence of the CENPC DH-inducer domain of Solanum lycopersicum, Nicotiana tabacum, Nicotiana tomentosiformis, Capsicum annuum, Solanum tuberosum and Solanum frutescence, and its amino acid sequence is shown in SEQ ID NO:5.

The "Solanum CENPC DH-inducer domain protein sequence" defines a specific region of the CENPC protein from species belonging to the Solanum plant genus. It is highly

conserved among Solanum species. The amino acid sequence of the consensus protein sequence of the CENPC DH-inducer domain of *Solanum lycopersicum, Solanum pimpinellifolium, Solanum peruvianum, Solanum chiemliewskii, Solanum cheesmaniae, Solanum neorickii, Solanum Arcanum, Solanum peruvianum, Solanum huaylasense, Solanum chilense, Solanum habrochaites, Solanum pennellii, Solanum galapagense and Solanum tuberosum* is shown as SEQ ID NO:6.

The term "indeterminate" as used herein refers to the type of growth habit of tomato plants, which is commonly classified as determinate or indeterminate. This classification preferably depends on the capacity of the shoot system for continued sympodial growth. The terms are used in their art-recognized meaning.

The terms "polynucleotide" and "nucleic acid" are used interchangeably herein.

A "chimeric gene" (or recombinant gene) refers to any gene, which is not normally found in nature in a species, in particular a gene in which one or more parts of the nucleic acid sequence are present that are not associated with each other in nature. For example the promoter is not associated in nature with part or all of the transcribed region or with another regulatory region. The term "chimeric gene" is understood to include expression constructs in which a promoter or transcription regulatory sequence is operably linked to one or more coding sequences or to an antisense (reverse complement of the sense strand) or inverted repeat sequence (sense and antisense, whereby the RNA transcript forms double stranded RNA upon transcription).

"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 referred to as "substantially identical" or "essentially similar" when they (when optimally aligned by for example the programs GAP or BESTFIT using default parameters) share at least a certain minimal percentage of sequence identity (as defined below). GAP uses 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 GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For nucleotides the default scoring matrix used is nwsgapdna and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 915-919). Sequence alignments and scores for percentage sequence identity may be determined using computer programs, such as the GCG Wisconsin Package, Version 10.3, available from Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752 USA, or EmbossWin version 2.10.0 (using the program "needle"). Alternatively percent similarity or identity may be determined by searching against databases, using algorithms such as FASTA, BLAST, etc.

A "host cell" or a "recombinant host cell" or “transformed cell” are terms referring to a new individual cell (or organism) arising as a result of introduction of at least one nucleic acid molecule, especially comprising a chimeric gene encoding a desired protein. The host cell is preferably a plant cell or a bacterial cell. The host cell may contain the nucleic acid molecule or chimeric gene as an extra-chromosomally (episomal) replicating molecule, or more preferably, comprises the nucleic acid molecule or chimeric gene integrated in the nuclear or plastid genome of the host cell.

As used herein, the term “plant” includes plant cells, plant tissues or organs, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant cell clumps, and plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, fruit (e.g. harvested tomatoes), flowers, leaves, seeds, roots, root tips and the like.

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. It encompasses the verbs “to essentially consist of” and “to consist of”.

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.

Detailed description of the invention

The present inventor found that elimination of an endogenous CENPC in combination with expression of a non-endogenous CENPC protein having an active mutation in a plant resulted in a plant that has useful properties for breeding. It was found that such plant can function as a haploid inducer line. When such haploid inducer line is crossed with a plant having an endogenous CENPC protein, a portion of the resulting progeny lacks the chromosomes derived from the hapoid inducer line, thereby allowing the production of haploid progeny or progeny with aberrant ploidy. Haploid plants are useful for improving breeding.

Equal distribution of DNA in mitosis requires the assembly of a large proteinaceous ensemble onto the centromeric DNA, called the kinetochore. Kinetochores are multisubunit complexes that assemble on centromeres to bind spindle microtubules and promote faithful chromosome segregation during cell division. A 16-subunit complex named the constitutive centromere-associated network (CCAN) creates the centromere-kinetochore interface.

CENPC, a CCAN subunit, is crucial for kinetochore assembly because it links centromeres with the microtubule-binding interface of kinetochores. The exact role of CENPC in CCAN organization is not yet fully understood, but certain data point to CENPC as a blueprint for kinetochore assembly. When CENPC is depleted, the proper formation of both centromeres and kinetochores is prevented.

CENPC proteins having an active mutation

The present invention provides a CENPC protein having an active mutation. Such CENPC protein having an active mutation comprises one or more active mutations. When a plant that expresses such CENPC protein having an active mutation and lacks expression of, or has suppressed expression of, endogenous CENPC protein, is crossed to a wild type plant expressing endogenous CENPC protein, haploid plants are formed at relatively high frequency. CENPC proteins having an active mutation can be created by a variety of means known to the skilled person. These include, without limitation, random mutagenesis, single or multiple amino acid targeted mutagenesis, generation of complete or partial protein domain deletions, fusion with heterologous amino acid sequences, and the like. Typically, the polynucleotide encoding endogenous CENPC protein will be knocked out or inactivated. Haploid plants are formed at a more than normal frequency, such as at least 0.1, 0.5, 1, 5, 10, 20% or more. CENPC proteins having an active mutation can, for example, be tested by recombinant expression of the CENPC protein having an active mutation in a plant lacking endogenous CENPC protein, crossing the transgenic plant to a plant expressing endogenous CENPC protein, and then screening for the production of haploid progeny.

Any number of mutations can be introduced into an endogenous CENPC protein to generate a CENPC protein having an active mutation. For example, the CENPC protein having an active mutation may be identical to the endogenous CENPC protein but for 1, 2, 3, 4, 5, 6, 7, 8, or more amino acids.

The active mutation preferably is not present in the CENPC motif.

The CENPC protein is preferably a plant CENPC protein. The plant may be any plant, but preferably belongs to the Solanaceae family, more preferably to the genus Solanum, even more preferably to the species Solanum lycopersicum.

In an embodiment, the one or more active mutations are made in the endogenous CENPC protein as represented by an amino acid sequence as shown in any of SEQ ID NO: 2, 3, 4, or 15-19, or a variant thereof having at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more, such as 100%, amino acid sequence identity to the amino acid sequence of any of SEQ ID NO: 2, 3, 4, or 15-19, preferably over the entire length. Amino acid sequence identity is determined by pairwise alignment using the Needleman and Wunsch algorithm and GAP default parameters as defined above.

The active mutation within the CENPC protein having an active mutation may be located throughout the protein. In an embodiment, the active mutation is located between amino acid residue Glu548 and Pro562 of the endogenous CENPC protein as represented by an amino acid sequence as shown in any of SEQ ID NO: 3 or 4, or a variant of the CENPC protein having an amino acid sequence as shown in SEQ ID NO: 3 or 4 as taught herein.

The active mutation within the CENPC protein having an active mutation may be located between amino acid residues Glu551 and His565 of SEQ ID NO:2, or a variant of the CENPC protein having an amino acid sequence as shown in SEQ ID NO: 2 as taught herein.

In an embodiment, one or more of the amino acid residues on positions corresponding to amino acid residues 552, 553, 554, 555, or 556 of the endogenous Solanum lycopersicum CENPC protein (the amino acid sequence of which is shown in SEQ ID NO:4) are mutated. For example, aspartic acid on position 554, asparagine on position 555, or the methionine on position 556 of SEQ ID NO:4, or a variant of the CENPC protein having an amino acid sequence as shown in SEQ ID NO: 4 as taught herein, or the corresponding amino acid residue in any of SEQ ID NO: 2 or 3, may be mutated.

In SEQ ID NO:2 or variants thereof as taught herein, Asp557 corresponds to Asp554 in the amino acid sequence of SEQ ID NO:4, Asn 558 corresponds to Asn555 in the amino acid sequence of SEQ ID NO:4, and Met559 corresponds to Met556 in the amino acid sequence of SEQ ID NO:4. In SEQ ID NO:3 or variants thereof as taught herein, Asp554 corresponds to Asp554 in the amino acid sequence of SEQ ID NO:4, Asn 555 corresponds to Asn555 in the amino acid sequence of SEQ ID NO:4, and Met556 corresponds to Met556 in the amino acid sequence of SEQ ID NO:4.

The amino acid residue on position 554 of the amino acid sequence of SEQ ID NO:4 or a variant thereof, or the amino acid residue corresponding to this amino acid residue in any of SEQ ID NO: 2 or 3 or variants thereof as taught herein, e.g., aspartic acid, may, for example, be mutated into a positively charged amino acid residue such as histidine, lysine, or arginine, preferably into lysine. The amino acid residue on position 555 of the amino acid sequence of SEQ ID NO:4 or a variant thereof, or the amino acid residue corresponding to this amino acid residue in any of SEQ ID NO: 2 or 3 or variants thereof as taught herein, e.g., asparagine, may, for example, be mutated into an aromatic amino acid residue such as phenylalanine, tryptophan, or tyrosine, preferably into tyrosine. The amino acid residue at position 556 of the amino acid sequence of SEQ ID NO:4 or a variant thereof, or the amino acid residue corresponding to this amino acid residue in any of SEQ ID NO: 2 or 3 or variant thereof as taught herein, e.g., methionine, may, for example, be mutated into an amino acid residue with a small side chain such as glycine, serine, cysteine, alanine, threonine, or valine, preferably into valine.

In an embodiment, an amino acid residue may be inserted in the domain between amino acid residues Glu548 and Pro562, particularly between amino acid residues 552 and 553, of the endogenous CENPC protein as represented by an amino acid sequence as shown in any of SEQ ID NO: 3 or 4, or a variant thereof as taught herein, or between amino acid residues Glu561 and Pro565 of the CENPC protein as represented by an amino acid sequence as shown in SEQ ID NO:2, or a variant thereof as taught herein.

For example, a positively charged residue such as a His residue may be inserted between amino acid residues 552 and 553 of the amino acid sequence as shown in SEQ ID NO: 3 or 4, or a variant thereof as taught herein, or between amino acid residues 555 and 556 of the amino acid sequence depicted in SEQ ID NO:2, or a variant thereof as taught herein.

In an embodiment, the amino acid residue inserted between amino acid residues 552 and 553 of the amino acid sequence as shown in SEQ ID NO: 4 is not a glutamine residue.

In an embodiment, the active mutations that are made in the endogenous CENPC protein having the amino acid sequence of any of SEQ ID NO:3 or 4, or a variant thereof, to yield a CENPC protein having an active mutation are selected from the group consisting of insertion of His between amino acid residues 552 and 553 (552_553insH), and the mutations D554K, N555Y, M556V, or any combinations thereof, such as 552_553insH /D554K/N555Y/M556V, 552_553insH/554K/N555Y, 552_553insH/D554K/M556V, 552_553insH/N555Y/M556V, 552_553insH/D554K, 552_553insH/N555Y, 552_553insH/M556V, D554K/N555Y, D554K/M556V, and N555Y/M556V, or the corresponding mutations in SEQ ID NO:2, or a variant thereof (D557K, N558Y, M559V and 555_556insH)..

<u>CENPC-encoding polynucleotides having an active mutation, chimeric genes, vectors, host cells</u>

Polynucleotides having nucleic acid sequences, such as cDNA, genomic DNA and RNA molecules, encoding any of the above proteins are also provided. Due to the degeneracy of the genetic code a variety of nucleic acid sequences may encode the same amino acid sequence. Any polynucleotides encoding CENPC proteins or variants thereof are herein referred to as “CENPC-encoding polynucleotides”. The polynucleotides provided include naturally occurring, artificial or synthetic nucleic acid sequences. 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).

The present invention further relates to a polynucleotide encoding a CENPC protein having an active mutation as taught herein. Said polynucleotide may be a synthetic,

recombinant and/or isolated polynucleotide. In an embodiment, said polynucleotide is derived from an endogenous CENPC-encoding polynucleotide that comprises the nucleic acid sequence of SEQ ID NO:11 or 14 or a variant thereof having at least 70%, preferably at least 75%, such as 80%, 85%, 90%, 95%, more preferably at least 97%, 98%, or 99% sequence identity to the nucleic acid sequence of SEQ ID NO:11 or 14, preferably over the full length, and which shares the endogenous CENPC activity of the polypeptide comprising the amino acid sequence of SEQ ID NO:4. In contrast, the CENPC-encoding polynucleotide having an active mutation taught herein comprises one or more active mutations that reduce(s) or eliminate(s) endogenous CENPC activity to less than 90, 80, 70, 60, 50, 40, 30, 20, 10% of CENPC activity of the endogenous CENPC protein from which it is derived. CENPC activity may be measured in vitro by measuring centromeric localization during separation of the chromosomes, for example, using a GFP fusion, where the level of fluorescence is a measure of CENPC activity. Alternatively, yeast-2-hybrid interactions may be measured in vitro using all known proteins and/or centromeric DNA that interact with CENPC protein. If the interaction is impaired, functionality of CENPC is impaired.

In an embodiment, one or more nucleotides at positions 1660-1668 of the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:14 are modified such that the polynucleotide encodes a CENPC protein in which the amino acid sequence of SEQ ID NO:4 has an altered amino acid residue at position 554 and/or at position 555 and/or at position 556.

In an embodiment, one or more nucleotides at positions 2449-2450 and/or 2454-2462 of the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:11 are modified such that the polynucleotide encodes a CENPC protein in which the amino acid sequence of SEQ ID NO:4 has an amino acid residue inserted between residues 552 and 553, and/or has an altered amino acid residue at position 554 and/or at position 555 and/or at position 556.

For example, aspartic acid on position 554, asparagine on position 555, or the methionine on position 556 of any ofSEQ ID NO:4, or a variant of the CENPC protein having an amino acid sequence as shown in SEQ ID NO: 4 as taught herein, or the corresponding amino acid residue in any of SEQ ID NO: 2 or 3, or variants thereof as taught herein, may be mutated. The amino acid residue on position 554 of the amino acid sequence of SEQ ID NO:4 or a variant thereof, or the amino acid residue corresponding to this amino acid residue in any of SEQ ID NO: 2 or 3, or variant thereof as taught herein, e.g., aspartic acid, may, for example, be mutated into a positively charged amino acid residue such as histidine, lysine, or arginine, preferably into lysine. The amino acid residue on position 555 of the amino acid sequence of SEQ ID NO:4 or a variant thereof, or the amino acid residue corresponding to this amino acid residue in any of SEQ ID NO: 2 or 3, or variants thereof as taught herein, e.g., asparagine, may, for example, be mutated into an aromatic amino acid residue such as

phenylalanine, tryptophan, or tyrosine, preferably into tyrosine. The amino acid residue at position 556 of the amino acid sequence of SEQ ID NO:4 or a variant thereof, or the amino acid residue corresponding to this amino acid residue in any of SEQ ID NO: 2 or 3, or variants thereof as taught herein, e.g., methionine, may, for example, be mutated into an amino acid residue with a small side chain such as glycine, serine, cysteine, alanine, threonine, or valine, preferably into valine.

In an embodiment, the active mutations that are made in the endogenous CENPC protein to yield an actively mutated CENPC protein are selected from the group consisting of 552_553insH, D554K, N555Y, M556V, or any combinations thereof as taught herein.

In certain embodiments, said polynucleotide comprises the nucleic acid sequence of any of SEQ ID NO:7, 9, 12, or 13.

In one embodiment of the invention, nucleic acid sequences encoding CENPC proteins (including CENPC proteins, or variants or fragments thereof, having an active mutation), as described above, are used to make chimeric genes, and/or vectors for transfer of the CENPC protein encoding polynucleotides into a host cell and production of the CENPC protein(s) in host cells, such as cells, tissues, organs or organisms derived from transformed cell(s). Vectors for the production of CENPC protein (or protein fragments or variants thereof) as taught herein in plant cells are herein referred to as "expression vectors".

Suitable host cells for expression of CENPC proteins include prokaryotes, yeast, or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al., Cloning vectors: A Laboratory Manual, Elsevier, N.Y., (1985). Cell-free translation systems could also be employed to produce the proteins of the present invention using RNAs derived from nucleic acid sequences disclosed herein.

Suitable prokaryotic host cells include gram-negative and gram-positive organisms, for example, Escherichia coli or Bacilli. Another suitable prokaryotic host cell is Agrobacterium, in particular Agrobacterium tumefaciens.

CENPC proteins as taught herein can also be expressed in yeast host cells, for example from the Saccharomyces genus (e.g., Saccharomyces cerevisiae). Other yeast genera, such as Pichia or Kluyveromyces, can also be employed.

Alternatively, CENPC proteins as taught herein may be expressed in higher eukaryotic host cells, including plant cells, fungal cells, insect cells, and mammalian, optionally non-human, cells.

One embodiment of the invention is a non-human organism modified to comprise a polynucleotide as taught herein. The non-human organism and/or host cell may be modified by any methods known in the art for gene transfer including, for example, the use of delivery devices such as lipids and viral vectors, naked DNA, electroporation, chemical methods and

particle-mediated gene transfer. In an advantageous embodiment, the non-human organism is a plant.

Any plant cell may be a suitable host cell. Suitable plant cells include those from monocotyledonous plants or dicotyledonous plants. For example, the plant may belong to the genus Solanum (including Lycopersicon), Nicotiana, Capsicum, Petunia and other genera. The following host species may suitably be used: Tobacco (Nicotiana species, e.g. N. benthamiana, N. plumbaginifolia, N. tabacum, etc.), vegetable species, such as tomato (L. esculentum, syn. Solanum lycopersicum) such as e.g. cherry tomato, var. cerasiforme or currant tomato, var. pimpinellifolium) or tree tomato (S. betaceum, syn. Cyphomandra betaceae), potato (Solanum tuberosum), eggplant (Solanum melongena), pepino (Solanum muricatum), cocona (Solanum sessiliflorum) and naranjilla (Solanum quitoense), peppers (Capsicum annuum, Capsicum frutescens, Capsicum baccatum), ornamental species (e.g. Petunia hybrida, Petunia axillaries, P. integrifolia), coffee (Coffea).

Alternatively, the plant may belong to any other family, such as to the Cucurbitaceae or Gramineae. Suitable host plants include for example maize/corn (Zea species), wheat (Triticum species), barley (e.g. Hordeum vulgare), oat (e.g. Avena sativa), sorghum (Sorghum bicolor), rye (Secale cereale), soybean (Glycine spp, e.g. G. max), cotton (Gossypium species, e.g. G. hirsutum, G. barbadense), Brassica spp. (e.g. B. napus, B. juncea, B. oleracea, B. rapa, etc), sunflower (Helianthus annus), safflower, yam, cassava, alfalfa (Medicago sativa), rice (Oryza species, e.g. O. sativa indica cultivar-group or japonica cultivar-group), forage grasses, pearl millet (Pennisetum spp. e.g. P. glaucum), tree species (Pinus, poplar, fir, plantain, etc), tea, coffea, oil palm, coconut, vegetable species, such as pea, zucchini, beans (e.g. Phaseolus species), cucumber, artichoke, asparagus, broccoli, garlic, leek, lettuce, onion, radish, turnip, Brussels sprouts, carrot, cauliflower, chicory, celery, spinach, endive, fennel, beet, fleshy fruit bearing plants (grapes, peaches, plums, strawberry, mango, apple, plum, cherry, apricot, banana, blackberry, blueberry, citrus, kiwi, figs, lemon, lime, nectarines, raspberry, watermelon, orange, grapefruit, etc.), ornamental species (e.g. Rose, Petunia, Chrysanthemum, Lily, Gerbera species), herbs (mint, parsley, basil, thyme, etc.), woody trees (e.g. species of Populus, Salix, Quercus, Eucalyptus), fibre species e.g. flax (Linum usitatissimum) and hemp (Cannabis sativa), or model organisms, such as Arabidopsis thaliana.

Preferred host cells are derived from “crop plants” or “cultivated plants”, i.e. plant species which is cultivated and bred by humans. A crop plant may be cultivated for food or feed purposes (e.g. field crops), or for ornamental purposes (e.g. production of flowers for cutting, grasses for lawns, etc.). A crop plant as defined herein also includes plants from which non-food products are harvested, such as oil for fuel, plastic polymers, pharmaceutical products, cork, fibres (such as cotton) and the like.

The construction of chimeric genes and vectors for, preferably stable, introduction of CENPC protein-encoding nucleic acid sequences as taught herein into the genome of host cells is generally known in the art. To generate a chimeric gene the nucleic acid sequence encoding a CENPC protein as taught herein is operably linked to a promoter sequence, suitable for expression in the host cells, using standard molecular biology techniques. The promoter sequence may already be present in a vector so that the CENPC protein encoding nucleic acid sequence is simply inserted into the vector downstream of the promoter sequence. The vector may then be used to transform the host cells and the chimeric gene may be inserted in the nuclear genome or into the plastid, mitochondrial or chloroplast genome and expressed using a suitable promoter (e. g., Mc Bride et al., 1995 Bio/Technology 13, 362; US 5,693, 507). In an embodiment the chimeric gene as taught herein comprises a suitable promoter for expression in plant cells or microbial cells (e.g. bacteria), operably linked to a nucleic acid sequence encoding a CENPC protein as taught herein, optionally followed by a 3'nontranslated nucleic acid sequence. The bacteria may subsequently be used for plant transformation (Agrobacterium-mediated plant transformation).

Plants expressing CENPC polypeptides having an active mutation

The present invention provides plants or plant cells expressing a CENPC polypeptide having an active mutation as taught herein. The present invention also provides plants comprising a polynucleotide as taught herein, a chimeric gene as taught herein, or a vector as taught herein. The plant preferably belongs to the family Solanaceae, more preferably to the genus Solanum, yet more preferably to the species Solanum lycopersicum.

The plants or plant cells preferably do not express, or express at reduced levels (e.g., less than 90, 80, 70, 60, 50, 40, 30, 20, 10% of wild type levels), an endogenous CENPC protein. For example, one can generate a mutation in an endogenous CENPC protein that reduces or eliminates endogenous CENPC protein activity or expression, or one can generate a knockout for endogenous CENPC protein. In this case, one may generate a plant heterozygous for the gene knockout or mutation and introduce an expression vector for expression of a CENPC protein having an active mutation in the plant. Progeny from the heterozygote can then be selected that are homozygous for the mutation or knockout but that comprise the CENPC protein having an active mutation.

Accordingly, in plants or plant cells taught herein preferably one or both endogenous CENPC alleles are knocked out or mutated such that said plants or plant cells significantly or essentially completely lack endogenous CENPC activity, i.e., sufficient to induce embryo lethality without complementary expression of a CENPC protein having an active mutation as taught herein. In plants having more than a diploid set of chromosomes, all endogenous

CENPC alleles may be inactivated, mutated or knocked out. Alternatively, the expression of endogenous CENPC protein may be silenced by any way known in the art, e.g. by introducing a siRNA or microRNA that reduces or eliminates expression of endogenous CENPC protein. Ideally, the silencing agent is selected to silence the endogenous CENPC protein but not the CENPC protein having an active mutation.

Methods for the generation of plants

It is an embodiment of the invention to modify an endogenous *CENPC* gene using targeted mutagenesis methods (also referred to as targeted nucleotide exchange (TNE) or oligo-directed mutagenesis (ODM)). Targeted mutagenesis methods include, without limitation, those employing zinc finger nucleases, Cas9-like, Cas9/crRNA/tracrRNA or Cas9/gRNA CRISPR systems, or targeted mutagenesis methods employing mutagenic oligonucleotides, possibly containing chemically modified nucleotides for enhancing mutagenesis with sequence complementarity to the *CENPC* gene, into plant protoplasts (e.g., KeyBase® or TALENs).

Alternatively, mutagenesis systems such as TILLING (Targeting Induced Local Lesions IN Genomics; McCallum *et al.*, 2000, Nat Biotech 18:455, and McCallum *et al.* 2000, Plant Physiol. 123, 439-442, both incorporated herein by reference) may be used to generate plant lines which comprise a *CENPC* gene encoding a CENPC protein having an active mutation. TILLING uses traditional chemical mutagenesis (e.g. EMS mutagenesis) followed by high-throughput screening for mutations. Thus, plants, seeds and tissues comprising a *CENPC* gene having the desired mutation may be obtained.

The method may comprise the steps of mutagenizing plant seeds (e.g. EMS mutagenesis), pooling of plant individuals or DNA, PCR amplification of a region of interest, heteroduplex formation and high-throughput detection, identification of the mutant plant, sequencing of the mutant PCR product. It is understood that other mutagenesis and selection methods may equally be used to generate such modified plants. Seeds may, for example, be radiated or chemically treated and the plants may be screened for a modified phenotype.

Modified plants may be distinguished from non-modified plants, i.e., wild type plants, by molecular methods, such as the mutation(s) present in the DNA, and by the modified phenotypic characteristics. The modified plants may be homozygous or heterozygous for the mutation.

Thus, a method for making a plant as taught herein is provided, which method comprises the steps of: i) modifying an endogenous plant CENPC-encoding polynucleotide within a plant cell to obtain a plant CENPC-encoding polynucleotide having an active mutation; ii) selecting a plant cell comprising the plant CENPC-encoding polynucleotide having an active mutation; and iii) optionally, regenerating a plant from said plant cell.

The present invention also provides a method for making a plant as taught herein, comprising the steps of: i) transforming a plant cell with a polynucleotide as taught herein, a chimeric gene as taught herein, or a vector as taught herein; ii) selecting a plant cell comprising said polynucleotide; and iii) optionally, regenerating a plant from said plant cell.

The methods for making a plant as taught herein may further comprise the step of modifying an endogenous plant CENPC protein-encoding polynucleotide or any other endogenous plant polynucleotide involved in expression of said polynucleotide within said plant cell to prevent expression of endogenous CENPC protein.

The CENPC protein-encoding polynucleotides, preferably a CENPC protein-encoding chimeric gene, as taught herein can be stably inserted in a conventional manner into the nuclear genome of a single plant cell, and the so-transformed plant cell can be used in a conventional manner to produce a transformed plant that has an altered phenotype due to the presence of the CENPC protein as taught herein in certain cells at a certain time. In this regard, a T-DNA vector, comprising a CENPC protein-encoding polynucleotide as taught herein, in Agrobacterium tumefaciens can be used to transform the plant cell, and thereafter, a transformed plant can be regenerated from the transformed plant cell using the procedures described, for example, in EP 0 116 718, EP 0 270 822, PCT publication WO84/02913 and published European Patent application EP 0 242 246 and in Gould et al. (1991, Plant Physiol. 95,426-434). The construction of a T-DNA vector for Agrobacterium mediated plant transformation is well known in the art. The T-DNA vector may be either a binary vector as described in EP 0 120 561 and EP 0 120 515 or a co-integrate vector which can integrate into the Agrobacterium Ti-plasmid by homologous recombination, as described in EP 0 116 718.

Likewise, selection and regeneration of transformed plants from transformed plant cells is well known in the art. Obviously, for different species and even for different varieties or cultivars of a single species, protocols are specifically adapted for regenerating transformants at high frequency.

The resulting transformed plant can be used in a conventional plant breeding scheme to produce haploid plants that may subsequently become doubled haploid plants.

Methods for the generation of haploid plants and/or doubled haploid plants

The invention also relates to a method of generating a haploid plant, a plant with aberrant ploidy or a doubled haploid plant, said method comprising the steps of:

crossing a plant expressing an endogenous CENPC protein to the plant as taught herein; and selecting a haploid plant, a plant with aberrant ploidy, or a doubled haploid plant.

The skilled person is capable of selecting a haploid plant. Exemplary techniques include flow cytometry, or validation by specific SNP calling.

Said plant expressing an endogenous CENPC protein may be an F1 plant.

The plant expressing an endogenous CENPC protein may be a pollen parent of the cross, or may be an ovule parent of the cross.

Crossing a plant as taught herein, lacking expression of an endogenous CENPC protein to take part in the kinetochore complex and expressing a CENPC protein having an active mutation as taught herein, to a wild-type plant will result in at least some progeny that is haploid and comprises only chromosomes from the plant that expresses the endogenous CENPC protein. Thus, the present invention allows for the generation of haploid plants having all of its chromosomes from a plant of interest by crossing the plant of interest with a plant expressing a CENPC protein having an active mutation as taught herein, and collecting the resulting haploid seed.

Thus, genome elimination can be engineered with a precise molecular change independent of parental genotype. CENPC protein is found in any plant species. This allows haploid plants to be made in species where conventional methods for haploid plant production, such as tissue culture of haploid cells and wide crosses, are unsuccessful.

The plant expressing a CENPC protein having an active mutation as taught herein may be crossed as either the male or female parent. The methods taught herein allow for transfer of paternal chromosomes into maternal cytoplasm. Thus, it can generate cytoplasmic male sterile lines with a desired genotype in a single step.

Additionally, the present disclosure teaches a method of generating a doubled haploid plant, said method comprising the steps of:

crossing a plant expressing an endogenous CENPC protein to the modified plant as taught herein; selecting a haploid plant; and converting said haploid plant into a doubled haploid plant.

Thus, once generated, haploid plants can be used for the generation of doubled haploid plants, which comprise an exact duplicate copy of chromosomes. A wide variety of methods are known for generating doubled haploid organisms from haploid organisms. For example, chemicals such as colchicine may be applied to convert the haploid plant into a doubled haploid plant. Alternatively, ploidy may double spontaneously during embryonal development or at a later developmental stage of a plant.

In an embodiment, the methods for generation of haploid plants, plants with aberrant ploidy and/or doubled haploid plants as taught herein do not comprise sexually crossing the whole genomes of said plant. Instead, one set of chromosomes is eliminated during the cross.

Doubled haploid plants can be further crossed to other plants to generate F1, F2, or subsequent generations of plants with desired traits.

Doubled haploids plants may be obtained that do not bear transgenic or mutagenized genes. Additionally, doubled haploid plants can rapidly create homozygous F2s from a hybrid F1.

Sequence Listing

SEQ ID NO:1: Consensus CENPC motif, protein sequence
SEQ ID NO:2: Consensus Solanaceae CENPC protein sequence
SEQ ID NO:3: Consensus Solanum CENPC protein sequence
SEQ ID NO:4: Solanum lycopersicum CENPC protein sequence (Solyc03g120340.2.1)
SEQ ID NO:5: Consensus Solanaceae CENPC DH-inducer domain protein sequence
SEQ ID NO:6: Consensus Solanum CENPC DH-inducer domain protein sequence
SEQ ID NO:7: Solanum lycopersicum CENPC-552_553insH-D554K-N555Y coding sequence
SEQ ID NO:8: Solanum lycopersicum CENPC-552_553insH-D554K-N555Y protein sequence
SEQ ID NO:9: Solanum lycopersicum CENPC-M556V coding sequence
SEQ ID NO:10: Solanum lycopersicum CENPC-M556V protein sequence
SEQ ID NO:11: Solanum lycopersicum CENPC genomic DNA sequence (Solyc03g120340.2.1)
SEQ ID NO:12: Solanum lycopersicum CENPC-552_553insH-D554K-N555Y genomic DNA sequence
SEQ ID NO:13: Solanum lycopersicum CENPC-M556V genomic DNA sequence
SEQ ID NO:14: Solanum lycopersicum CENPC coding sequence (Solyc03g120340.2.1)
SEQ ID NO: 15: Consensus Cucurbitaceae CENPC protein sequence
SEQ ID NO:16: Consensus Brassicaceae CENPC protein sequence
SEQ ID NO:17: Consensus Fabaceae CENPC protein sequence
SEQ ID NO:18: Consensus Poaceae CENPC protein sequence
SEQ ID NO:19: Consensus Rosaceae CENPC protein sequence

Brief description of the Figure

Figure 1 shows a tetrad of a CENPC-552_553insH-D554K-N555Y mutant. The arrow indicates a micronucleus.

Examples

Material and Methods

*Plant material*

Two tomato cultivars were used, namely "MoneyBergTMV+"and "MicroTom". From a tomato MoneyBergTMV+ mutant population two somatic non-synonymous mutants were selected, following methods described in WO 2007/037678 and WO2009/041810 in the gene CENPC, namely CENPC-552_553insH-D554K-N555Y and CENPC-M556V. The CENPC-552_553insH-D554K-N555Y mutations are in amino acid residues 554 and 555 of the endogenous CENPC protein, with an insertion of an additional amino acid residue between protein positions 552 and 553 due to a splice acceptor site mutation. The CENPC-M556V is mutated at amino acid residue 556 of the endogenous CENPC protein. From the same tomato MoneyBerg TMV+ mutant population a somatic synonymous mutant was selected, following methods described in WO 2007/037678 and WO2009/041810 in the gene Msi2, namely Msi2_D337D, which is mutated at amino acid position 337. The selected mutant plants were self-pollinated and in the offspring, plants were selected that were homozygous for the mutated loci or locus, respectively.

*Method*

Uni-parental genome elimination and the resulting production of a haploid plant was provoked by making a cross between a so called haploid inducer line and another not haploid inducer line, for example a breeding line or MicroTom. Crosses of tomato lines for uni-parental genome elimination were performed at relatively high temperatures (26-28°C), since it is known that an elevated temperature can, but only in some cases, have a positive effect on the occurrence of uni-parental genome elimination (Sanei et al. PNAS 108.33 (2011): E498-E505).

*Results*

The non-synonymous mutations of G to T, G to A, C to A and A to T in the CENPC-552_553insH-D554K-N555Y mutant resulted in three changes at the protein level. (1) The splice acceptor site just before exon 7 with the wild type sequence "tagcag^GTT" (intron 6 in lower case type setting, exon 7 first codon in uppercase type setting, splice site is indicated by the caret symbol) has a G to T mutation, which resulted in the creation of a novel splice acceptor site: "tag^CATGTT". Hereby a "CAT" codon encoding for a histidine is inserted between amino acid positions 552 and 553, extending exon 7 at the 5'-end (SEQ ID NO:7). (2) The G to A and C to A mutation resulted in an amino acid modification of an aspartic acid to a lysine at protein position 554. (3) The A to T mutation resulted in an amino acid modification of an asparagine to a tyrosine at protein position 555. The non-synonymous mutation of A to G in the CENPC-M556V mutant resulted in an amino acid modification of a methionine to a valine at protein position 556. The synonymous mutation of C to T in the Msi2_D337D mutant did not result in an amino acid modification. Furthermore this plant has

been equally treated during selection from the mutant population and did not have any mutation in the CENPC gene. Therefore the Msi2_D337D mutant was used as a control for the uni-parental genome elimination crosses.

Mutant plants homozygous for the CENPC-552_553insH-D554K-N555Y, the CENPC-M556V or the Msi2_D337D mutation were used as pollen donor and as female in crosses at relatively high temperatures (26-28°C) using non-mutated wild type MicroTom plants as female or pollen donor, respectively. Table 1 lists an overview of all crosses made and the number of offspring plants which were evaluated for the MicroTom phenotype.

*Table 1. List of crosses made. Genetic background of all listed mutant plants is MoneyBergTMV+. Shown are the number of offspring plants tested and the number of offspring plants which showed MicroTom dwarf phenotype and the year the cross was performed.*

| **Plant used as female** | **Plant used as male** | **Number of plants tested** | **Number of plants with MicroTom phenotype** | **Year** |
|---|---|---|---|---|
| CenPC-552_553insH-D554K-N555Y | MicroTom | 188 | 2 | 2014 |
| MicroTom | CenPC-552_553insH-D554K-N555Y | 65 | 1 | 2014 |
| CenPC-552_553insH-D554K-N555Y | MicroTom | 564 | 3 | 2015 |
| MicroTom | CenPC-552_553insH-D554K-N555Y | 306 | 2 | 2015 |
| MicroTom | CenPC-M556V | 188 | 4 | 2014 |
| MicroTom | CenPC-M556V | 317 | 1 | 2015 |
| Msi2_D337D | MicroTom | 160 | 0 | 2014 |
| MicroTom | Msi2_D337D | 36 | 0 | 2014 |
| MoneyBergTMV+ | MicroTom | 188 | 0 | 2015 |
| MicroTom | MoneyBergTMV+ | 188 | 0 | 2015 |

Seeds derived from CENPC-552_553insH-D554K-N555Y, CENPC-M556V, Msi_D337D and MoneyBerg TMV+ crosses listed in table 1 were sown and the plants were evaluated for their

DNA content by means of flow cytometry. The flow cytometry analysis resulted in a determination of only normal diploid ploidy levels for all plants tested, similar to wild type tomato cultivars such as MoneyBergTMV+, with the exception of one plant derived from a CENPC-M556V x MicroTom cross from 2015; this plant was found to be aneuploid. The cultivar MicroTom has a dwarf phenotype, which is known to be recessive (Marti *et al*, J Exp Bot, Vol. 57, No. 9, pp. 2037–2047, 2006). After a cross of MicroTom to or with, for instance a MoneyBergTMV+ wild type cultivar, one only finds offspring with the indeterminate non-dwarf phenotype of the MoneyBergTMV+ wild type cultivar. The same was found for crosses with the Msi2_D337D synonymous mutant and MicroTom; all offspring of a MicroTom and Msi2_D337D mutant crosses showed the indeterminate non-dwarf phenotype of the MoneyBergTMV+ parent. Remarkably, using the CENPC-552_553insH-D554K-N555Y mutant as male or female parent, in total 8 plants were found which showed a MicroTom phenotype. Furthermore, using the CENPC-M556V as male parent, in total 5 plants were found which showed a MicroTom phenotype. For all mentioned 13 plants with MicroTom phenotype, their phenotype indicates that the MoneyBergTMV+ parent genetic material is not part of the resulting offspring and this indicates that these 13 offspring plants are of haploid MicroTom origin. The ploidy of all mentioned 13 plants with MicroTom phenotype was found to be diploid, which indicates that spontaneous doubling had occurred, a phenomena which has been described to have an exceptional high frequency of appearance for tomato (Report of the Tomato Genetics Cooperative Number 62- December 2012).

For CENPC-552_553insH-D554K-N555Y offspring with MicroTom phenotype, in order to determine whether and to what extent uni-parental genome elimination had occurred, a single nucleotide polymorphism (SNP) assay was run for in total 44 positions for the 2014 offspring, spread across each of the 12 tomato chromosomes (4 SNPs on chromosome 1, 2, 3, 4, 5, 6, 10 and 12; 3 SNPs on chromosome 8 and 11; 2 SNPs on chromosome 9). The same analysis was performed for the 2015 offspring, now on 22 positions (2 SNPs on chromosome 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12; 1 SNP on chromosome 9 and 11). The single nucleotide polymorphisms selected were homozygous for one base pair for the MicroTom parent and homozygous for all but not the MicroTom base pair in the MoneyBergTMV+ parent. A regular cross between a wild type MicroTom cultivar and the MoneyBergTMV+ cultivar would result in a heterozygous single nucleotide polymorphism score. However, when the process of uni-parental genome elimination has occurred, one expects the loss of the haploid inducer line genome.

The single nucleotide polymorphism test resulted in calling of only homozygous base pair scores from the MicroTom parent for each of the 8 offspring plants which also showed the MicroTom phenotype and none of the MoneyBergTMV+ parent were called. Based on the single nucleotide polymorphism scores it was concluded that the complete genome of the

CENPC-552_553insH-D554K-N555Y mutant was no longer present in the offspring. Therefore, it can be concluded that the CENPC-552_553insH-D554K-N555Y mutant functions as a highly efficient haploid inducer line. In the crosses in which the CENPC-552_553insH-D554K-N555Y mutant was used as female parent, a selfing of MicroTom can be ruled out. It is highly unlikely that in the experiment using MicroTom as female parent selfing took place, given the very low number of offspring showing the MicroTom phenotype (only 1 seed out of 65 and 2 seeds out of 306), and the fact that only homozygous base pairs were scored.

For CENPC-M556V offspring with MicroTom phenotype, in order to determine whether and to what extent uni-parental genome elimination had occurred, a single nucleotide polymorphism (SNP) assay was run for in total 24 positions, spread across each of the 12 tomato chromosomes, with 2 SNPs per chromosome in 2014 and 22 positions were tested in 2015 (2 SNPs on chromosome 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12; 1 SNP on chromosome 9 and 11). The single nucleotide polymorphisms selected were homozygous for one base pair for the MicroTom parent and homozygous for all but not the MicroTom base pair in the MoneyBergTMV+ parent. A regular cross between a wild type MicroTom cultivar and the MoneyBergTMV+ cultivar would result in a heterozygous single nucleotide polymorphism score. However, when the process of uni-parental genome elimination has occurred, one expects the loss of the haploid inducer line genome.

The single nucleotide polymorphism test resulted in calling of only homozygous base pair scores from the MicroTom parent for each of the 5 offspring plants which also showed the MicroTom phenotype and none of the MoneyBergTMV+ parent were called. Based on the single nucleotide polymorphism scores it was concluded that the complete genome of the CENPC-M556V mutant was no longer present in the offspring. Therefore, it can be concluded that the CENPC-M556V mutant functions as a highly efficient haploid inducer line. In the crosses in which the CENPC-M556V mutant was used as female parent, a selfing of MicroTom can be ruled out. It is highly unlikely that in the experiment using MicroTom as female parent selfing took place, given the very low number of offspring showing the MicroTom phenotype (only 4 seed out of 188 and 1 out of 317), and the fact that only homozygous base pairs were scored.

*Phenotype of the CENPC-552_553insH-D554K-N555Y mutant*

Pollen tetrads of the CENPC-552_553insH-D554K-N555Y mutant and of not mutated RZ52201 control plants were checked for occurrence of aberrancies. From four different flower trusses at least one flower was taken, its anthers were stained with DAPI (4',6-diamidino-2-fenylindool) and stained anthers squashed in order to look at pollen tetrads. For the CENPC-552_553insH-D554K-N555Y mutant, micronuclei were observed with an average

frequency of 2.34±0.90% (Figure 1). For the control plant flowers, micronuclei were observed with an average frequency of 0.58±0.36%. The difference between control and mutant frequencies was evaluated with a Mann-Whitney rank sum test and it was found that the difference in the median values between the two groups is greater than would be expected by chance, therefore it is concluded that there was a statistically significant difference (P = 0.016).

It can therefore be concluded that the separation of chromosomes during meiosis is much more frequently disturbed as a result of the CENPC-552_553insH-D554K-N555Y mutation compared to the control. Aberrant mitosis, for instance observations of micronuclei, are often used as direct evidences of chromosome elimination and haploid production in inter-, intra-specific hybridizations in crops. For example, aberrant mitosis as well as aberrant meiosis, for instance micronuclei, were found in a study of a maize DH-inducer line (Qiu, Fazhan, et al. Current Plant Biology 1 (2014): 83-90). The observations of meiosis micronuclei in the CENPC-552_553insH-D554K-N555Y mutant, suggest that during mitosis similar processes occur. It is likely that the process of uniparental genome elimination during the first mitotic divisions after fusion of wild type and CENPC-552_553insH-D554K-N555Y zygotes takes place and that this results in the observed induction of haploids.

**CLAIMS**

1. CENPC protein of plant origin comprising one or more active mutations, wherein the one or more active mutations are present in a protein comprising at least 75% sequence identity to the amino acid sequence of any one of SEQ ID NO: 2, 3, 4 and 15-19 wherein the protein, when present in a plant in the absence of its endogenous CENPC protein, allows generation of haploid progeny, or progeny with aberrant ploidy, at a higher frequency than normal when said plant is crossed with a wild-type plant, and wherein the one or more active mutations are present in a doubled haploid inducer domain represented by the amino acid sequences as depicted in any of SEQ ID NO: 5 or 6.

2. CENPC protein according to claim 1, wherein the one or more active mutations are chosen from the group consisting of a mutation between amino acid residues at position 552 and 553, a mutation of an amino acid residue at position 554, a mutation of an amino acid residue at position 555 and a mutation of an amino acid residue at position 556, or any combination thereof, in the amino acid sequence of any of SEQ ID NO: 3 or 4; or of a mutation between amino acid residues at position 555 and 556, a mutation of an amino acid residue at position 557, a mutation of an amino acid residue at position 558 and a mutation of an amino acid residue at position 559, or any combination thereof, in the amino acid sequence of SEQ ID NO: 2.

3. CENPC protein according to claim 2, wherein the amino acid that is mutated at position 554 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is aspartic acid and/or the amino acid that is changed at position 555 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 558 in the amino acid sequence of SEQ ID NO: 2, is asparagine and/or the amino acid that is changed at position 556 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 559 in the amino acid sequence of SEQ ID NO: 2, is methionine.

4. CENPC protein according to claim 2 or 3, wherein the amino acid at position 554 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is changed into a positively charged amino acid residue, and/or the amino acid at position 555 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 558 in the amino acid sequence of SEQ ID NO: 2, is changed into a tyrosine and/or the amino acid at position 556 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is changed into valine, and/or wherein a positively charged residue, is inserted between amino acid residues 552 and 553 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or between amino acid residues 555 and 556 in the amino acid sequence of SEQ ID NO: 2.

5. CENPC protein according to any one of claims 2-4, wherein a histidine is inserted between amino acid residues 552 and 553 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or between amino acid residues 555 and 556 in the amino acid sequence of SEQ ID NO: 2, and/or an aspartic acid at position 554 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 557 in the amino acid sequence of SEQ ID NO: 2, is changed into a lysine and/or an asparagine at position 555 of the amino acid sequence of any of SEQ ID NO: 3 or 4, or at position 558 in the amino acid sequence of SEQ ID NO: 2, is changed into a tyrosine.

6. CENPC protein according to claim 1, which comprises the amino acid sequence of SEQ ID NO: 8 or 10.

7. The CENPC protein according to any one of claims 1 – 6, wherein the active mutation is not an insertion of a glutamine residue between amino acid residues 552 and 553 of the amino acid sequence as shown in SEQ ID NO: 4.

8. Polynucleotide encoding the CENPC protein according to any one of the preceding claims.

9. Polynucleotide comprising the nucleic acid sequence selected from the group consisting of SEQ ID NO: 7, 9, 12 and 13.

10. Chimeric gene, vector or host cell comprising the polynucleotide according to claim 8 or 9.

11. Plant, seed or plant cell comprising a polynucleotide according to claim 8 or 9, or a chimeric gene or vector according to claim 10.

12. Plant, seed or plant cell according to claim 11, wherein the endogenous CENPC protein is not expressed.

13. Plant, seed or plant cell according to claim 11 or 12, which is a *Solanum* plant.

14. Method for making a plant or plant cell according to any one of claims –11-13, said method comprising the steps of:

a) modifying an endogenous plant CENPC protein-encoding polynucleotide within a plant cell to obtain a plant CENPC protein-encoding polynucleotide encoding a CENPC protein having an active mutation, which, when present in a plant in the absence of its endogenous CENPC protein, allows generation of haploid progeny, or progeny with aberrant ploidy, at a higher frequency than normal when said plant is crossed with a wild-type plant;

b) selecting a plant cell comprising the plant CENPC protein-encoding polynucleotide having the mutation; and

c) optionally, regenerating a plant from said plant cell.

15. Method for making a plant or plant cell according to claim –11-13, said method comprising the steps of:

a) transforming a plant cell with a polynucleotide according to claim 8 or 9, or a chimeric gene or vector according to claim 10;

b) selecting a plant cell comprising said polynucleotide, chimeric gene or vector; and

c) optionally, regenerating a plant from said plant cell.

16. A method according to claim 14 or 15, said method further comprising the step of:
modifying said plant cell to prevent expression of endogenous CENPC protein.

17. A method of generating a haploid plant, or a plant with aberrant ploidy, said method comprising the steps of:

a) crossing a plant expressing an endogenous plant CENPC protein to the plant of any one of claims –11-13, wherein the plant of any one of claims –11-13 does not express an endogenous CENPC protein at least in its reproductive parts and/or during embryonic development;

b) harvesting seed;

c) growing at least one seedling, plantlet or plant from said seed; and

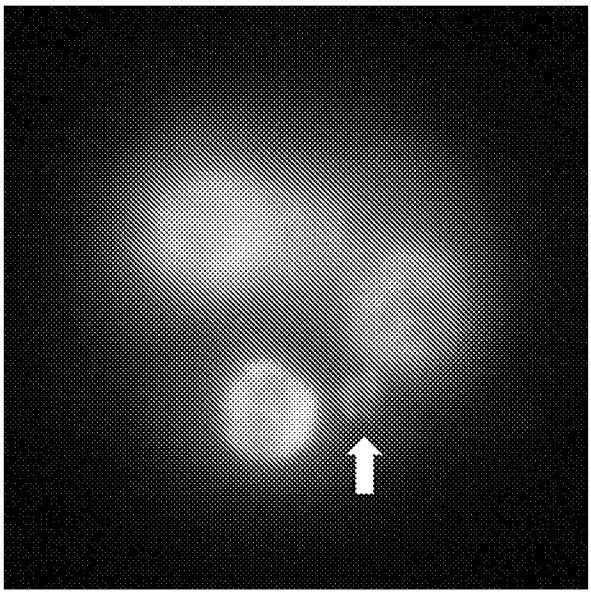
d) selecting a haploid seedling, plantlet or plant; a seedling, plantlet or plant with aberrant ploidy; or a doubled haploid seedling, plantlet or plant.

18. A method of generating a doubled haploid plant, said method comprising the steps of: converting the haploid plant obtained in step d) of claim 17 into a doubled haploid plant.

19. Method according to claim 18, wherein the conversion is performed by treatment with colchicine.

20. Use of a polynucleotide of claim 8 or 9 for producing a haploid inducer line.

Fig. 1



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Ser Glu Val Leu Ala Glu Thr Leu Pro Ser Leu Gln Ala Gln Gly Lys
515 520 525

Ala Val Asp Thr Ala Asn Tyr Thr Ile Glu Thr Ala Val Glu Asp Phe
530 535 540

Gly Ser Thr Glu Ile Asp Gln Leu Val Asp Asn Met Leu Pro Glu Thr
545 550 555 560

Ala Pro Ser Ala Glu Gln Asp His Tyr Phe Glu Asp Ser Val Lys Asp
565 570 575

Leu Asn Ser Asp Gln Leu Asn Ser Val Gly Val Glu Val Pro Ser Arg
580 585 590

Asp Val Arg Pro Lys Phe Pro Glu Met Ser Pro Gln His His Lys Gln
595 600 605

Ala Lys Asp Lys Gln Gln Lys Ala Lys Lys Leu Ala Val Gly Arg Arg
610 615 620

Glu Arg Lys His Leu Ser Ser Arg Pro Ser Leu Ala Asp Ala Gly Thr
625 630 635 640

Ser Phe Glu Ser Gly Val Arg Arg Ser Lys Arg Met Lys Thr Arg Pro
645 650 655

Leu Glu Tyr Trp Lys Gly Glu Arg Leu Leu Tyr Gly Arg Val Asp Glu
660 665 670

Gly Leu Lys Leu Val Gly Leu Lys Tyr Ile Ser Pro Gly Lys Gly Ser

675 680 685

Phe Lys Val Lys Ser Tyr Ile Pro Asp Asp Tyr Lys Asp Leu Val Asp
690 695 700

Leu Ala Ala Arg Tyr
705

<210> 4
<211> 709
<212> PRT
<213> Solanum lycopersicum

<400> 4

Met Val Asn Glu Ala Leu Ile Ser Asp Pro Val Asp Pro Leu His Ser
1 5 10 15

Leu Ala Gly Leu Ser Leu Leu Pro Thr Thr Val Arg Val Ser Thr Gly
20 25 30

Ala Ser Val Ser Val Asp Ser Lys Asp Leu Glu Ser Ile His Asn Phe
35 40 45

Met Lys Ser Met Glu Thr Lys Gly Pro Gly Leu Leu Glu Glu Ala Arg
50 55 60

Glu Ile Val Asp Asn Gly Ala Glu Leu Leu Asn Thr Lys Phe Thr Ser
65 70 75 80

Phe Ile Arg Ser Lys Gly Ile Asp Gly Asp Leu Ala Ile Lys Gly Lys
85 90 95

Glu Lys Val Gln Glu Arg Arg Pro Gly Leu Gly Arg Lys Arg Ala Arg
100 105 110

Phe Ser Leu Lys Pro Ser Thr Ser Gln Pro Thr Val Ser Ile Ala Pro
115 120 125

Arg Leu Asp Ile Asp Gln Leu Ser Asp Pro Val Glu Phe Phe Ser Val
130 135 140

Ala Glu Lys Leu Glu Val Ala Glu Lys Glu Ile Glu Arg Gln Lys Gly
145 150 155 160

Gly Ser Ile His Asp Pro Asp Val Asn Asn Pro Pro Ala Asn Ala Arg
165 170 175

Arg Arg Arg Pro Gly Ile Leu Gly Lys Ser Val Lys Tyr Lys His Arg
180 185 190

Phe Ser Ser Ser Gln Pro Glu Asn Asp Asp Ala Phe Ile Ser Ser Gln
195 200 205

```
Glu Thr Leu Glu Asp Asp Ile Leu Val Glu His Gly Ser Gln Leu Pro
    210                 215                 220

Glu Glu Leu His Gly Leu Asn Val Glu Leu Gln Glu Ala Glu Leu Thr
225                 230                 235                 240

Gly Pro Ile Lys Lys Ser Glu Asn Arg Ile Asn Lys Ile Leu Asp Glu
                245                 250                 255

Leu Leu Ser Gly Ser Gly Glu Asp Leu Asp Arg Asp Met Ala Val Ser
            260                 265                 270

Lys Leu Gln Glu Gln Leu Lys Ile Lys Pro Ile Glu Leu Gly Thr Leu
        275                 280                 285

Cys Ile Pro Glu Phe Pro Val Thr Gly Lys Phe Asp Gly Lys Ala Leu
    290                 295                 300

Gly Glu Arg Ile Gln Lys Pro Ser Lys Phe Phe Leu Glu Ile Ala Glu
305                 310                 315                 320

Leu Val Lys Ser Ala Thr Glu Gly Thr Pro Ser Ser His Lys Gln His
                325                 330                 335

Glu Glu Ser Pro Ala Ser Lys Leu Ala Ser Pro Thr Pro Pro Lys Ser
            340                 345                 350

Pro Phe Gly Ser Leu Ser Leu Leu Lys Lys Lys Leu Met Gln Ser Asn
        355                 360                 365

Pro Leu Arg Asp Pro Phe Ser Pro Leu Asn Ile Asp Leu Gln Ser Glu
    370                 375                 380

His Pro Asp Trp Ser Ala Lys Lys Lys Ser Gln Cys Val Asn Asn Asn
385                 390                 395                 400

Val Gly Pro Ile Glu Ser Arg Gly Cys Glu Asn Thr Asn Ile Met Val
                405                 410                 415

Pro Leu Arg Gly Ser Asp Leu Val His Glu Gln Pro Ile Glu Lys Asn
            420                 425                 430

Pro Gly Arg Asp Ser Val Lys Thr Gly Pro Asn Gly Ser Arg Ser Gly
        435                 440                 445

Met Glu Gln His Asn Gly Tyr Asp Asp Ile Asp Ala Asn Thr Asn Asp
    450                 455                 460

Asn Leu Asn Met Arg Asn Val Asp Ser His His Glu Ser Asp Gly Leu
465                 470                 475                 480
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Asp Lys Val Lys Asp Asp Ser Val Ile Lys Asn Val Leu Lys Ala Leu
                485                 490                 495

Gln Gly Leu Glu Thr Lys Ser Tyr Ile Asp Cys Gln Lys Leu Gln Asp
            500                 505                 510

Ser Glu Val Leu Ala Glu Thr Leu Pro Ser Leu Gln Ala Gln Gly Lys
        515                 520                 525

Ala Val Asp Thr Ala Asn Tyr Thr Ile Glu Thr Ala Val Glu Asp Phe
    530                 535                 540

Gly Ser Thr Glu Ile Asp Pro Leu Val Asp Asn Met Leu Pro Glu Thr
545                 550                 555                 560

Ala Pro Ser Ala Glu Gln Asp His Tyr Phe Glu Asp Ser Val Lys Asp
                565                 570                 575

Leu Asn Ser Asp Gln Leu Asn Ser Val Gly Val Glu Val Pro Ser Arg
            580                 585                 590

Asp Val Arg Pro Lys Phe Pro Glu Met Ser Pro Gln His His Lys Gln
        595                 600                 605

Ala Lys Asp Lys Gln Gln Lys Ala Lys Glu Leu Ala Val Gly Arg Arg
    610                 615                 620

Glu Arg Lys His Leu Ser Ser Arg Pro Ser Leu Ala Asp Ala Gly Thr
625                 630                 635                 640

Ser Phe Glu Ser Gly Val Arg Arg Ser Lys Arg Met Lys Thr Arg Pro
                645                 650                 655

Leu Glu Tyr Trp Lys Gly Glu Arg Leu Leu Tyr Gly Arg Val Asp Glu
            660                 665                 670

Gly Leu Lys Leu Val Gly Leu Lys Tyr Ile Ser Pro Gly Lys Gly Ser
        675                 680                 685

Phe Lys Val Lys Ser Tyr Ile Pro Asp Asp Tyr Lys Asp Leu Val Asp
    690                 695                 700

Leu Ala Ala Arg Tyr
705
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<210> 5
<211> 15
<212> PRT
<213> Solanaceae

<220>
<221> MISC_FEATURE
<222> (3)..(3)
<223> Xaa is Asp or Gly

<220>
<221> MISC_FEATURE
<222> (4)..(4)
<223> Xaa is  Gln or Gly or Pro

<220>
<221> MISC_FEATURE
<222> (5)..(5)
<223> Xaa is  Gln or Leu

<220>
<221> MISC_FEATURE
<222> (8)..(8)
<223> Xaa is  Asp or Asn

<220>
<221> MISC_FEATURE
<222> (10)..(10)
<223> Xaa is  Leu or Pro

<220>
<221> MISC_FEATURE
<222> (12)..(12)
<223> Xaa is  Gln or Glu

<220>
<221> MISC_FEATURE
<222> (13)..(13)
<223> Xaa is  Arg or Thr

<220>
<221> MISC_FEATURE
<222> (15)..(15)
<223> Xaa is Asn or His or Pro

<400> 5

Glu Ile Xaa Xaa Xaa Val Asp Xaa Met Xaa Pro Xaa Xaa Ala Xaa
1               5                  10                  15


<210> 6
<211> 15
<212> PRT
<213> Solanum


<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> Xaa is Ile or Thr

<220>
<221> MISC_FEATURE
<222> (4)..(4)
<223> Xaa is Gln or Pro

<400> 6

Glu Xaa Asp Xaa Leu Val Asp Asn Met Leu Pro Glu Thr Ala Pro
1               5                  10                  15

<210> 7
<211> 2133
<212> DNA
<213> Solanum lycopersicum

<400> 7
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tctctactcc caacaacagt tagggtttcc accggcgcct cagtatcagt agactccaaa 120

gacctagagt caattcacaa tttcatgaag tccatggaaa ccaagggtcc tgggctcttg 180

gaggaggcta gggaaattgt ggataacggt gcggagcttt tgaataccaa gtttacaagt 240

ttcatacgat ctaaaggcat tgatggggat cttgcaatca agggaaagga gaaggtccag 300

gagagaagac caggacttgg gcgtaaaagg gctcgatttt ccctcaagcc tagtacaagt 360

caacctactg tcagcatagc tcctcgttta gatattgatc aactatcaga tccagtggaa 420

tttttctcag ttgctgaaaa gctagaagtt gctgagaagg aaatagaaag acagaaaggt 480

ggcagtatcc atgatccaga tgtgaataat cccccagcca atgctcggcg tcgtcgacca 540

ggcatcttgg gcaaatcggt gaagtataag caccgtttct catcctccca gcctgagaat 600

gatgatgcat ttatatcatc tcaagagaca ttggaggatg atattctggt ggaacatggt 660

tctcagttgc cagaagagct ccatggcctc aatgttgaac tacaagaagc agaactcaca 720

gggccaataa agaaatcaga aaacagaatc aacaagatac tggatgaatt actgtctggt 780

agtggtgaag atctagaccg ggacatggca gtatccaaat tgcaagagca gttgaagatc 840

aagcccattg aactaggaac tttatgtatt cctgagttcc ctgtgactgg aaagtttgat 900

ggtaaggctc ttggagagag aattcagaag cctagtaagt ttttttgga gatagcggag 960

ttggttaaaa gtgcaactga gggaacgcca tcctctcaca aacagcatga agaaagtcct 1020

gccagtaaat tagcatcacc cactccacca aaaagtccat ttggttcatt gtctttgttg 1080

aaaaagaaac ttatgcagtc aaatccactg agagatcctt tttcgcctct caacattgat 1140

ctgcaatcag agcatccaga ttggtccgcg aagaagaaat cacaatgtgt aaataataat 1200

gttggaccta ttgaatctcg tggttgtgaa aatacaaata ttatggtccc tttaagaggt 1260

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gggccaaatg gatctcgatc tggaatggag caacataatg gctatgatga tattgatgcc 1380

aatactaacg acaacttaaa tatgagaaat gtggattccc accatgagtc tgatgggctt 1440

gacaaagtga aagatgattc agttataaag aatgttttga aggctctaca aggtcttgag 1500

accaaatcct acattgactg tcaaaagctg caggatagtg aagttcttgc tgaaacacta 1560

ccttctcttc aagcccaagg aaaagctgtc gatacagcca attataccat agaaacagct 1620

gttgaggatt ttggatcaac tgaaattgat ccgctgcatg ttaaatatat gctaccagaa 1680

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gagatgagcc ctcagcatca caagcaggca aaagataagc aacagaaagc aaaggaactt 1860

gctgttggaa ggcgtgaaag aaaacatctt tcttctaggc caagtctagc agatgctggc 1920

acatcatttg aaagtggggt tagacgaagc aaacgaatga agacaaggcc tttggaatat 1980

tggaaaggcg aaagattatt atatggacgg gttgatgagg gtttgaagct tgttggcttg 2040

aaatacatct ctccgggaaa gggatcattt aaggtgaagt cttacattcc tgatgactac 2100

aaagacctag ttgatttggc agctcgctat tga 2133

<210> 8
<211> 710
<212> PRT
<213> Solanum lycopersicum

<400> 8

Met Val Asn Glu Ala Leu Ile Ser Asp Pro Val Asp Pro Leu His Ser
1 5 10 15

Leu Ala Gly Leu Ser Leu Leu Pro Thr Thr Val Arg Val Ser Thr Gly
20 25 30

Ala Ser Val Ser Val Asp Ser Lys Asp Leu Glu Ser Ile His Asn Phe
35 40 45

Met Lys Ser Met Glu Thr Lys Gly Pro Gly Leu Leu Glu Glu Ala Arg
50 55 60

Glu Ile Val Asp Asn Gly Ala Glu Leu Leu Asn Thr Lys Phe Thr Ser
65 70 75 80

Phe Ile Arg Ser Lys Gly Ile Asp Gly Asp Leu Ala Ile Lys Gly Lys
85 90 95

Glu Lys Val Gln Glu Arg Arg Pro Gly Leu Gly Arg Lys Arg Ala Arg
100 105 110

Phe Ser Leu Lys Pro Ser Thr Ser Gln Pro Thr Val Ser Ile Ala Pro
115 120 125

Arg Leu Asp Ile Asp Gln Leu Ser Asp Pro Val Glu Phe Phe Ser Val
130 135 140

Ala Glu Lys Leu Glu Val Ala Glu Lys Glu Ile Glu Arg Gln Lys Gly
145 150 155 160

Gly Ser Ile His Asp Pro Asp Val Asn Asn Pro Pro Ala Asn Ala Arg
165 170 175

Arg Arg Arg Pro Gly Ile Leu Gly Lys Ser Val Lys Tyr Lys His Arg
180 185 190

Phe Ser Ser Ser Gln Pro Glu Asn Asp Asp Ala Phe Ile Ser Ser Gln
195 200 205

```
Glu Thr Leu Glu Asp Asp Ile Leu Val Glu His Gly Ser Gln Leu Pro
    210                 215                 220

Glu Glu Leu His Gly Leu Asn Val Glu Leu Gln Glu Ala Glu Leu Thr
225                 230                 235                 240

Gly Pro Ile Lys Lys Ser Glu Asn Arg Ile Asn Lys Ile Leu Asp Glu
                245                 250                 255

Leu Leu Ser Gly Ser Gly Glu Asp Leu Asp Arg Asp Met Ala Val Ser
            260                 265                 270

Lys Leu Gln Glu Gln Leu Lys Ile Lys Pro Ile Glu Leu Gly Thr Leu
        275                 280                 285

Cys Ile Pro Glu Phe Pro Val Thr Gly Lys Phe Asp Gly Lys Ala Leu
    290                 295                 300

Gly Glu Arg Ile Gln Lys Pro Ser Lys Phe Phe Leu Glu Ile Ala Glu
305                 310                 315                 320

Leu Val Lys Ser Ala Thr Glu Gly Thr Pro Ser Ser His Lys Gln His
                325                 330                 335

Glu Glu Ser Pro Ala Ser Lys Leu Ala Ser Pro Thr Pro Pro Lys Ser
            340                 345                 350

Pro Phe Gly Ser Leu Ser Leu Leu Lys Lys Lys Leu Met Gln Ser Asn
        355                 360                 365

Pro Leu Arg Asp Pro Phe Ser Pro Leu Asn Ile Asp Leu Gln Ser Glu
    370                 375                 380

His Pro Asp Trp Ser Ala Lys Lys Lys Ser Gln Cys Val Asn Asn Asn
385                 390                 395                 400

Val Gly Pro Ile Glu Ser Arg Gly Cys Glu Asn Thr Asn Ile Met Val
                405                 410                 415

Pro Leu Arg Gly Ser Asp Leu Val His Glu Gln Pro Ile Glu Lys Asn
            420                 425                 430

Pro Gly Arg Asp Ser Val Lys Thr Gly Pro Asn Gly Ser Arg Ser Gly
        435                 440                 445

Met Glu Gln His Asn Gly Tyr Asp Asp Ile Asp Ala Asn Thr Asn Asp
    450                 455                 460

Asn Leu Asn Met Arg Asn Val Asp Ser His His Glu Ser Asp Gly Leu
465                 470                 475                 480
```

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Asp Lys Val Lys Asp Asp Ser Val Ile Lys Asn Val Leu Lys Ala Leu
                485                 490                 495

Gln Gly Leu Glu Thr Lys Ser Tyr Ile Asp Cys Gln Lys Leu Gln Asp
            500                 505                 510

Ser Glu Val Leu Ala Glu Thr Leu Pro Ser Leu Gln Ala Gln Gly Lys
        515                 520                 525

Ala Val Asp Thr Ala Asn Tyr Thr Ile Glu Thr Ala Val Glu Asp Phe
    530                 535                 540

Gly Ser Thr Glu Ile Asp Pro Leu His Val Lys Tyr Met Leu Pro Glu
545                 550                 555                 560

Thr Ala Pro Ser Ala Glu Gln Asp His Tyr Phe Glu Asp Ser Val Lys
                565                 570                 575

Asp Leu Asn Ser Asp Gln Leu Asn Ser Val Gly Val Glu Val Pro Ser
            580                 585                 590

Arg Asp Val Arg Pro Lys Phe Pro Glu Met Ser Pro Gln His His Lys
        595                 600                 605

Gln Ala Lys Asp Lys Gln Gln Lys Ala Lys Glu Leu Ala Val Gly Arg
    610                 615                 620

Arg Glu Arg Lys His Leu Ser Ser Arg Pro Ser Leu Ala Asp Ala Gly
625                 630                 635                 640

Thr Ser Phe Glu Ser Gly Val Arg Arg Ser Lys Arg Met Lys Thr Arg
                645                 650                 655

Pro Leu Glu Tyr Trp Lys Gly Glu Arg Leu Leu Tyr Gly Arg Val Asp
            660                 665                 670

Glu Gly Leu Lys Leu Val Gly Leu Lys Tyr Ile Ser Pro Gly Lys Gly
        675                 680                 685

Ser Phe Lys Val Lys Ser Tyr Ile Pro Asp Asp Tyr Lys Asp Leu Val
    690                 695                 700

Asp Leu Ala Ala Arg Tyr
705                 710
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<210> 9
<211> 2130
<212> DNA
<213> Solanum lycopersicum

<400> 9

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gacctagagt caattcacaa tttcatgaag tccatggaaa ccaagggtcc tgggctcttg 180

gaggaggcta gggaaattgt ggataacggt gcggagcttt tgaataccaa gtttacaagt 240

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gagagaagac caggacttgg gcgtaaaagg gctcgatttt ccctcaagcc tagtacaagt 360

caacctactg tcagcatagc tcctcgttta gatattgatc aactatcaga tccagtggaa 420

tttttctcag ttgctgaaaa gctagaagtt gctgagaagg aaatagaaag acagaaaggt 480

ggcagtatcc atgatccaga tgtgaataat cccccagcca atgctcggcg tcgtcgacca 540

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gatgatgcat ttatatcatc tcaagagaca ttggaggatg atattctggt ggaacatggt 660

tctcagttgc cagaagagct ccatggcctc aatgttgaac tacaagaagc agaactcaca 720

gggccaataa agaaatcaga aaacagaatc aacaagatac tggatgaatt actgtctggt 780

agtggtgaag atctagaccg ggacatggca gtatccaaat tgcaagagca gttgaagatc 840

aagcccattg aactaggaac tttatgtatt cctgagttcc ctgtgactgg aaagtttgat 900

ggtaaggctc ttggagagag aattcagaag cctagtaagt ttttttgga gatagcggag 960

ttggttaaaa gtgcaactga gggaacgcca tcctctcaca aacagcatga agaaagtcct 1020

gccagtaaat tagcatcacc cactccacca aaaagtccat ttggttcatt gtctttgttg 1080

aaaaagaaac ttatgcagtc aaatccactg agagatcctt tttcgcctct caacattgat 1140

ctgcaatcag agcatccaga ttggtccgcg aagaagaaat cacaatgtgt aaataataat 1200

gttggaccta ttgaatctcg tggttgtgaa aatacaaata ttatggtccc tttaagaggt 1260

tcagacttag tgcatgagca accaattgaa aagaatccag gcagagatag tgtaaaaact 1320

gggccaaatg gatctcgatc tggaatggag caacataatg gctatgatga tattgatgcc 1380

aatactaacg acaacttaaa tatgagaaat gtggattccc accatgagtc tgatgggctt 1440

gacaaagtga aagatgattc agttataaag aatgttttga aggctctaca aggtcttgag 1500

accaaatcct acattgactg tcaaaagctg caggatagtg aagttcttgc tgaaacacta 1560

ccttctcttc aagcccaagg aaaagctgtc gatacagcca attataccat agaaacagct 1620

gttgaggatt ttggatcaac tgaaattgat ccgctggttg acaatgtgct accagaaaca 1680

gcgccttctg cagaacaaga tcattacttt gaggattctg tcaaagactt aaatagtgat 1740

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aaaggcgaaa gattattata tggacgggtt gatgagggtt tgaagcttgt tggcttgaaa 2040

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gacctagttg atttggcagc tcgctattga 2130

<210> 10
<211> 709
<212> PRT
<213> Solanum lycopersicum

<400> 10

Met Val Asn Glu Ala Leu Ile Ser Asp Pro Val Asp Pro Leu His Ser
1 5 10 15

Leu Ala Gly Leu Ser Leu Leu Pro Thr Thr Val Arg Val Ser Thr Gly
20 25 30

Ala Ser Val Ser Val Asp Ser Lys Asp Leu Glu Ser Ile His Asn Phe
35 40 45

Met Lys Ser Met Glu Thr Lys Gly Pro Gly Leu Leu Glu Glu Ala Arg
50 55 60

Glu Ile Val Asp Asn Gly Ala Glu Leu Leu Asn Thr Lys Phe Thr Ser
65 70 75 80

Phe Ile Arg Ser Lys Gly Ile Asp Gly Asp Leu Ala Ile Lys Gly Lys
85 90 95

Glu Lys Val Gln Glu Arg Arg Pro Gly Leu Gly Arg Lys Arg Ala Arg
100 105 110

Phe Ser Leu Lys Pro Ser Thr Ser Gln Pro Thr Val Ser Ile Ala Pro
115 120 125

Arg Leu Asp Ile Asp Gln Leu Ser Asp Pro Val Glu Phe Phe Ser Val
130 135 140

Ala Glu Lys Leu Glu Val Ala Glu Lys Glu Ile Glu Arg Gln Lys Gly
145 150 155 160

Gly Ser Ile His Asp Pro Asp Val Asn Asn Pro Pro Ala Asn Ala Arg
165 170 175

Arg Arg Arg Pro Gly Ile Leu Gly Lys Ser Val Lys Tyr Lys His Arg
180 185 190

Phe Ser Ser Ser Gln Pro Glu Asn Asp Asp Ala Phe Ile Ser Ser Gln
195 200 205

Glu Thr Leu Glu Asp Asp Ile Leu Val Glu His Gly Ser Gln Leu Pro
210 215 220

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Glu Glu Leu His Gly Leu Asn Val Glu Leu Gln Glu Ala Glu Leu Thr
225                 230                 235                 240

Gly Pro Ile Lys Lys Ser Glu Asn Arg Ile Asn Lys Ile Leu Asp Glu
                245                 250                 255

Leu Leu Ser Gly Ser Gly Glu Asp Leu Asp Arg Asp Met Ala Val Ser
            260                 265                 270

Lys Leu Gln Glu Gln Leu Lys Ile Lys Pro Ile Glu Leu Gly Thr Leu
        275                 280                 285

Cys Ile Pro Glu Phe Pro Val Thr Gly Lys Phe Asp Gly Lys Ala Leu
    290                 295                 300

Gly Glu Arg Ile Gln Lys Pro Ser Lys Phe Phe Leu Glu Ile Ala Glu
305                 310                 315                 320

Leu Val Lys Ser Ala Thr Glu Gly Thr Pro Ser Ser His Lys Gln His
                325                 330                 335

Glu Glu Ser Pro Ala Ser Lys Leu Ala Ser Pro Thr Pro Pro Lys Ser
            340                 345                 350

Pro Phe Gly Ser Leu Ser Leu Leu Lys Lys Lys Leu Met Gln Ser Asn
        355                 360                 365

Pro Leu Arg Asp Pro Phe Ser Pro Leu Asn Ile Asp Leu Gln Ser Glu
    370                 375                 380

His Pro Asp Trp Ser Ala Lys Lys Lys Ser Gln Cys Val Asn Asn Asn
385                 390                 395                 400

Val Gly Pro Ile Glu Ser Arg Gly Cys Glu Asn Thr Asn Ile Met Val
                405                 410                 415

Pro Leu Arg Gly Ser Asp Leu Val His Glu Gln Pro Ile Glu Lys Asn
            420                 425                 430

Pro Gly Arg Asp Ser Val Lys Thr Gly Pro Asn Gly Ser Arg Ser Gly
        435                 440                 445

Met Glu Gln His Asn Gly Tyr Asp Asp Ile Asp Ala Asn Thr Asn Asp
    450                 455                 460

Asn Leu Asn Met Arg Asn Val Asp Ser His His Glu Ser Asp Gly Leu
465                 470                 475                 480

Asp Lys Val Lys Asp Asp Ser Val Ile Lys Asn Val Leu Lys Ala Leu
                485                 490                 495
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Gln Gly Leu Glu Thr Lys Ser Tyr Ile Asp Cys Gln Lys Leu Gln Asp
            500                 505                 510

Ser Glu Val Leu Ala Glu Thr Leu Pro Ser Leu Gln Ala Gln Gly Lys
        515                 520                 525

Ala Val Asp Thr Ala Asn Tyr Thr Ile Glu Thr Ala Val Glu Asp Phe
    530                 535                 540

Gly Ser Thr Glu Ile Asp Pro Leu Val Asp Asn Val Leu Pro Glu Thr
545                 550                 555                 560

Ala Pro Ser Ala Glu Gln Asp His Tyr Phe Glu Asp Ser Val Lys Asp
                565                 570                 575

Leu Asn Ser Asp Gln Leu Asn Ser Val Gly Val Glu Val Pro Ser Arg
            580                 585                 590

Asp Val Arg Pro Lys Phe Pro Glu Met Ser Pro Gln His His Lys Gln
        595                 600                 605

Ala Lys Asp Lys Gln Gln Lys Ala Lys Glu Leu Ala Val Gly Arg Arg
    610                 615                 620

Glu Arg Lys His Leu Ser Ser Arg Pro Ser Leu Ala Asp Ala Gly Thr
625                 630                 635                 640

Ser Phe Glu Ser Gly Val Arg Arg Ser Lys Arg Met Lys Thr Arg Pro
                645                 650                 655

Leu Glu Tyr Trp Lys Gly Glu Arg Leu Leu Tyr Gly Arg Val Asp Glu
            660                 665                 670

Gly Leu Lys Leu Val Gly Leu Lys Tyr Ile Ser Pro Gly Lys Gly Ser
        675                 680                 685

Phe Lys Val Lys Ser Tyr Ile Pro Asp Asp Tyr Lys Asp Leu Val Asp
    690                 695                 700

Leu Ala Ala Arg Tyr
705
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<210> 11
<211> 6220
<212> DNA
<213> Solanum lycopersicum

<400> 11

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gacctagagt caattcacaa tttcatgaag tccatggtaa ttttccgttt gtttttcttc      180
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acttctttag atttctagct cttttataga tcttacatgt taatttagag gagaagtagt 240

gttaatgtcg ttactatgga gttttcgctg ttcttcggtt cgttcctgca ctgaactcca 300

acttacaatt agtacaatta aaatggagtc taacttacat aattgttgta gtttgtccgt 360

ttacaattgg ctagttatat gattttggat gatgttgttc tccttcttta tgataataaa 420

aggaaaccaa gggtcctggg ctcttggagg aggctaggga aattgtggat aacggtgcgg 480

agcttttgaa taccaagttt acaagtttca tacgatctaa aggcattgat ggggatcttg 540

caatcaaggg aaaggagaag gtccaggaga gaagaccagg acttgggcgt aaaagggctc 600

gattttccct caagcctagt acaaggtaaa ttatagacag tttttcttta ttctctaatt 660

agatcctatt gaaaccatgg tgttagctta ccttgctatg cagtcaacct actgtcagca 720

tagctcctcg tttagatatt gatcaactat cagatccagt ggaatttttc tcagttgctg 780

aaaagctaga aggtgatttt gtttcttttg ttatctctta actatctgct tgcaaatttg 840

atccgtcggc aactaaaaac agtatatatt atacttagaa aaataacagt tttagtgttt 900

tacatatggt gaacttacag ttgctgagaa ggaaatagaa agacagaaag gtggcagtat 960

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Gln Ala Arg Ser Ile Leu Asp Gly Asn Ser Asn Ser Met Ile Ser Glu
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Ala Ala Thr Phe Leu Val Lys Asn Glu Lys Asn Glu Glu Ala Thr Val
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Lys Ala Glu Glu Asn Pro Gln Glu Arg Arg Pro Ala Leu Asn Arg Lys
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Arg Ala Arg Phe Ser Leu Lys Pro Asp Ala Arg Gln Pro Pro Val Asn
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Leu Glu Pro Thr Phe Asp Ile Lys Gln Leu Lys Asp Pro Glu Glu Phe
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Phe Leu Ala Tyr Glu Lys His Glu Asn Ala Lys Lys Glu Ile Gln Lys
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Gln Thr Gly Ala Val Leu Lys Asp Leu Asn Gln Gln Asn Pro Ser Thr
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Asn Thr Arg Gln Arg Arg Pro Gly Ile Leu Gly Arg Ser Val Arg Tyr
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Lys His Gln Tyr Ser Ser Ile Xaa Thr Glu Asp Asp Gln Asn Val Asp
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Pro Ser Gln Val Thr Phe Asp Ser Gly Ile Phe Ser Pro Leu Lys Leu
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Gly Thr Glu Thr His Pro Ser Pro His Ile Ile Asp Ser Glu Lys Lys
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Thr Asp Glu Asp Val Ala Phe Glu Glu Glu Glu Glu Glu Glu Glu Leu
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Val Ala Ser Ala Thr Lys Ala Glu Asn Arg Val Asn Asp Ile Leu Asp
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Glu Phe Leu Ser Gly Asn Cys Glu Asp Leu Glu Gly Asp Arg Ala Ile
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Asn Ile Leu Gln Glu Arg Leu Gln Ile Lys Pro Leu Thr Leu Glu Lys
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Leu Gln Lys Ile Glu Xaa Leu Lys Ser Lys Gln Asp Asn Glu Asn Leu
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Val Asn Pro Val Ser Thr Pro Ser Ser Met Arg Ser Pro Leu Ala Ser
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Leu Ser Ala Leu Asn Arg Arg Ile Ser Leu Ser Asn Ser Ser Xaa Asp
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Ser Phe Ser Ala His Gly Ile Asp Gln Ser Pro Ala Arg Asp Pro Tyr
385                 390                 395                 400

Leu Phe Glu Leu Gly Asn His Leu Ser Asp Ala Val Gly Ile Thr Glu
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Gln Ser Ser Val Ser Lys Leu Lys Pro Leu Leu Thr Arg Asp Gly Gly
            420                 425                 430

Thr Val Ala Asn Gly Ile Lys Pro Ser Lys Ile Leu Ser Gly Asp Asp
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Ser Met Ser Lys Ile Ser Ser Ser Asn Ile Leu Asn Val Pro Gln Val
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Gly Gly Asn Thr Ala Leu Ser Gly Thr Tyr Ala Ser Thr Glu Ala Lys
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Asn Val Ser Gly Ser Ser Thr Asp Val Glu Ile Asn Glu Lys Leu Ser
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Cys Leu Glu Ala Gln Ala Asp Ala Val Ala Asn Met Gln Ile Glu Asp
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His Glu Gly Ser Ala Ser Glu Gln Pro Lys Leu Ser Glu Val Asp Leu
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Ile Lys Glu Tyr Pro Val Gly Ile Arg Ser Gln Leu Asp Gln Ser Thr
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Thr Thr Thr Cys Ala Glu Asn Ile Val Asp Gly Xaa Ser Arg Ser Ser
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Gly Thr Asp His His Asp Gly Glu Gln Val Lys Pro Lys Ser Arg Ala
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Gly Ala Gly Thr Thr Trp Gln Ser Gly Val Arg Arg Ser Thr Arg Phe
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Lys Thr Arg Pro Leu Glu Tyr Trp Lys Gly Glu Arg Leu Leu Tyr Gly
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Arg Val His Glu Ser Leu Ala Thr Val Ile Gly Leu Lys Tyr Val Ser
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35 40 45

His Thr Leu Leu Glu Ser Met Pro Phe Glu Ile Gln Lys Glu His Gln
50 55 60

Glu Gln Ala Lys Ala Ile Leu Glu Asp Val Asn Val Asp Val Lys Leu
65 70 75 80

Asn Pro Ile Pro Asn Lys Arg Glu Arg Arg Pro Gly Leu Asp Arg Lys
85 90 95

Arg Lys Ser Phe Ser Leu Xaa Leu Thr Thr Ser Gln Pro Pro Pro Val
100 105 110

Ala Pro Ser Phe Asp Pro Ser Lys Tyr Pro Lys Pro Glu Asp Tyr Phe
115 120 125

Ala Ala Tyr Asp Lys Phe Glu Leu Ala Asn Arg Glu Trp Gln Lys Gln
130 135 140

Thr Gly Ser Ser Val Ile Asp Thr Gln Gln Asn Pro Pro Ser Arg Arg
145 150 155 160

Pro Arg Arg Pro Gly Ile Pro Gly Arg Lys Arg Gly Pro Tyr Lys His
165 170 175

Thr Tyr Thr Asp Ser Tyr Phe Thr Asp Ala Ile Asn Leu Glu Ala Ser
180 185 190

Glu Lys Glu Asn Pro Ile Pro Ser Glu Gln Ser Leu Glu Xaa Thr Thr
195 200 205

Ala Ala His Val Thr Thr Xaa Asp Arg Glu Val Asp Asp Ser Thr Val
210 215 220

Asp Thr Asp Lys Asp Leu Asn Asn Ile Leu Thr Glu Leu Leu Ala Cys
225 230 235 240

Ser Xaa Asp Glu Leu Glu Gly Asp Ala Ala Val Lys Leu Leu Glu Xaa
245 250 255

Arg Leu Gln Ile Lys Pro Phe Asn Glu Lys Phe Asp Ile Pro Xaa Phe
260 265 270

Gln Asp Val Arg Xaa Met Asp Leu Lys Ala Ser Gly Ser Asn Pro Ser
275 280 285

Asn Arg Lys Ser Ala Leu Ser Xaa Ile Gln Asn Leu Leu Lys Gly Ile
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Asn Arg Asp Ala Val Arg Lys Asn Ser His Ser Pro Ser Pro Lys His

305 310 315 320

Phe Ser Ser Pro Asn Pro Pro Glu Asp Gln Phe Ser Phe Pro Asp Ile
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His Asn Leu Leu Pro Gly Asp Gln Gln Pro Ser Glu Val Asp Ile Gln
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Pro Ala Lys Asp Leu Asn Ile Gly Ser Ser Xaa Ala Xaa Asp Ala Asp
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Lys Asp Ile Pro Asn Thr Ser Pro Ser Asn Val Gly Thr Val Asp Val
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Arg Pro Gly Leu Gly Leu Xaa Arg Pro Arg Phe Ser Xaa Lys Pro Thr
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Lys Lys Pro Ser Val Glu Asp Leu Leu Pro Ile Leu Asp Leu Xaa Ala
65 70 75 80

Leu Lys Asp Pro Ala Glu Phe Phe Xaa Ala His Glu Arg Leu Glu Asn
85 90 95

Ala Lys Arg Glu Ile Gln Lys Gln Leu Gly Ala Ser Glu Ser Asp Gln
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Asp Ser Thr Xaa Pro Arg Xaa Arg Arg Pro Gly Leu Xaa Gly Asn Arg
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Xaa Pro Ile Arg Tyr Xaa His Arg Tyr Xaa Thr Glu Thr Xaa Asp Asp
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Val Leu Ser Ser Gln Glu Ala Xaa Xaa Ser Xaa Ser Leu Asp Pro Val
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Xaa Xaa Asn Thr Asp Lys Xaa Xaa Ala Ser Xaa Ala Ser Xaa Glu Lys
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Glu Val Xaa Asp Ser Ser Ala Ile Xaa Gly Asn Glu Leu Asp Glu Leu
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Leu Asp Gly Leu Leu Xaa Cys Asn Ser Glu Asp Leu Glu Gly Asp Gly
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Ala Met Xaa Leu Leu Gln Glu Arg Leu Gln Ile Lys Pro Ile Ala Leu
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Glu Lys Phe Ser Val Pro Asp Phe Pro Asp Lys Gln Xaa Xaa Asp Leu
225 230 235 240

Lys Ser Leu Gln Gly Asn Lys Ser Lys Pro Arg Lys Ala Leu Ser Asp
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Ile Asp Asn Leu Leu Lys Gly Met Asn Xaa Lys Lys Thr Pro Leu Lys
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Arg Xaa Val Gln Cys Pro Val Gln Gln Leu Ala Ser Pro Thr Pro Pro
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Arg Ser Pro Phe Ala Ser Leu Leu Ser Leu Gln Lys His Ile Ser Arg
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Ser Lys Gln Ser Val Asp Pro Phe Ser Xaa His Glu Ile Asp His Xaa
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Ser Xaa Lys Asn Tyr Ser Pro Thr His Met Val Asn Pro Glu Xaa Asn
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Leu Val Gly Ser Xaa Xaa Leu Ser Asn Glu Leu Asn Ala Xaa Met Ile
            340                 345                 350

Glu Asp Val Ile Ala Xaa Gly Lys Thr Ser Leu Asp Thr Asp Arg Asn
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Gly Thr Ser Thr Ser Glu Ile Pro Arg Phe Asp Val Ser Asn Glu Pro
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His Asp Ile Asn Thr His Thr Ser Glu Ile Ser Lys Glu Asp Asn Ser
385                 390                 395                 400

Gly Lys Ser Ser Asn Asn Leu Asn Val Xaa Xaa Ile Glu Asp Ile Xaa
                405                 410                 415

Ala Val Gly Thr Ser Leu Ala Glu Asp Xaa Ala Arg Asn Cys Thr Ser
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Thr Pro Gln Lys Ser Met Val Asp Asn Ser Arg Glu Pro Arg Phe Asp
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Ala Asn Val Asp Ser Asn Glu Pro Val Xaa Met Asp Val Asp Ile Gly
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Gly Ile Ser Gly Met Gly Lys Arg Val Met Asp Asp Thr Glu Xaa Arg
465                 470                 475                 480

Gln Asn Ile Glu Pro Asn Glu Xaa Cys Gln Ser Glu Asp Lys Thr Asn
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Met Gln Thr Phe Thr Ala Ser Ile Pro Thr Asp Asp Thr Asn Leu Asn
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Val Val Pro Leu Ala Asp Gln Ser Asn Pro Xaa Gly Tyr Gln Ala Ser
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Ser Val Asp Lys Arg Xaa Arg Arg Ser Asp Asp Gly Pro Glu His Gln
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Cys Leu Gln Glu Lys Thr Asp Gly Ser Met Ala Pro Val Asn Gly Gln
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Lys Arg Val Lys Ser Arg Val Lys Ser Lys Xaa Lys Lys Leu Ser Arg
565 570 575

Lys Ser Leu Ala Asp Ala Gly Thr Ser Trp Xaa Ser Gly Leu Arg Arg
580 585 590

Ser Thr Arg Ile Arg Thr Arg Pro Leu Glu Tyr Trp Lys Gly Glu Arg
595 600 605

Xaa Val Tyr Gly Arg Val His Gln Ser Leu Ala Thr Val Ile Gly Val
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Val Glu Gln Ala Arg Met Val Leu Lys Glu His Gly Asp Ile Gln Lys
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Met Lys Asp Thr Ala Ser Lys Pro Val Pro Val Val Asp Xaa Ser Lys
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Leu Leu Asn Ile Ser Asp Pro Glu Glu Tyr Phe Met Thr Leu Asp Xaa
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Leu Glu Glu Ala Xaa Lys Glu Ile Lys Arg Leu Asn Gly Glu Val Xaa
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Lys Ala Val Leu Asn Phe Asp Pro Xaa Asp Pro Pro Lys Arg Arg Pro
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Gly Leu Leu Gly Arg Lys Ser Val Arg Ser Phe Lys Phe Ile Asp Ala
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Asp Thr Gln Asp Pro Ile Glu Val Pro Ala Ser Gln Thr Glu Thr Xaa
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Thr Gly Ser Gln Xaa Ser Gln Asp Asp Met His Ala Ser Val Ala Glu
195 200 205

Lys Asn Glu Gln Ser Val Pro Ser Ser Ser Ser Xaa Ala Ile Ser Asp
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Val Ser Gly Lys Glu Asp Ser Leu Ala Glu Lys Asp Gly Arg Asp Asp
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Leu Xaa Tyr Leu Leu Xaa Ser Leu Xaa Asn Leu Asp Glu Ser Glu Glu
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Glu Asn Phe Leu Arg Lys Thr Leu Xaa Ile Lys Glu Ile Arg Lys Ile
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Glu Lys Val Cys Leu Pro Asn Ser Ile Pro Gly Asp Arg Xaa Leu Arg
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Ser Asn Thr Glu Gln Lys Xaa Ser Met Arg Xaa His Pro Pro Glu Ser
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Leu Pro Gln Ser His Gln Xaa Arg Ile Ser Glu Leu Glu Lys His Leu
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His Asp Ser Ser Asp Val Leu Met Thr Asp Glu Ala Asn Thr Ala Ser
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Glu Ile Asp Arg Glu Thr Pro Asn Leu Gly Xaa Lys Ala Ala Asp His
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Val Leu Asp Pro Glu Pro Asn Asp His Ala Tyr Glu Arg Gln Pro Gly
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Ser Ser Leu Gly Leu Asp Thr Glu Val Ala Lys Glu Lys Glu Ala Xaa
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Gln Ser Gln Met His Gly Gly Ser Thr Lys Lys Leu Ala Xaa Asp Ser
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Ile His Ile Arg Leu Lys Ser Met Ala Ser Gln His Ser Pro Ser Lys
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Glu Lys Ala Val Glu Xaa Pro Arg Gln Arg Arg Pro Ala Leu Gly Leu
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Lys Arg Ala Arg Phe Ser Met Lys Pro Xaa Ser Ser Gln Pro Asp Thr
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Leu Phe Lys Glu Xaa Glu Xaa Tyr Glu Asn Ala Lys Lys Glu Leu Glu
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Gln Glu Thr Ser Ile Ser Pro Leu Asn His Ser Thr Xaa Ile Glu Pro
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Asn Xaa Asn Val Ala Leu Glu Glu Met Asp Leu Ala Gly Ser Leu Ala
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Xaa Cys Leu Arg Met Lys Pro Ile Glu Pro Glu Lys Ser Asp Leu Pro
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Glu Phe Xaa Ser Ile Asp Ser Ser Arg Arg Thr Leu Pro Lys Gly Asp
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Cys Leu Thr Asp Ile Asp Asn Leu Leu Lys Gly Leu Ile Ser Ser Thr
        355                 360                 365

Xaa Xaa Lys His Arg Lys Xaa Gly Ala Glu Gly Ser Ile His Xaa Ala
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Ser Pro Thr Pro Pro Lys Ser Pro Phe Ala Ser Ile Leu Asp Leu His
385                 390                 395                 400

Lys Arg Ile Leu His Ser Ser Pro Ser Ser Asp Pro Phe Ser Ala His
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Asp Ile Asp Xaa Phe Leu Pro Glu Thr Asn Pro Ser Ser Val Glu Asn
            420                 425                 430

Arg Asn Lys Gln Ser Glu Leu Val Asp Met Arg Glu Gln Ile Thr Val
        435                 440                 445

Ser Xaa Xaa Leu Lys Ser Pro Xaa Ile Xaa Xaa Asn Asp Asn Ile Glu
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Val Ala Xaa Gly Gly Ser Ser Xaa Val Asp Ile Xaa Glu Phe Ser Tyr
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