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(54) **DROUGHT TOLERANT PLANTS**

TROCKENHEITSRESISTENTE PFLANZEN

PLANTES TOLÉRANTES À LA SÉCHERESSE

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Description

FIELD OF THE INVENTION

[0001] The present invention relates to the field of plant biotechnology and plant breeding. Provided are drought tolerant plants, especially drought tolerant vegetable species such as tomato (*Solanum lycopersicum*), and methods for making genetically modified or mutant drought tolerant plants. The invention provides a novel gene, referred to as *SlPP2C1,* encoding he S1PP2C protein which is a negative regulator of absisic acid (ABA) response. Down-regulation, knock-out or silencing of the *SlPP2C1* gene results in plants having significantly increased drought tolerance. Provided are also plants, seeds, fruit and plant parts, comprising a mutant *SlPP2C1* allele in their genome and having significantly increased drought tolerance. In another embodiment methods for making drought tolerant plants comprising one or more mutant *SlPP2C1* alleles in their genome are provided herein.

BACKGROUND OF THE INVENTION

[0002] The phytohormone abscisic acid (ABA) is important for the regulation of abiotic stress responses (such as drought, salinity, cold shock, wounding, pathogen attack) and seed development and dormancy. Screens for mutants with altered abiotic stress responses or seed dormancy have been used frequently and resulted in the identification of genes important for ABA biosynthesis and ABA signal transduction. Via such a screen the *Arabidopsis* ABA-insensitive mutants *abi1-1* and *abi2-1* have been identified (Koornneef et al. 1984, Physiol Plantarum 61: 377-383).

[0003] Cloning and characterization of the *AtABI1* gene revealed that it encodes for a serine/threonine protein phosphatase type 2C (PP2C, Leung et al. 1994, Science 264: 1448-1452; Meyer et al. 1994, Science 264: 1452-1455). *AtABI2* also encodes for a protein phosphatase type 2C. *abi1-1* and *abi2-1* mutants carry mutations in the *AtABI1* and *AtABI2* genes, which result in identical Gly-to-Asp substitutions at equivalent positions (Leung et al. 1997, Plant Cell 9: 759-771). Both mutants were shown to have reduced phosphatase activity (Bertauche et al. 1996, Eur J Biochem 241: 193-200; Leung *et al.* 1997, supra), which would suggest that AtABI1 and AtABI2 are positive regulators of ABA sensitivity. However, constitutive over-expression of *AtABI1* inhibited ABA action in maize protoplast, and reduction-of-function mutants of *AtABI1* and *AtABI2* were shown to have hypersensitive responses to ABA (Scheen 1998, PNAS 95:975-980; Gosti et al. 1999, Plant Cell 11: 1897-1909 ; Merlot et al. 2001, Plant J 25:295-303). Altogether, it was therefore concluded that AtABI1 and AtABI2 are negative regulators of the ABA response. The exact mechanism by which the mutations in *abi1-1* and *abi2-1* induce ABA insensitivity is still unknown, although it might be related to the preferential nuclear localization of the mutated proteins (Moes et al. 2008, Plant J 54: 806-819). AtABI1 and AtABI2 are important for seed dormancy but also for seedling growth and regulation of stomatal aperture, suggesting that these proteins act before major branch points that control tissue-specific ABA signaling cascades (Leung *et al.* 1997, supra).

[0004] Seventy-six *PP2Cs* have been identified in *Arabidopsis,* of which one subgroup subgroup PP2C-A), consisting of nine genes, has been associated with ABA signal transduction (Schweighofer et al. 2004, Trends in Plant Science Vol 9: 236-243). Several of the *Arabidopsis* genes belonging to this group were also found to encode for negative regulators of the ABA response. For example *AtP2C-HA* (Rodriguez et al. 1998, Plant Mol Biol 38: 879-883) (also named *AtHAB1,* Saez et al. 2004, Plant J 37: 354-369) is a repressor of the ABA signalling pathway that regulates numerous ABA responses such as stomatal closure, seed germination and inhibition of vegetative growth. Also HAB2 seems to have a similar regulatory role. *AtPP2CA* (Kuhn et al. 2006, Plant Physiol 140:127-139) has also been described as being a negative regulator of ABA, with the mutant showing ABA hypersensitivity. Interestingly, the gene disruption mutant showed an ABA hypersensitive stomatal closure response, while transpiration (water loss) of the mutant was no different than in wild type plants. Also Yoshida et al. (2006, Plant Physiology 140: 115-126) studied a missense loss-of-function mutation in *AtPP2CA,* which had 1/100th protein phosphatase activity of the wild type, and whereby the mutant *Arabidopsis* plants showed ABA hypersensitivity during seed germination, but mutant plants did not show any change in drought tolerance compared to wild type (page 124, LH Column, last paragraph).

[0005] ABI1 and ABI2 are repressors of ABA signalling pathways that regulate many ABA responses, such as stomatal closure, osmotic water permeability of plasma membranes, drought-induced resistance and rhizogenesis, response to glucose, high light stress, seed germination and inhibition of vegetative growth. AHG1 (At5g51760) is a negative regulator of ABA during seed germination (Nishimura et al. 2007, Plant J 50: 935-949) and AHG3 (At3g11410) is a negative regulator of ABA during seed germination and cold acclimation. Three other, At5g9220, At2g29380 and Atlg07430, may after all not be involved in ABA signalling as null mutations did not reveal any change in sensitivity to ABA (Yoshida et al, 2006: Plant Physiology 140:115-126).

[0006] ABA biosynthesis and signalling is, thus, extremely complex, and although various genes have been identified in *Arabidopsis* which appear to be involved (group PP2C-A), their role in ABA dependent responses, such as abiotic stress, seed dormancy and seedling growth is to a large extent unclear. In addition, protein sequences show little conservation and amino acid sequences share little sequence identity. The genes are grouped together phylogenetically,

based on protein domains (or motifs) such as the catalytic domain (protein serine/threonine phosphatase like domain) which is typically located at the C-terminal of the proteins. The N-terminal varies considerably and may play a role in substrate binding or provide specific attachment sites to signalling complexes. In addition to the *in vivo* function being unclear, also little is known about the subcellular localization, substrates and specificity of the enzymes or how these monomeric enzymes are regulated *in vivo.*

[0007] WO2007/088234 describes that combined inactivation of ABI1 and HAB1 strengthens the response to ABA leads to *Arabidopsis* plants which are resistant to salinity and hydric stress. Tomato orthologs of ABI1 (SGN- U231558) and HAB1 (SGN-U217609) are presented in Fig. 6 and 7. See also Saez et al. 2006, Plant Physiol 141:1389-1399.

[0008] Although *abi1 hab1* double mutants lead to drought tolerance in *Arabidopsis thaliana,* there remains a need for providing genes which are suitable for generating drought tolerance in crop plants, especially in field crops (e.g. rice, maize, soybean, wheat, barley, rye, sorghum, Brassica, etc.) and vegetable crops (e.g. tomato, cucumber, onion, carrot, cabbage, cauliflower, broccoli, watermelon, melon, lettuce, leek, spinach, radish, potato, artichoke, corn salad, pumpkin, squash, bean, peas, pepper, etc.). Especially in vegetable crops water shortage can be a big problem, as roots of many crops are shallow and as products are often sold fresh and water shortage can lead to reduced vegetable quality and reduced yield. Fruit and seed vegetables, such as tomatoes, are sensitive to water shortage during flowering and during fruit- and seed development. Fruit-set can be seriously reduced by water shortage during fruit development. Common practice to deal with potential water stress situations is to irrigate crops and/or to plant cultivars or varieties with drought tolerance, in as far as these are available. Also mulches and row covers may be used.

[0009] Despite breeding efforts, tomato plants remain sensitive to drought and no commercial cultivar with drought tolerance is available.

[0010] The present invention provides new genes, referred to as *SlPP2C1,* suitable for generating drought tolerant crop plants, especially tomato plants and other vegetable plants. The present invention also provides methods of generating drought tolerant plants. Also provided are the drought tolerant plants, seeds and plant parts (harvested fruit, etc.) themselves.

GENERAL DEFINITIONS

[0011] The term "nucleic acid sequence" (or nucleic acid molecule) refers to a DNA or RNA molecule in single or double stranded form, particularly a DNA encoding a protein or protein fragment according to the invention. An "isolated nucleic acid sequence" refers to a nucleic acid sequence which is no longer in the natural environment from which it was isolated, e.g. the nucleic acid sequence in a bacterial host cell or in the plant nuclear or plastid genome.

The terms "protein" or "polypeptide" are used interchangeably and refer to molecules consisting of a chain of amino acids, without reference to a specific mode of action, size, 3 dimensional structure or origin. A "fragment" or "portion" of a S1PP2C1 protein may thus still be referred to as a "protein". An "isolated protein" is used to refer to a protein which is no longer in its natural environment, for example *in vitro* or in a recombinant bacterial or plant host cell.

[0012] The term "gene" means a DNA sequence comprising a region (transcribed region), which is transcribed into an RNA molecule (e.g. an mRNA) in a cell, operably linked to suitable regulatory regions (e.g. a promoter). A gene may thus comprise several operably linked sequences, such as a promoter, a 5' leader sequence comprising e.g. sequences involved in translation initiation, a (protein) coding region (cDNA or genomic DNA) and a 3'non-translated sequence comprising e.g. transcription termination sites.

A "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). A "cis-gene" is a chimeric gene wherein preferably all of the gene sequences, but at least the transcribed sequence, are/is from a plant species which is sexually compatible with the species into which the gene is introduced.

"Expression of a gene" refers to the process wherein a DNA region, which is operably linked to appropriate regulatory regions, particularly a promoter, is transcribed into an RNA, which is biologically active, i.e. which is capable of being translated into a biologically active protein or peptide (or active peptide fragment) or which is active itself (e.g. in post-transcriptional gene silencing or RNAi). The coding sequence may be in sense-orientation and encodes a desired, biologically active protein or peptide, or an active peptide fragment. In gene silencing approaches, the DNA sequence is preferably present in the form of an antisense DNA or an inverted repeat DNA, comprising a short sequence of the target gene in antisense or in sense and antisense orientation (inverted repeat). "Ectopic expression" refers to expression in a tissue in which the gene is normally not expressed.

An "active protein" or "functional protein" is a protein which has protein activity as measurable *in vitro,* e.g. by an *in vitro*

activity assay, and/or *in vivo,* e.g. by the phenotype conferred by the protein. A "wild type" protein is a fully functional protein, as present in the wild type plant. A "mutant protein" is herein a protein comprising one or more mutations in the nucleic acid sequence encoding the protein, whereby the mutation results in (the mutant nucleic acid molecule encoding) a "reduced-function" or "loss-of-function" protein, as e.g. measurable by the protein activity *in vitro* compared to the activity of the wild type protein, e.g. by an activity assay, and/or *in vivo,* e.g. by the phenotype conferred by the mutant allele.

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

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

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

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

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

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

"Silencing" refers to a down-regulation or completely inhibition of gene expression of the target gene or gene family.

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

A "sense" RNA transcript is generally made by operably linking a promoter to a double stranded DNA molecule wherein the sense strand (coding strand) of the DNA molecule is in 5' to 3' orientation, such that upon transcription a sense RNA is transcribed, which has the identical nucleotide sequence to the sense DNA strand (except that T is replaced by U in the RNA). An "antisense" RNA transcript is generally made by operably linking a promoter to the complementary strand (antisense strand) of the sense DNA, such that upon transcription an antisense RNA is transcribed.

A "transcription regulatory sequence" is herein defined as a nucleic acid sequence that is capable of regulating the rate of transcription of a (coding) sequence operably linked to the transcription regulatory sequence. A transcription regulatory sequence as herein defined will thus comprise all of the sequence elements necessary for initiation of transcription (promoter elements), for maintaining and for regulating transcription, including e.g. attenuators or enhancers. Although mostly the upstream (5') transcription regulatory sequences of a coding sequence are referred to, regulatory sequences found downstream (3') of a coding sequence are also encompassed by this definition.

As used herein, the term "promoter" refers to a nucleic acid fragment that functions to control the transcription of one or more genes, located upstream with respect to the direction of transcription of the transcription initiation site of the gene, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter. A "constitutive" promoter is a promoter that is active in most tissues under most physiological and developmental conditions. An "inducible" promoter is a promoter that is physiologically (e.g. by external application of certain compounds) or developmentally regulated. A "tissue specific" promoter is only active in specific types of tissues or cells.

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

The term "domain" as used herein means any part(s) or domain(s) of the protein with a specific structure or function that

can be transferred to another protein for providing a new hybrid protein with at least the functional characteristic of the domain. Specific domains can also be used to identify protein members belonging to the S1PP2C1 group of proteins, such as S1PP2C 1 orthologs from other plant species. Examples of domains found in S1PP2C1 proteins are the serine/threonine phosphatase 2C (PP2C or PP2C-like) catalytic domain comprises within about amino acid 84-391 of SEQ ID NO: 2 or the N-terminal region of the S1PP2C protein (amino acid 1-83 of SEQ ID No: 2).

The terms "target peptide" refers to amino acid sequences which target a protein to intracellular organelles such as plastids, preferably chloroplasts, mitochondria, or to the extracellular space (secretion signal peptide). A nucleic acid sequence encoding a target peptide may be fused (in frame) to the nucleic acid sequence encoding the amino terminal end (N-terminal end) of the protein.

A "nucleic acid construct" or "vector" is herein understood to mean a man-made nucleic acid molecule resulting from the use of recombinant DNA technology and which is used to deliver exogenous DNA into a host cell. The vector backbone may for example be a binary or superbinary vector (see e.g. US5591616, US2002138879 and WO9506722), a co-integrate vector or a T-DNA vector, as known in the art and as described elsewhere herein, into which a chimeric gene is integrated or, if a suitable transcription regulatory sequence is already present, only a desired nucleic acid sequence (e.g. a coding sequence, an antisense or an inverted repeat sequence) is integrated downstream of the transcription regulatory sequence. Vectors usually comprise further genetic elements to facilitate their use in molecular cloning, such as e.g. selectable markers, multiple cloning sites and the like (see below).

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 at least one nucleic acid molecule, especially comprising a chimeric gene encoding a desired protein or a nucleic acid sequence which upon transcription yields an antisense RNA or an inverted repeat RNA (or hairpin RNA) for silencing of a target gene/gene family, having been introduced into said cell. The host cell is preferably a plant cell or a bacterial cell. The host cell may contain the nucleic acid construct as an extra-chromosomally (episomal) replicating molecule, or more preferably, comprises the chimeric gene integrated in the nuclear or plastid genome of the host cell.

The term "selectable marker" is a term familiar to one of ordinary skill in the art and is used herein to describe any genetic entity which, when expressed, can be used to select for a cell or cells containing the selectable marker. Selectable marker gene products confer for example antibiotic resistance, or more preferably, herbicide resistance or another selectable trait such as a phenotypic trait (e.g. a change in pigmentation) or a nutritional requirements. The term "reporter" is mainly used to refer to visible markers, such as green fluorescent protein (GFP), eGFP, luciferase, GUS and the like.

The term "ortholog" of a gene or protein refers herein to the homologous gene or protein found in another species, which has the same function as the gene or protein, but (usually) diverged in sequence from the time point on when the species harbouring the genes diverged (i.e. the genes evolved from a common ancestor by speciation). Orthologs of the tomato *SlPP2C1* gene may thus be identified in other plant species based on both sequence comparisons (e.g. based on percentages sequence identity over the entire sequence and/or over specific domains) and/or functional analysis.

The terms "homologous" and "heterologous" refer to the relationship between a nucleic acid or amino acid sequence and its host cell or organism, especially in the context of transgenic organisms. A homologous sequence is thus naturally found in the host species (e.g. a tomato plant transformed with a tomato gene), while a heterologous sequence is not naturally found in the host cell (e.g. a tomato plant transformed with a sequence from potato plants). Depending on the context, the term "homolog" or "homologous" may alternatively refer to sequences which are descendent from a common ancestral sequence (e.g. they may be orthologs).

"Stringent hybridisation conditions" can be used to identify nucleotide sequences, which are substantially identical to a given nucleotide sequence. Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequences at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridises to a perfectly matched probe. Typically stringent conditions will be chosen in which the salt concentration is about 0.02 molar at pH 7 and the temperature is at least 60°C. Lowering the salt concentration and/or increasing the temperature increases stringency. Stringent conditions for RNA-DNA hybridisations (Northern blots using a probe of e.g. 100nt) are for example those which include at least one wash in 0.2X SSC at 63°C for 20min, or equivalent conditions. Stringent conditions for DNA-DNA hybridisation (Southern blots using a probe of e.g. 100nt) are for example those which include at least one wash (usually 2) in 0.2X SSC at a temperature of at least 50°C, usually about 55°C, for 20 min, or equivalent conditions. See also Sambrook *et al.* (1989) and Sambrook and Russell (2001).

"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 or the Emboss program "Needle" (using default parameters, see below) share at least a certain minimal percentage of sequence identity (as defined further below). These programs use the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length, maximizing the number of matches and minimises the number of gaps. Generally, the

default parameters are used, with a gap creation penalty = 10 and gap extension penalty = 0.5 (both for nucleotide and protein alignments). 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 for example 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 EMBOSS (http://www.ebi.ac.uk/Tools/webservices/services/ emboss). Alternatively percent similarity or identity may be determined by searching against databases such as FASTA, BLAST, etc., but hits should be retrieved and aligned pairwise to compare sequence identity.

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

As used herein, the term "plant" includes the whole plant or any parts or derivatives thereof, such as plant organs (e.g., harvested or non-harvested storage organs, bulbs, tubers, fruits, leaves, etc.), plant cells, plant protoplasts, plant cell tissue cultures from which whole 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, fruits (e.g., harvested tissues or organs, such as harvested tomatoes, etc.), tubers (e.g. potatoes) flowers, leaves, seeds, tubers, bulbs, clonally propagated plants, roots, root-stocks, stems, root tips and the like. Also any developmental stage is included, such as seedlings, immature and mature, etc.

[0013] "Plant variety" is a group of plants within the same botanical taxon of the lowest grade known, which (irrespective of whether the conditions for the recognition of plant breeder's rights are fulfilled or not) can be defined on the basis of the expression of characteristics that result from a certain genotype or a combination of genotypes, can be distinguished from any other group of plants by the expression of at least one of those characteristics, and can be regarded as an entity, because it can be multiplied without any change. Therefore, the term "plant variety" cannot be used to denote a group of plants, even if they are of the same kind, if they are all characterized by the presence of 1 locus or gene (or a series of phenotypical characteristics due to this single locus or gene), but which can otherwise differ from one another enormously as regards the other loci or genes.

[0014] "F1, F2, etc." refers to the consecutive related generations following a cross between two parent plants or parent lines. The plants grown from the seeds produced by crossing two plants or lines is called the F1 generation. Selfing the F1 plants results in the F2 generation, etc. "F1 hybrid" plant (or F1 seed) is the generation obtained from crossing two inbred parent lines. An "M1 population" is a plurality of mutagenized seeds / plants of a certain plant line or cultivar. "M1, M2, M3, M4, etc." refers to the consecutive generations obtained following selfing of a first mutagenized seed / plant (M1).

[0015] The term "allele(s)" means any of one or more alternative forms of a gene at a particular locus, all of which alleles relate to one trait or characteristic at a specific locus. In a diploid cell of an organism, alleles of a given gene are located at a specific location, or locus (loci plural) on a chromosome. One allele is present on each chromosome of the pair of homologous chromosomes. A diploid plant species may comprise a large number of different alleles at a particular locus. These may be identical alleles of the gene (homozygous) or two different alleles (heterozygous).

The term "locus" (loci plural) means a specific place or places or a site on a chromosome where for example a gene or genetic marker is found. The *SlPP2C1* locus is thus the location in the genome where the *SlPP2C1* gene is found.

"Wild type allele" (WT) refers herein to a version of a gene encoding a functional protein (wild type protein). The wild type *SlPP2C1* allele is, for example, depicted in SEQ ID NO: 1. "Mutant allele" refers herein to an allele comprising one or more mutations in the coding sequence (mRNA or cDNA) or genomic sequence compared to the wild type allele. Such mutation(s) (e.g. insertion, inversion, deletion and/or replacement of one or more nucleotides) may lead to the encoded protein having reduced *in vitro* and/or *in vivo* functionality (reduced function) or no *in vitro* and/or *in vivo* functionality (loss of function), e.g. due to the protein e.g. being truncated or having an amino acid sequence wherein one or more amino acids are deleted, inserted or replaced. Such changes may lead to the protein having a different 3D conformation, being targeted to a different sub-cellular compartment, having a modified catalytic domain, having a modified substrate affinity and/or specificity, etc.

"Drought tolerance" or "significantly enhanced drought tolerance" or "drought tolerant plant" refers herein to a (on average) significantly enhanced ability of a plant line, cultivar or variety to withstand water shortage / water stress / drought compared to suitable control plants (e.g. wild type plants), i.e. the symptoms associated with water stress (e.g. wilting of the leaves) are (statistically) significantly reduced in drought tolerant plants compared to the controls exposed to the same water stress conditions and/or (average) recovery and/or survival rate and/or harvestable yield of the plants after exposure to water shortage / water stress / drought conditions is significantly increased compared to the control plants, such as wild type plants. There are various methods for determining whether a plant is drought tolerant, as will be

explained elsewhere herein. Preferably a drought tolerant plant has significantly enhanced drought tolerance during all developmental stages, but at least during one of the following developmental stages: as mature plant, during flowering or anthesis, during fruit set and fruit development, during seed development, during fruit ripening. Preferably the plant has in addition also drought tolerance at least at the seed stage and/or during the seedling stage and/or from seedling stage to (and including) mature plant.

"Wild type plant" refers herein to a plant comprising a wild type (WT) *SlPP2C1* allele encoding a functional protein (e.g. in contrast to "mutant plants", comprising a mutant *SlPP2C1* allele). Such plants are suitable controls in phenotypic assays. Preferably wild type and/or mutant plants are "cultivated plants", i.e. varieties, breeding lines or cultivars of a species, cultivated by humans and having good agronomic characteristics; preferably such plants are not "wild plants", i.e. plants which generally have much poorer yields and poorer agronomic characteristics than cultivated plants and e.g. grow naturally in wild populations. "Wild plants" include for example ecotypes, PI (Plant Introduction) lines, landraces or wild accessions or wild relatives of a species, or so-called heirloom varieties or cultivars, i.e. varieties or cultivars commonly grown during earlier periods in human history, but which are not used in modern agriculture.

"Drought" refers preferably both to short term water shortage or stress (artificial, e.g. no irrigation of the soil and/or natural, e.g. no rain fall), e.g. equal to or less than 30 days, 20 days, 15, days, 14 days, 10 days, 9, 8, 7, 6, 5 days, or less and/or long term water shortage or stress (artificial, e.g. no irrigation of the soil and/or natural, e.g. no rain fall), e.g. equal to or more than 31 days, 35 days, 40 days, 45 days, 50 days, 60 days, 70 days, 80 days, 90 days or more.

"Variants" of the *SlPP2C1* gene or protein include both natural allelic variants found within the species *S. lycopersicum,* as well as orthologs found in other plant species, such as other dicotyledonous plant species, or monocotyledonous species.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The present inventors set out to study genes which are differentially expressed during tomato fruit set, by carrying out transcriptome analysis (cDNA-AFLP) of pollinated ovaries and GA_3 (gibberellic acid) treated ovaries (Vriezen et al. 2008, New Phytologist 177:60-76). One gene, which was relatively highly expressed in unpollinated ovaries and less expressed in pollinated ovaries, correlating with ABA (abscisic acid) levels (high in unpollinated ovaries and low after pollination) was characterized further by generating transgenic plants, in order to study the role of this gene in ABA signalling. The tomato gene was named *SlPP2C1* (*Solanum lycopersicum PP2C1),* as it encodes a protein of 397 amino acids which comprises a serine/threonine phosphatase 2C catalytic domain in the C-terminal region between about amino acid 84 to 391 (as determined by InterProScan).

[0017] It was found that *SlPP2C1* is involved in ABA signalling and that over-expression of this gene under control of the CaMV 35S promoter leads to plants which are less sensitive to the phytohormone ABA than wild type plants, while plants in which the gene was co-suppressed (having significantly lower *SlPP2C1* transcript levels in at least leaf tissue than wild type plants) had a higher ABA sensitivity (see Examples). Reduced levels of the (wild type) S1PP2C1 protein, thus, enhanced ABA sensitivity, meaning that the S1PP2C1 protein is a negative regulator of ABA signalling.

[0018] Surprisingly, it was further found that the tomato plants in which *SlPP2C1* transcript levels were significantly reduced compared to wild type plants showed no wilting at all after 9 days of water deprivation, while wild type plants had wilted leaves and *SlPP2C1* over-expressing plants showed severe signs of wilting. This was even more surprising as the *SlPP2C1* gene and S1PP2C1 protein did not have any significant sequence identity with any known negative regulators of ABA that have been shown to play a role in drought tolerance in *Arabidopsis,* such as ABI1 and HAB1, or with any of the proteins of group A, see Table 1 below.

Table 1 - Sequence identity between *Arabidopsis* proteins (putatively) involved in ABA signalling (Schweigerhofer *et al.* 2004, supra, see Figure 1, group A therein) and S1PP2C1 protein

Arabidposis PP2C subgroup A	S1PP2C1 protein (% sequence identity)
At5g51760 (AHG1)	32.3
At3g11410 (AtPP2CA, AHG3)	38.8
At5g59220	38.6
Atlg07430 (AIP1)	37.5
At2g29380	39.8
At1g17550 (HAB2)	33.5
Atlg72770 (HAB1)	32.0

(continued)

Arabidposis PP2C subgroup A	S1PP2C1 protein (% sequence identity)
At4g26080 (ABI1)	36.0
At5g57050 (ABI2)	37.6
Sequence identity is determined using pairwise alignments over the entire length with Needle (Emboss), Blossum 62 matrix, GAP opening penalty = 10, GAP extension penalty = 0.5	

[0019] Sequence identity of S1PP2C1 to *Arabidopsis* PP2C, subgroup A, proteins is, thus, low. Notably, null mutations (T-DNA insertions, whereby no mRNA was detectable by RT-PCR) in the protein to which the highest similarity is found, *At2g29380,* did not show any changes in ABA sensitivity in *Arabidopsis* (Yoshida et al, 2006, Plant Physiology 140:115-126). In addition, this gene is expressed in roots and siliques of *Arabidopsis,* but not in leaves and inflorescences (see Fig.5 of Xue et al. 2008. BMC Genomics 9:550).

[0020] The finding that *SlPP2C1* is involved in drought tolerance in tomato can be used to generate transgenic and/or non-transgenic plants with enhanced drought tolerance and, preferably, desired agronomic characteristics. The different embodiments of the invention are described herein below and in the non-limiting Examples. Parts described herein as being applicable to transgenic approaches are generally also applicable to non-transgenic approaches and *vice versa,* unless indicated otherwise.

In one embodiment transgenic plants of the species *Solanum lycopersicum* in which endogenous *SlPP2C1* expression is down-regulated or silenced, at least in leaf tissue, and which are drought tolerant are provided. In another embodiment non-transgenic plants of the species *Solanum lycopersicum* comprising one or more mutant *SlPP2C1* alleles (either in homozygous or heterozygous form) and wherein said mutant allele(s) encode(s) an S1PP2C1 protein which has reduced functionality *in vitro* and/or *in vivo* compared to the wild type protein, or even no functionality, and whereby the mutation results in the plants (mutant line or progeny thereof) having significantly enhanced drought tolerance compared to plants lacking the mutant allele(s) (wild type plants), are provided herein. Such non-transgenic, drought tolerant plants are in one embodiment of the invention generated using TILLING or Eco-TILLING, but can also be generated using other known mutagenesis methods combined with breeding methods. Thus, in one embodiment the mutant *SlPP2C1* allele is induced and identified by humans, using mutagenesis techniques ("induced mutant"), while in another embodiment of the invention the mutant *SlPP2C1* allele is a "natural mutant", meaning it is found in natural plant populations. "Induced mutants" are preferably generated in cultivated germplasm and are thus directly present in agronomically valuable lines. On the other hand "natural mutants" or "spontaneous mutants" or "natural variants" or "natural allelic variants / variation" are based on natural variation (polymorphisms / mutations) found in a species and are, thus, likely present in plant material of inferior agronomic quality, not cultivated in modern agriculture, e.g. wild plants. The later alleles then need to be transferred into a cultivated plant having good agronomic characteristics, which is an embodiment of the invention.

[0021] Drought stress or dehydration stress is one of the most serious abiotic stresses plants have to cope with world-wide. Four-tenths of the world's agricultural land lies in arid or semi-arid regions. Apart from that, also plants grown in regions with relatively high precipitation may suffer spells of drought throughout the growing season. Many agricultural regions have low rain-fall and rely on irrigation to maintain yields and product quality. Conferring or enhancing the tolerance of crop plants to short and long spells of drought and reducing the water requirement of crops grown in irrigated agriculture is clearly important. The plants provided herein have a lower irrigation requirement and/or higher yield and/or higher percentage survival when grown in regions experiencing short drought spells or longer periods of drought.

Nucleic acid sequences and proteins according to the invention

[0022] In one disclosure nucleic acid sequences and amino acid sequences of S1PP2C1 are provided, as well as methods for isolating or identifying "variants" thereof for example allelic variants within the species (*Solanum lycopersicum*) or within the genus *Solanum,* or orthologs of S1PP2C1 of other plant species, such as other vegetable species or field crop species.

The wild type S1PP2C1 protein (derived from tomato cultivar Moneymaker) is depicted in SEQ ID NO: 2. It is a protein of 397 amino acids which comprises a (putative) serine/threonine phosphatase 2C catalytic domain in the C-terminal region between about amino acid 84 to 391. In particular, the domain comprises amino acids Asp-Xaa-Phe-Leu-Ile-Leu-Ala-Ser-Asp-Gly-Leu-Trp-Asp-Val (with Xaa being any amino acid, but preferably Glu, see amino acid 307-320 of SEQ ID NO: 2). There is also a (putative) manganese/magnesium aspartate binding site from amino acid 147 to 149 (DGH or Asp-Gly-His, where Asp binds magnesium or manganese). Alternatively, the domain comprising or consisting of amino acids 144 to 150, i.e. GVXDGHG (where X is any amino acid, preferably Y) may be involved in interaction with protein kinases. An "S1PP2C1 protein" (including "variants" thereof, such as proteins encoded by allelic variants of the gene or

by orthologs of the gene) may be defined by their amino acid sequence identity to SEQ ID NO: 2 over the entire length, i.e. proteins having a sequence identity of at least about 90%, 95%, 98%, 99% or more to SEQ ID NO: 2 (as determined by pairwise alignment using Emboss "Needle", Blossum 62 matrix, GAP opening penalty = 10, GAP extension penalty = 0.5) and having an *in vivo* function which is essentially similar to that of SlPP2C1.

Also included herein are loss-of-function mutants of the wild type S1PP2C1 proteins (or of variants thereof, as defined above) or reduced-function mutants of wild type S1PP2C1 proteins (or of variants thereof), as described elsewhere, and plants or plant parts comprising one or more nucleotides encoding such mutants and having enhanced drought tolerance compared to plants comprising a nucleic acid sequence encoding a wild type protein.

[0023] Preferably, a S1PP2C1 protein according to the invention also comprises at least a catalytic serine/threonine phosphatase domain in the C-terminal region, i.e. a domain comprising at least 60%, 70%, 80%, 90% or preferably at least 95%, 98%, 99% or more amino acid sequence identity to amino acid 84 to 391 of SEQ ID NO: 2 (as determined by pairwise alignment using Emboss "Needle", Blossum 62 matrix, GAP opening penalty = 10, GAP extension penalty = 0.5). In one embodiment an S1PP2C1 protein according to the invention comprises the sequence Asp-Xaa-Phe-Leu-Ile-Leu-Ala-Ser-Asp-Gly-Leu-Trp-Asp-Val (with Xaa being any amino acid, but preferably Glu) or a sequence which has 90% or 95% or 98% or more sequence identity to this sequence (as determined by pairwise alignment using Emboss "Needle", Blossum 62 matrix, GAP opening penalty = 10, GAP extension penalty = 0.5). Preferably an S1PP2C1 protein further comprises at least one DGH motif, i.e. at least one DGH sequence or GVXDGHG sequence (where X is any amino acid, preferably Y).

[0024] A "function which is essentially similar to the function of S1PP2C1" refers herein to the protein having a proven function in sensitivity/tolerance to drought stress and/or in determining ABA sensitivity of the plant tissue. Plants over-expressing *SlPP2C1,* or a variant thereof, in at least leaf tissue, are significantly more susceptible to drought stress than wild type plants and/or have a significantly reduced ABA sensitivity compared to controls (e.g. wild type plants or plants transformed with an empty vector). *Vice versa,* plants with reduced levels of fully functional (wild type) SlPP2C1 protein, or a variant thereof, in at least the leaf tissue, are significantly less susceptible to drought stress than wild type plants and/or have a significantly enhanced ABA sensitivity compared to controls.

[0025] Thus, the function of a (putative) S1PP2C1 protein can be tested using a variety of known methods, preferably by comparing the phenotype of transformants constitutively expressing the protein being tested to the phenotype of SlPP2C1 over-expressing transformants of the same host species (and variety) (preferably comprising a chimeric S1PP2C1 encoding gene stably integrated into the host's genome), allowing a direct comparison of the functional effect on the phenotype of the transformants.

[0026] Similarly, transformants in which the *SlPP2C1* gene (or variant) is silenced or down-regulated (e.g. mRNA of *SlPP2C1* is significantly reduced at least in leaf tissue compared to wild type or control transformants) can be used to determine the function. A "significant reduction" of the mRNA of *SlPP2C1* transcript refers to the target mRNA being present at a level of less than or equal to 90%, 80%, 70%, 60%, 50% 40%, 30%, 20% or less (10%, 5% or 0%) of the transcript level found in the wild type or control transformants (e.g. empty vector transformant). It is understood that in any transformation experiments a certain degree of variation in the phenotype of transformants is seen, normally due to position effects in the genome and/or due to copy number. A skilled person will know how to compare transformants to one another, e.g. by selecting single copy number events and analysing their phenotypes. Other methods of determining or confirming *in vivo* gene/protein function include the generation of knock-out mutants or reduced-function mutants or transient expression studies. Promoter-reporter gene expression studies may also provide information as to the spatio-temporal expression pattern and the role of the protein.

[0027] Constitutive (over)expression of a *SlPP2C1* gene, or a gene encoding a variant thereof, should result in one or more of the following phenotypic changes compared to the wild type plants or control transformants:

- Significantly increased sensitivity to water stress (i.e. significantly reduced drought tolerance) and/or
- Significantly reduced ABA sensitivity.

[0028] A "significantly increased sensitivity to water stress" or "significantly reduced drought tolerance" refers to an average, statistically significant increase of leaf wilting symptoms of a plurality of plants comprising the *SlPP2C1* allele by at least 10% compared to control levels and can, for example, be tested as described in the Examples or equivalent experiments. In short, a plurality (e.g. at least 10, 15, 20 or more of a transgenic line) of transformed plants and control plants of the same age (e.g. mature plants) are saturated with water at the start of the experiment and are then not watered for an extended period of time, e.g. at least about 7, 8, 9, 10, 11, 12, 13, 14 days or more, e.g. 3 weeks or 4 weeks or more (depending on the plant species). When the control plants start to show wilting of the leaves ("slight wilting" or "moderate wilting"), all plants are assessed for signs of wilting of the leaves using e.g. visual assessment. Wilting symptoms of leaves can, for example, be scored on a scale of 4-1, as "highly/severely wilted" (4), "moderate wilted" (3), "slightly wilted" (2) or "no wilting" (1). Plants are said to have a significantly reduced drought tolerance if (average) wilting is increased by at least 10%, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%

compared to the wild type (or empty vector transformed) control. Alternatively or in addition, field trials can be carried out to test and/or confirm whether or not a significantly reduced drought tolerance is seen in the field under spells of water-stress.

[0029] Other assays may be used, of course. For example, a period of water deprivation may be followed by a period of recovery (watering) and the percentage of plant survival may be assessed, see e.g. Zheng et al. (2009, Biochemical and Biophysical Research Communications 379: 986, LH Column). Plants are said to have a significantly reduced drought tolerance if the percentage survival is reduced by at least 10%, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to the wild type (or empty vector transformed) control. See also Xiong et al. 2006 (Plant Physiology 142: 1065-1074) and Yu et al. 2008 (The Plant Cell 20: 1134-1151).

[0030] "Significantly reduced ABA sensitivity" can also be tested as described in the Examples by testing seed germination percentages on one or more different ABA concentrations and/or root elongation on one or more different ABA concentrations. In summary, seed germination on medium comprising ABA is at least 5%, 10%, 20%, 30%, 40%, 50% or more, higher for the *SlPP2C1* overexpressing transformant than for the control seeds on the same ABA concentration. For example, on 1 or 3 μM ABA, 50% of wild type seeds germinate, while 55%, 60% or more of transformed (*SlPP2C1* overexpressing) seeds germinate. Thus, seed germination of overexpressing transformants is inhibited less by ABA. Root growth / elongation of overexpressing transformants is also inhibited less by ABA.

[0031] Down-regulation or silencing of a *SlPP2C1* gene, or a gene encoding a variant member, should result in one or more of the following phenotypic changes compared to the wild type plants or control transformants:

- Significantly reduced sensitivity to water stress (i.e. significantly enhanced drought tolerance) and/or
- significantly enhanced ABA sensitivity.

[0032] A "significantly reduced sensitivity to water stress" or "significantly enhanced drought tolerance" refers to an average, statistically significant decrease of leaf wilting symptoms of a plurality of plants, in which the *SlPP2C1* gene is down-regulated or silenced, by at least 10% compared to control levels (e.g. wild type plants or empty vector transformants) and can, for example, be tested as described in the Examples or using equivalent methods. In short, a plurality of transformed plants (e.g. at least 10, 15, 20 or more of a transgenic line) and controls of the same age are saturated with water at the start of the experiment and are then not watered for an extended period of time, e.g. at least about 7, 8, 9, 10, 11, 12, 13, 14 days or more, e.g. 3 weeks or 4 weeks or more (depending on the plant species). When the control plants start to show wilting of the leaves ("slight wilting" or "moderate wilting"), all plants are assessed for signs of wilting of the leaves using e.g. visual assessment. Wilting symptoms of leaves can, for example, be scored on a scale of 1 to 4, as "highly/severely wilted" (4), "moderately wilted" (3), "slightly wilted" (2) or "no wilting" (1). Plants are said to have a significantly enhanced drought tolerance if (average) wilting is reduced by at least 10%, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to the control plants. Alternatively or in addition field trials can be used to determine whether a significantly enhanced drought tolerance is conferred by the down-regulation or silencing of endogenous *SlPP2C1* gene(s).

[0033] Other assays may be used, of course. For example, a period of water deprivation may be followed by a period of recovery (watering) and the percentage of plant survival may be assessed, see e.g. Zheng et al. (2009, Biochemical and Biophysical Research Communications 379: 986, LH Column). Plants are said to have a significantly enhanced drought tolerance if the percentage survival is enhanced by at least 10%, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to the wild type (or empty vector transformed) control. See also Xiong et al. 2006 (Plant Physiology 142: 1065-1074) and Yu et al. 2008 (The Plant Cell 20: 1134-1151).

[0034] The above assays, or equivalent assays, can also be used to determine whether plant comprising a mutant *SlPP2C1* allele (e.g. a loss-of-function or a reduced-function mutant) has significantly enhanced drought tolerance, as will be described further down.

[0035] "Significantly enhanced ABA sensitivity" can also be tested as described in the Examples. In summary, average seed germination on medium comprising ABA is at least 10%, 20%, 30%, 40%, 50%, or more, lower for the *SlPP2C1* down-regulated or silenced transformants than for the control plants (e.g. wild type) on the same ABA concentration. For example, on 1 or 3 μM ABA, 50% of wild type seeds germinate, while less than 40%, 35%, 30%, or less, of transformed (*SlPP2C1* silenced) seeds germinate. Thus, seed germination of down-regulated or silenced transformants is inhibited more by ABA. Root growth of down-regulated or silenced transformants is also inhibited more by ABA.

[0036] The exact methods used may vary, depending for example on the plant species. Assays for determining drought tolerance and/or ABA sensitivity in vegetable species or in field crops are known in the art. It is also understood that alternative methods exist to assess the phenotypes. Such methods are within the scope of the skilled person.

[0037] The above methods can be used to test whether any putative *SlPP2C1* gene, such as an allele from a wild or cultivated tomato plant or from a tomato breeding line or PI (plant introduction) line or from a different species (e.g. tobacco, or other vegetable species or from field crop species) is indeed a *SlPP2C1* variant, which can then be used to generate transgenic and/or non-transgenic plants having (significantly) enhanced drought tolerance compared to suitable

controls, such as the wild type plant. It is understood that regarding transgenic plants, preferably plants having good agronomic characteristics are transformed and regenerated, i.e. cultivated plants (for example high yielding cultivars or breeding lines) and that the most suitable controls are empty vector transformants of the same line or a plurality of plants of the non-transformed line as such.

[0038] In addition, *in vitro* phosphatase activity assays can be carried out to test protein activity/functionality, see e.g. Gosti et al. 1999, Plant Cell 11: 1897-1910, Material and Methods - PP2C Activities, page 1907, where the protein is expressed in *E. coli* and the enzyme activity is assayed using ^{32}P labeled casein as substrate. Such activity assays are also suitable for determining whether specific types of mutant S1PP2C1 proteins (e.g. generated by TILLING or other methods, see elsewhere herein) or chimeric proteins retain all or partial functionality. See also Bertauche et al., 1996, Eur. J. Biochem 241: page 195 for a phosphates assay and page 194 for expression in *E. coli*.

[0039] A S1PP2C1 protein has "reduced function *in vitro"* if the percentage of de-phosphorylation of ^{32}P -casein by the mutant protein is equal to or less than 70% of the wild type protein (under the same assay conditions, e.g. 1 or 2 μg protein incubated with ^{32}P -casein for 2 hours at 30 degrees Celsius in the presence of 20mM magnesium acetate, and in the presence of okadaic acid), preferably equal to or less than about 60%, 50%, 40%, 30%, 20%, 10% ("reduction of function") or about 0% of the wild type protein (i.e. complete "loss of function"). A protein having reduced function *in vitro* can be used to infer that the protein also has "reduced function or no function *in vivo",* i.e. *in planta,* e.g. at least in the leaf tissue and/or fruit tissue. For example, a plant comprising one (heterozygous) or two (homozygous) alleles encoding a mutant protein having reduced or no function *in vitro* results in the plant having significantly enhanced drought tolerance (compared to plants lacking the mutant allele / having wild type alleles) and therefore also reduced function or no function *in vivo.* The *in vivo* reduced-function or loss-of-function of the protein is confirmed by drought tolerance assays (e.g. in the field or as described in the Examples) of plants homozygous or heterozygous for the mutant allele.

[0040] Other putative S1PP2C1 genes/proteins can be identified *in silico,* e.g. by identifying nucleic acid or protein sequences in existing nucleic acid or protein database (e.g. GENBANK, SWISSPROT, TrEMBL) and using standard sequence analysis software, such as sequence similarity search tools (BLASTN, BLASTP, BLASTX, TBLAST, FASTA, etc.). Putative amino acid sequences or nucleic acid sequences comprising or encoding an S1PP2C1 protein (as defined above) are selected, cloned or synthesized *de novo* and tested for *in vivo* functionality by e.g. overexpression or silencing in a plant host. It is noted that the designation SlPP2C1 is also used herein for proteins which are derived from species other than *Solanum lycopersicum,* i.e. the prefix *Sl* does herein not limit the protein as being from a particular species.

[0041] One putative ortholog of the tomato *SlPP2C1* gene and protein is the potato protein of SEQ ID NO: 15 (StPP2C1), encoded by the cDNA of SEQ ID NO: 14 (*StPP2C1*). The StPP2C1 protein has 89.1% amino acid sequence identity with S1PP2C1 and 91.5% nucleotide sequence identity with *SlPP2C1* (using Emboss-Needle, Gap opening = 10.0, Gap extension = 0.5, and Blosum62 for proteins or dnafull for nucleic acids).

[0042] The S1PP2C1 proteins according to the invention may be isolated from natural sources, synthesized *de novo* by chemical synthesis (using e.g. a peptide synthesizer such as supplied by Applied Biosystems) or produced by recombinant host cells (e.g. *E. coli*). The SlPP2C1 proteins may be used to raise mono- or polyclonal antibodies, which may for example be used for the detection of S1PP2C1 proteins in tissue samples, such as leaves (immunochemical analysis methods and kits).

[0043] In one disclosure reduced-function or loss-of-function mutant S1PP2C1 proteins are provided and plants and plant parts comprising one or more *SlPP2C1* alleles, which encode reduced-function or loss-of-function mutants. Any type of mutation may lead to a reduction in function or loss of function of the encoded protein, e.g. insertion, deletion or replacement of one or more nucleotides in the cDNA (SEQ ID NO: 1, or variants) or in the corresponding genomic *SlPP2C1* sequence (SEQ ID NO: 11, or variants). The "corresponding genomic sequence" is the endogenous DNA sequence (depicted in SEQ ID NO: 11, or variants thereof) from which SEQ ID NO: 1 mRNA (cDNA), or variant mRNA is transcribed. The wild type genomic region which is transcribed into mRNA comprises nucleotides 2591 to 5050, which includes the 5' UTR (nucleotides 2591-2675 of SEQ ID NO: 11), two introns and the 3'UTR (nucleotides 4976-5050 of SEQ ID NO: 11). The *in vitro* and/or *in vivo* function of such proteins can be tested as described above. Plants comprising a nucleic acid sequence encoding such mutant reduced-function or loss-of-function proteins and having enhanced drought tolerance, can for example be generated using TILLING or identified using EcoTILLING, as described further below.

[0044] In one embodiment of the invention (cDNA or genomic) nucleic acid sequences encoding such mutant proteins comprise one or more non-sense and/or mis-sense mutations, e.g. transitions (replacement of purine with another purine (A ↔ G) or pyrimidine with another pyrimidine (C ↔ T)) or transversions (replacement of purine with pyrimidine, or *vice versa* (C/T ↔ A/G). In one embodiment the non-sense and/or mis-sense mutation(s) is/are in the nucleotide sequence encoding the C-terminal region, preferably in the (putative) catalytic domain of amino acid 84 to 391 of SEQ ID NO: 2 (or of an essentially similar domain in a variant S1PP2C1 protein, i.e. in a domain comprising at least 80%, 90%, 95%, 98%, 99% amino acid identity to this domain). In another embodiment the non-sense and/or mis-sense mutation(s) is/are in the nucleic acid sequence encoding amino acid 307-320 of SEQ ID NO: 2 (or a variant thereof). In another embodiment the non-sense and/or mis-sense mutation(s) is/are in the nucleic acid sequence encoding the putative magnesium

binding site, i.e. Asp-Gly-His (amino acid 147-149 of SEQ ID NO:2 or variant thereof) and/or in the nucleic acid sequence encoding the putative protein kinase interaction domain (amino acids 144-150 of SEQ ID NO: 2 or of an essentially similar domain in a variant S1PP2C1 protein).

[0045] In a specific embodiment nucleic acid sequences encoding such mutant proteins comprise one or more mis-sense mutations in amino acids Gly148, Ser171, Ala155, Gly132 of SEQ ID NO: 2 (or the equivalent amino acid in a variant S1PP2C1 protein). Also mutant plants, seeds and plant parts comprising one or more of the above mis-sense mutations and having significantly enhanced stress tolerance, especially drought tolerance, are encompassed herein.

[0046] In a specific embodiment of the invention drought tolerant plants comprising a mutant loss-of-function or reduced-function *SlPP2C1* allele are provided, for example whereby the mutant allele comprises a mis-sense mutation in the nucleotide sequence encoding GVXDGHG (with X being any amino acid, preferably Y), or an essentially similar sequence (comprising at least 80%, 90%, 95%, 98%, 99% amino acid identity to this domain), resulting in at least one amino acid being replaced, e.g. resulting in for example DVXDGHG or GVXDGHD or DVXDGHD or GVXDDHG.

[0047] The function of specific domains, such as the N-terminal or the catalytic domain or the magnesium binding and/or protein kinase interaction domain, can be analyzed by deleting all or part of the domain(s) in a S1PP2C1 protein or the introduction of one or more mutations into the domain, and analysis of the resulting effect on the function of the SlPP2C1 protein. Likewise, plants comprising spontaneous or induced mutations (e.g. generated by TILLING or identified by EcoTILLING) can be analyzed for the mutation and the phenotype of the plant comprising the mutation, in particular drought tolerance.

[0048] In one embodiment, the loss-of-function or reduced-function S1PP2C1 protein is a truncated protein, i.e. a protein fragment of any one of the S1PP2C1 proteins defined further above (including variants thereof). In general EMS (Ethyl methanesulfonate) induces substitutions of guanine/cytosine to adenin/thymine. In case of a glutamine (Gln or Q, encoded by the nucleotides CAA or CAG) or arginine (Arg or R, encoded by the nucleotides CGA) codon, a substitution of the cytosine for thymine can lead to the introduction of a stop codon in the reading frame (for example CAA/CAG/CGA to TAA/TAG/TGA) resulting in a truncated protein. The truncated protein may, for example, comprise amino acids 1 to any one of the Gln (encoded by CAA or CAG) or Arg amino acids (encoded by CGA) downstream of the startcodon of SEQ ID NO: 2, or of a variant of SEQ ID NO: 2. Alternatively, the truncated protein may, for example, comprise or consist of amino acids 1-5, 1-1-20, 1-28, 1-33. 1-35, 1-36, 1-40, 1-43, 1-44, 1-46, 1-48, 1-49, 1-5-. 1-51, 1-54, 1-57, 1-66, 1-70, 1-71, 1-72, 1-73, 1-83 of SEQ ID NO: 2, or 1-85, 1-88, 1-91, 1-93, 1-94, 1-97, 1-104, 1-106, 1-110, 1-120 1-130, 1-141, 1-149, 1-151, 1-160, 1-170, 1-200, 1-301, 1-302, 1-305, 1-307, or other truncated proteins of S1PP2C1 (SEQ ID NO: 2), or of a variant thereof.

[0049] Also provided are nucleic acid sequences (genomic DNA, cDNA, RNA) encoding S1PP2C1 proteins, such as for example S1PP2C1 depicted in SEQ ID NO:2 or variants thereof as defined above (including any chimeric or hybrid proteins or mutated proteins or truncated proteins described), or any SlPP2C1 protein from another species. Due to the degeneracy of the genetic code various nucleic acid sequences may encode the same amino acid sequence. Any nucleic acid sequence encoding an S1PP2C1 protein (as defined above, including variants thereof) is herein referred to as *SlPP2C1*. The nucleic acid sequences provided include naturally occurring, artificial or synthetic nucleic acid sequences. A nucleic acid sequences encoding S1PP2C1 is provided for in SEQ ID NO: 1 (cDNA sequence from tomato) and SEQ ID NO: 11 (genomic DNA from tomato encoding wild type S1PP2C1 protein). The corresponding genomic sequence can be isolated using routine methods, such as PCR using specific or degenerate primers based on SEQ ID NO: 1 or SEQ ID NO: 11.

[0050] It is understood that when sequences are depicted in 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 replace by uracil (U).

[0051] Also provided are nucleic acid sequences (genomic DNA, cDNA, RNA) encoding mutant S1PP2C1 proteins, i.e. reduced function or loss-of-function S1PP2C1 proteins, as described above. For example, *SlPP2C1* nucleic acid sequences comprising one or more non-sense and/or mis-sense mutations in the wild type SlPP2C1 coding sequence, rendering the encoded protein non-functional or having a reduced function *in vivo* and/or *in vitro.* Also sequences with other mutations are provided, such as splice-site mutants, i.e. mutations in the genomic S1PP2C1 sequence leading to aberrant splicing of the pre-mRNA, and/or frame-shift mutations, and/or insertions and/or deletions of one or more nucleic acids.

[0052] Also included are variants and fragments of *SlPP2C1* nucleic acid sequences, such as nucleic acid sequences hybridizing to *SlPP2C1* nucleic acid sequences, e.g. to SEQ ID NO: 1, under stringent hybridization conditions as defined. Variants of *SlPP2C1* nucleic acid sequences also include nucleic acid sequences which have a sequence identity to SEQ ID NO: 1 or SEQ ID NO: 11 (nucleotides 2676 - 4975) of at least 50% or more, preferably at least 55%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, 99% or more (as determined by Emboss "needle" using default parameters, i.e. gap creation penalty = 10, gap extension penalty = 0.5, scoring matrix nwsgapdna). It is clear that many methods can be used to identify, synthesise or isolate variants or fragments of *SlPP2C1* nucleic acid sequences, such as nucleic acid hybridization, PCR technology, *in silico* analysis and nucleic acid synthesis, and the like. Variants of SEQ ID NO: 1 or SEQ ID NO: 11 (nucleotides 2676 - 4975) may either encode wild type, functional S1PP2C1 proteins (e.g. alleles from other tomato

varieties or breeding lines or wild accessions, or orthologs from other species than tomato), or they may encode reduced-function or loss-of function mutant alleles of any of these, as for example generated or identified by methods such as TILLING or EcoTILLING, or other methods.

[0053] Fragments include parts of any of the above *SlPP2C1* nucleic acid sequences (or variants), which may for example be used as primers or probes or in gene silencing constructs. Parts may be contiguous stretches of at least about 10, 15, 19, 20, 21, 22, 23, 25, 50, 60, 100, 200, 300, 450, 500, 600, 700, 800, 900, or more, nucleotides in length, of either the coding strand (sense strand) or the complementary strand (anti-sense strand,). Also included are, therefore, fragments of *SlPP2C1* nucleic acid sequences, whereby a fragment of at least about 20, 30, 40, 50, 60, 100, 150, 200 300, 450, 500, 600, 700, 800, 900 nucleotides in length comprises at least 50, 60, 70, 75%, more preferably at least 80, 90, 95, 98, 99% or more (100%) nucleic acid sequence identity to another fragment of a *SlPP2C1* nucleic acid sequence of about the same length (as determined by pairwise alignment using Emboss "needle" using default parameters, i.e. gap creation penalty = 10, gap extension penalty = 0.5, scoring matrix nwsgapdna).

[0054] Primer pairs which can be used for PCR amplification of *SlPP2C1* transcripts (mRNA or corresponding cDNA) from plant tissue DNA sample are, for example, depicted in SEQ ID NO: 3 and 4 and in primer pair of SEQ ID NO: 9 and 10. Such primer pairs can be used to detect and quantify *SlPP2C1* expression in plant tissue, e.g. in tomato leaf tissue. Likewise other specific or degenerate primers can be designed based on SEQ ID NO: 1 or SEQ ID NO: 11 (nucleotides 2676 - 4975) and used to amplify variants alleles of *SlPP2C1* from other tomato lines or from other species.

[0055] Once a specific mutant *SlPP2C1* allele has been generated and/or identified (e.g. by TILLING or EcoTILLING), also primers or probes specific for the mutant allele can be designed and an assay can be developed which detects the presence and/or absence of the mutant allele in a plant or plant part (using allele specific detection assays). Molecular marker assays for detection and/or transfer (e.g. by MAS, marker assisted selection) of the mutant allele can be developed. E.g. a SNP detection assay or a CAPS marker can be developed which detects the presence of mutant *SlPP2C1* nucleic acid sequence in DNA of plants and/or which can be used for transfer of the allele into other plants.

[0056] In one disclosure mutant nucleic acid sequences are provided, whereby the *SlPP2C1* nucleic acid sequence comprises one or more mutations leading to either a loss-of-function mutant of the S1PP2C1 protein or a reduced-function mutant of the S1PP2C1 protein. This aspect will be described in more detail elsewhere herein.

[0057] Plants can also be identified or generated (e.g. by homologous recombination, or by insertion, deletion or replacement of one or more nucleotides, etc.) which have one or more mutations in the *SlPP2C1* regulatory region(s), e.g. the promoter, whereby *SlPP2C1* gene expression, i.e. mRNA levels (of SEQ ID NO: 1 or variants) is/are significantly reduced in the plant compared to the wild type and whereby the plant has significantly enhanced drought tolerance.

[0058] The nucleic acid sequence described above, or fragments thereof, particularly DNA sequence, encoding the S1PP2C1 proteins of this invention (or variants of these) can be inserted in expression vectors (in co-suppression approaches) or into gene silencing vectors to generate drought tolerant plants.

[0059] In one embodiment of the invention *SlPP2C1* gene expression is downregulated in a host cell, plant or specific tissue(s), by e.g. RNAi approaches, as described elsewhere.

[0060] In another embodiment plants comprising one or more mutant *SlPP2C1* alleles are provided, whereby the mutation(s) confer enhanced drought tolerance on the plant compared to plants lacking the mutant allele(s). Mutant alleles are preferably generated by mutagenesis of the plant or seed and by identifying those plants or seeds which comprise one or more mutations in the target *PP2C1* allele(s) and whereby the mutation results in abolishment of transcription or translation (so that no S1PP2C1 protein is produced), or in translation of a reduced-function or loss-of-function S1PP2C1 protein. The reduction of functional, wild type S1PP2C1 protein, at least in leaf tissue, confers enhanced drought tolerance onto the plant, plant part or seed.

[0061] In another disclosure PCR primers and/or probes and kits for detecting the *SlPP2C1* DNA sequences are provided. Degenerate or specific PCR primer pairs to amplify *SlPP2Cl* DNA from samples can be synthesized based on SEQ ID NO: 1 or SEQ ID NO: 11 (e.g. based on nucleotides 2676 - 4975 or 2591- 5050) as known in the art (see Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and McPherson at al. (2000) PCR-Basics: From Background to Bench, First Edition, Springer Verlag, Germany). Likewise, DNA fragments of SEQ ID NO: 1 or SEQ ID NO: 11 (or variants thereof) can be used as hybridization probes. A *SlPP2C1* detection kit may comprise either *SlPP2C1* specific primers and/or *SlPP2C1* specific probes, and an associated protocol to use the primers or probe to detect *SlPP2C1* DNA in a sample. Such a detection kit may, for example, be used to determine, whether a plant has been transformed with an *SlPP2C1* gene (or part thereof) of the invention or whether a plant comprises one or more mutant *SlPP2C1* alleles. Because of the degeneracy of the genetic code, some amino acid codons can be replaced by others without changing the amino acid sequence of the protein.

[0062] In another disclosure antibodies that bind specifically to a S1PP2C1 protein, or mutant S1PP2C1 protein, according to the invention are provided. In particular monoclonal or polyclonal antibodies that bind to S1PP2C1, or to fragments or variants thereof (e.g. mutant proteins), are encompassed herein. An antibody can be prepared by using a S1PP2C1 protein according to the invention as an antigen in an animal using methods known in the art, as e.g. described in Harlow and Lane "Using Antibodies: A laboratory manual"(New York: Cold Spring Harbor Press 1998) and in Liddell

and Cryer "A Practical Guide to Monoclonal Antibodies" (Wiley and Sons, 1991). The antibodies can subsequently be used to isolate, identify, characterize or purify the SlPP2C1 protein to which it binds, for example to detect the S1PP2C1 protein in a sample, allowing the formation of an immunocomplex and detecting the presence of the immunocomplex by e.g. ELISA (enzyme linked immunoassay) or immunoblot analysis. Also provided are immunological kits, useful for detecting the S1PP2C1 proteins, protein fragments or epitopes in a sample provided. Samples may be cells, cell supernatants, cell suspensions, tissues, etc. Such a kit comprises at least an antibody that binds to a S1PP2C1 protein and one or more immunodetection reagents. The antibodies can also be used to isolate/identify other S1PP2C1 proteins, for example by ELISA or Western blotting.

[0063] It is clear that many methods can be used to identify, synthesise or isolate variants or fragments of *SlPP2C1* nucleic acid sequences, such as nucleic acid hybridization, PCR technology, *in silico* analysis and nucleic acid synthesis, and the like. Thus, an S1PP2C1-protein encoding nucleic acid sequence may be a sequence which is chemically synthesized or which is cloned from any plant species.

Transgenic drought tolerant plants

[0064] Transgenic plants, seeds and plant parts are provided in which *SlPP2C1* is silenced, preferably at least in leaf tissue or aerial tissue, and which have enhanced drought tolerance compared to wild type (non-transgenic) control plants or other control plants (e.g. empty vector transformants).

[0065] In one embodiment of the invention a homologous or heterologous nucleic acid sequence is used to silence the endogenous *SlPP2C1* gene(s) of the *Solanum lycopersicum* host species to be transformed. For example, a potato *SlPP2C1* gene, such as *StPP2C1* of SEQ ID NO: 14 (or variant or fragment thereof) may be used to silence *SlPP2C1* gene expression in transgenic tomato plants. Alternatively, homologous *SlPP2C1* nucleic acid sequences may be used. For example a sequence originating from a tomato plant species is reintroduced into said species (tomato). Thus, in one embodiment, the *SlPP2C1* DNA corresponds to, or is a modification/variant of, the endogenous *SlPP2C1* DNA of the species which is used as host species in transformation. Thus, a tomato *SlPP2C1* cDNA or genomic DNA (or a variant or fragment thereof) is preferably used to transform tomato plants. In addition (for regulatory approval and public acceptance reasons) the homologous or heterologous nucleic acid sequence may be operably linked to a transcription regulatory sequence, especially a promoter, which also originates from a plant species or even from the same plant which is to be transformed.

[0066] To generate plants comprising a chimeric gene, which upon expression results in silencing of the expression of an endogenous *SlPP2C1* gene or gene family, methods known in the art can be used.

[0067] "Gene silencing" refers to the down-regulation or complete inhibition of gene expression of one or more target genes, e.g. *SlPP2C1* genes, in a host cell or tissue. It is understood that in any transformation experiments a certain degree of variation in the phenotype of transformants is seen, normally due to position effects in the genome and/or due to copy number. Generally, "weak" and "strong" gene silencing plants are distinguished herein (all of which are embodiments of the invention), wherein "weak" gene silencing (RNAi) events refer to plants or plant parts wherein the endogenous target gene expression is reduced by about 15, 20 or 30% compared to the control tissue and "strong" gene silencing (RNAi) events refer to plants or plant parts wherein the endogenous target gene expression is reduced by at least about 50, 60, 70, 80, 90% or more compared to the control tissue (e.g. wild type). Silencing can be quantified by, for example, quantifying the transcript level of the target gene (e.g. using quantitative RT-PCR) and/or by determining and optionally quantifying the enzymatic activity of the target S1PP2C1 protein and/or by assessing and optionally quantifying resulting phenotype (enhanced drought tolerance and/or enhanced ABA sensitivity).

[0068] Without limiting the scope of the invention, plants having an optimal silencing level can be selected, so that resulting plants have significantly enhanced drought tolerance under the climatic conditions to which they are exposed in the field, while having minimal negative side-effects, such as reduced yield, reduced number of fruits, etc. compared to controls. Preferably survival and/or yield are increased in the drought tolerant plants.

[0069] The use of inhibitory RNA to reduce or abolish gene expression is well established in the art and is the subject of several reviews (e.g Baulcombe 1996, Plant Cell 8(2): 179-188; Depicker and Van Montagu, 1997, Curr Opin Cell Biol. 9(3): 373-82). There are a number of technologies available to achieve gene silencing in plants, such as chimeric genes which produce antisense RNA of all or part of the target gene (see e.g. EP 0140308 B1, EP 0240208 B1 and EP 0223399 B1), or which produce sense RNA of the target gene (also referred to as "co-suppression"), see EP 0465572 B1.

[0070] The most successful approach so far has however been the production of both sense and antisense RNA of the target gene ("inverted repeats"), which forms double stranded RNA (dsRNA) or a stem-loop structure (hairpin RNA, hpRNA) in the cell and silences the target gene(s) upon transcription from an upstream promoter. Methods and vectors for dsRNA and hpRNA production and gene silencing have been described in EP 1068311, EP 983370 A1, EP 1042462 A1, EP 1071762 A1 and EP 1080208 A1.

[0071] A chimeric gene for plant transformation may, therefore, comprise a transcription regulatory region which is active in plant cells operably linked to a sense and/or antisense DNA fragment (or a complete nucleic acid sequence)

of, or complementary or substantially similar to, a *SLPP2C1* target gene or gene family.

[0072] Generally short (sense and anti-sense) stretches of the target gene sequence, such as 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 nucleotides of coding and/or non-coding sequence of the target gene are sufficient. Longer sequences can also be used, such as at least about 50, 100, 200, 250, 500, 1000 or more nucleotides. Even DNA corresponding to, or being complementary to, the complete transcript RNA or mRNA may be used to make a sense and/or antisense construct. Preferably, the sense and antisense fragments/sequences are separated by a spacer sequence, such as an intron, which forms a loop (or hairpin) upon dsRNA formation.

[0073] In principle, any *SlPP2C1* gene or gene family can be targeted. For example, one or several specific *SlPP2C1* alleles may be silenced by choosing a nucleic acid region of their primary or mRNA transcripts specific for these alleles (see Byzova et al. Plant 2004 218: 379-387 for allele specific silencing in an organ specific manner). Similarly, a whole gene family may be targeted for silencing by choosing one or more conserved regions of the transcripts for making the silencing construct. As mentioned above, the DNA region used in sense and/or antisense orientation does not need to be part of the coding region, but may also correspond to, or be complementary to, parts of the primary transcript (comprising a 5' and 3' untranslated sequence and introns, as depicted in nucleotides 2591-5050 of SEQ ID NO: 11) or to parts of the mRNA transcript (where any introns have been removed and a poly A tail has been added). It is understood that in a DNA sequence which corresponds to an RNA sequence the U is replaced by a T. It is also noted that in a chimeric gene which transcribes a dsRNA or hpRNA targeting capable of silencing *SlPP2C1* gene expression upon transcription in a host cell, the sense and antisense regions need not be of equal length and one region may be longer than the other.

Thus, for example SEQ ID NO: 1 or variants thereof as described above, or fragments of any of these, or the genomic sequence or primary transcript sequence of SEQ ID NO: 1 (as depicted in SEQ ID NO: 11 from nucleotide 2591 to 5050), may be used to make a *SlPP2C1* gene silencing gene and vector and a transgenic plant in which one or more *SlPP2C1* genes are silenced in all or some tissues or organs, or upon induction (see e.g. Wielopolska et al. Plant Biotechnol J. 2005 6:583-90). A convenient way of generating hairpin constructs is to use generic vectors such as pHANNIBAL, pHELLSGATE, pSTARGATE vectors based on the Gateway® technology (see Wesley et al. 2004, Methods Mol Biol. 265:117-30; Wesley et al. 2003, Methods Mol Biol. 236:273-86 and Helliwell & Waterhouse 2003, Methods 30(4):289-P95). See also http://www.pi.csiro.au/rnai/ for other gene silencing vectors, such as inducible silencing vectors and vectors for silencing of multiple target genes and for the program MatchPoint which can be used to find the best sequence to use for silencing the target gene.

By choosing conserved nucleic acid sequences all *SlPP2C1* gene family members in a host plant can be silenced. The silencing of all family members of a host plant is a specific embodiment.

[0074] In one embodiment the promoter, which is operably linked to the sense and/or antisense nucleic acid sequence (to make a chimeric silencing / RNAi gene) is selected from a constitutive promoter, an inducible promoter (e.g. stress inducible, light inducible, chemically inducible, etc.), a hormone inducible promoter (e.g. ethylene or ABA inducible, etc.) a leaf specific promoter or promoter active in aerial tissue. Also early dehydration responsive promoters, such as RD2, are encompassed herein, as well as other stress inducible promoters, such as RD29 (Yamaguchi-Shinozaki and Shinozaki 1993, Mol Gen Genet 236: 331-340).

[0075] In certain embodiments a fruit specific promoter may be suitable. Also, the promoter of an *SlPP2C* gene itself may be used for silencing approaches. The promoter from tomato is comprised in SEQ ID NO: 11 from nucleotide 1-2675, especially the promoter comprises or consists of about 2000 nucleotides upstream of the ATG translation start codon at position 2676-2679 of SEQ ID NO: 11) or functional fragments thereof (e.g. 1500bp, 1000bp or less upstream of ATG). Optionally a 3' UTR may be operably linked to the 3' end of the chimeric gene, so that the operably linked DNA elements include promoter - *SlPP2C1* RNAi gene - 3'UTR.

[0076] Preferred constitutive promoters include: the strong constitutive 35S promoters or enhanced 35S promoters (the "35S promoters") of the cauliflower mosaic virus (CaMV) of isolates CM 1841 (Gardner et al., 1981, Nucleic Acids Research 9, 2871-2887), CabbB-S (Franck et al., 1980, Cell 21, 285-294) and CabbB-JI (Hull and Howell, 1987, Virology 86,482-493); the 35S promoter described by Odell et al. (1985, Nature 313, 810-812) or in US5164316, promoters from the ubiquitin family (e.g. the maize ubiquitin promoter of Christensen et al., 1992, Plant Mol. Biol. 18,675-689, EP 0 342 926, see also Cornejo et al. 1993, Plant Mol.Biol. 23, 567-581), the gos2 promoter (de Pater et al., 1992 Plant J. 2, 834-844), the emu promoter (Last et al., 1990, Theor. Appl. Genet. 81,581-588), Arabidopsis actin promoters such as the promoter described by An et al. (1996, Plant J. 10, 107.), rice actin promoters such as the promoter described by Zhang et al.(1991, The Plant Cell 3, 1155-1165) and the promoter described in US 5,641,876 or the rice actin 2 promoter as described in WO070067; promoters of the Cassava vein mosaic virus (WO 97/48819, Verdaguer et al. 1998, Plant Mol. Biol. 37,1055-1067), the pPLEX series of promoters from Subterranean Clover Stunt Virus (WO 96/06932, particularly the S7 promoter), a alcohol dehydrogenase promoter, e.g., pAdhIS (GenBank accession numbers X04049, X00581), and the TR1' promoter and the TR2' promoter (the "TR1'promoter" and "TR2'promoter", respectively) which drive the expression of the 1' and 2' genes, respectively, of the T-DNA (Velten et al., 1984, EMBO J 3, 2723-2730), the Figwort Mosaic Virus promoter described in US6051753 and in EP426641, histone gene promoters, such as the Ph4a748

promoter from Arabidopsis (PMB 8: 179-191), or others.

[0077] Alternatively, a promoter can be utilized which is not constitutive but rather is specific for one or more tissues or organs of the plant (tissue preferred / tissue specific, including developmentally regulated promoters). For example, a promoter active in leaf tissue or aerial plant parts, or epidermis specific promoters or guard cell specific promoters may be used.

[0078] Epidermal specific promoters, such as for example the Arabidopsis *LTP1* promoter (Thoma et al, 1994, Plant Physiol. 105(1):35-45.), the *CER1* promoter (Aarts et al 1995. Plant Cell. 7:2115-27), and the *CER6* promoter (Hooker et al 2002, Plant Physiol 129:1568-80.) and the orthologous tomato LeCER6 (Vogg et al, 2004, J. Exp Bot. 55: 1401-10), provide specific expression in above ground epidermal surfaces.

[0079] Also suitable are leaf or photosynthetic tissue specific promoters, such as the light inducible ribulose 1,5-bisphosphate carboxylase small subunit promoter (Pssu) from Arabidopsis as described in US5034322 or from sunflower, from pea (US 5254799) or from Zea mays; the potato ST-LS1 promoter which is stem and leaf specific (Stockhaus et al. 1987, Nucleic Acids Res.15(8):3479-91); the promoter of the chlorophyll a/b binding protein (CAB).

[0080] Guard cell specific promoters, such as the DGP1 promoter (Li et al., Sci China C Life Sci. 2005 48(2):181-6) may be used or drought-stress inducible promoters like RD29 (Yamaguchi-Shinozaki and Shinozaki 1993, supra), which is active in almost all the organs and tissues of vegetative plants during water deficiency.

[0081] The skilled person can easily test various promoters for their specificity and suitability in the methods according to the invention. In addition, the specificity of promoters may be modified by deleting, adding or replacing parts of the promoter sequence. Such modified promoters can be operably linked to reporter genes in order to test their spatio-temporal activity in transgenic plants.

[0082] Another alternative is to use a promoter whose expression is inducible. Examples of inducible promoters are chemical inducible promoters, such as dexamethasone as described by Aoyama and Chua (1997, Plant Journal 11: 605-612) and in US6063985 or by tetracycline (TOPFREE or TOP 10 promoter, see Gatz, 1997, Annu Rev Plant Physiol Plant Mol Biol. 48: 89-108 and Love et al. 2000, Plant J. 21: 579-88). Other inducible promoters are for example inducible by a change in temperature, such as the heat shock promoter described in US 5,447, 858, by anaerobic conditions (e.g. the maize ADH1S promoter), by light (US6455760), by pathogens (e.g. EP759085 or EP309862) or by senescence (SAG12 and SAG13, see US5689042) or by drought (see above). Obviously, there are a range of other promoters available. Examples of other inducible promoters are the Adh1 promoter which is inducible by hypoxia or cold stress, the Hsp70 promoter which is inducible by heat stress, and the PPDK promoter which is inducible by light. One preferred promoter is the ethanol-inducible promoter system, as described in Ait-ali et al. (2001, Plant Biotechnology Journal 1, 337-343), wherein ethanol treatment activates alcR, which in turn induces expression of the alc:35S promoter. See also Deveaux et al. (2003, The ethanol switch: a tool for tissue-specific gene induction during plant development. Plant J. 36, 918-930).

[0083] Optionally, the promoter-*SlPP2C1* RNAi gene may further comprise a 3'end transcription regulation signals ("3'end" or "3' UTR") (i.e. transcript formation and polyadenylation signals). Polyadenylation and transcript formation signals include those of, the nopaline synthase gene ("3' nos") (Depicker et al., 1982 J. Molec. Appl. Genetics 1, 561-573.), the octopine synthase gene ("3'ocs") (Gielen et al., 1984, EMBO J 3, 835-845) and the T-DNA gene 7 ("3' gene 7") (Velten and Schell, 1985, Nucleic Acids Research 13, 6981-6998), which act as 3'-untranslated DNA sequences in transformed plant cells, and others.

[0084] The chimeric *SlPP2C1* silencing gene (i.e. the promoter operably linked to a nucleic acid sequence which upon transcription in a plant cell is capable of silencing the endogenous *SlPP2C1* gene expression) 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 *SlPP2C1* silencing in certain cells at a certain time. In this regard, a T-DNA vector, comprising a promoter operably linked to a sense and/or antisense *SlPP2C1* sequence (and optionally a 3'UTR), may be introduced into *Agrobacterium tumefaciens* and 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.

[0085] Preferred T-DNA vectors each contain a promoter operably linked to *SlPP2C1* silencing gene between T-DNA border sequences, or at least located to the left of the right border sequence. Border sequences are described in Gielen et al. (1984, EMBO J 3,835-845). Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example in EP 0 223 247), pollen mediated transformation (as described, for example in EP 0 270 356 and WO85/01856), protoplast transformation as, for example, described in US 4,684, 611, plant RNA virus- mediated transformation (as described, for example in EP 0 067 553 and US 4,407, 956), liposome-mediated transformation (as described, for example in US 4,536, 475), and other methods such as those

described methods for transforming certain lines of corn (e. g., US 6,140, 553; Fromm et al., 1990, Bio/Technology 8, 833-839; Gordon-Kamm et al., 1990, The Plant Cell 2, 603-618) and rice (Shimamoto et al., 1989, Nature 338, 274-276; Datta et al. 1990, Bio/Technology 8, 736-740) and the method for transforming monocots generally (PCT publication WO92/09696). For cotton transformation see also WO 00/71733, and for rice transformation see also the methods described in WO92/09696, WO94/00977 and WO95/06722. For sorghum transformation see e.g. Jeoung JM et al. 2002, Hereditas 137: 20-8 or Zhao ZY et al. 2000, Plant Mol Biol.44:789-98). For tomato or tobacco transformation see also An G. Biology Manual A5, Dordrecht, Netherlands, Kluwer Academic Publishers, pp 1-9; Koornneef M. et al., 1986, In: Nevins D.J. and R.A. Jones, eds. Tomato Biotechnology, New York, NY, USA, Alan R. Liss, Inc. pp 169-178). For potato transformation see e.g. Sherman and Bevan (1988, Plant Cell Rep. 7: 13-16). Tomato transformation and regeneration can also be carried out according to De Jong et al. (2008) Plant Journal 57:160-170 and Sun et al. (2006) Plant Cell Physiol. 47: 426-431.

[0086] Likewise, selection and regeneration of transformed plants from transformed 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.

[0087] Besides transformation of the nuclear genome, also transformation of the plastid genome, preferably chloroplast genome, is included in the invention. One advantage of plastid genome transformation is that the risk of spread of the transgene(s) can be reduced. Plastid genome transformation can be carried out as known in the art, see e.g. Sidorov VA et al. 1999, Plant J.19: 209-216 or Lutz KA et al. 2004, Plant J. 37(6):906-13.

[0088] Disclosed hosts are of the family *Solanaceae,* such as species of the genus *Solanum,* e.g. tomato (*S. lycopersicum*).

[0089] In a preferred disclosure the host is of the family *Solanaceae.* In a more preferred disclosure the host is of the genus *Solanum.* The host according the present invention is of the species *S. lycopersicum.* Preferably, the host is a cultivated tomato of the species *S. lycopersicum,* i.e. a line or variety yielding high yields, such as fruit of at least 50 g average fresh weight or more, e.g. at least about 80g, 90g, 100g, 200g, 300g, or even up to 600 g (beef tomato types). Also small types, such as cherry or cocktail tomato are encompassed, as are full-flesh tomatoes such as the Nunhems variety Intense, e.g. lacking gel in the seed cavities. The host tomato plant may be determinate or indeterminate, of various fruit sizes and shapes, such as Roma type, cluster type, round. It may be a processing type tomato or a fresh market type. Also both open pollinated and hybrids are encompassed herein. In one embodiment the drought tolerant tomato plant is an F1 hybrid plant, grown from an F1 hybrid seed. To make F1 hybrid seeds of a transgenic plant according to the invention, two inbred parent lines may be made, each comprising a copy of the transgene in their genomes. When these plants are cross-fertilized, the F1 seeds are collected, which produce transgenic F1 hybrid plants with high yield and drought tolerance due to the transgene.

[0090] The embodiments described herein for 'host' plants also apply to non-transgenic mutant plants described elsewehere herein, whereby instead of a transgene a mutant *SlPP2C1* allele is present endogenously.

[0091] Thus, in one embodiment of the invention transgenic plants comprising a transcription regulatory element (especially a promoter as described above) operably linked to nucleic acid molecule which upon transcription is capable of silencing the endogenous *SlPP2C1* gene expression in the host cells.

[0092] The construction of chimeric genes and vectors for, preferably stable, introduction of *SlPP2C1* silencing gene into the genome of host cells is generally known in the art. To generate a chimeric gene the sense and/or antisense *SlPP2C* sequence 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 nucleic sequence is simply inserted into the vector downstream of the promoter sequence. The vector is then used to transform the host cells and the chimeric gene is inserted in the nuclear genome or into the plastid, mitochondrial or chloroplast genome and expressed there using a suitable promoter (e. g., Mc Bride et al., 1995 Bio/Technology 13, 362; US 5,693, 507).

[0093] The resulting transformed plant can be used in a conventional plant breeding scheme to produce more transformed plants with the same characteristics or to introduce the gene part into other varieties of the same or related plant species. An "elite event" can be selected, which is a transformation event having the transgene inserted in a particular location in the genome, which results in good expression of the desired phenotype (e.g. optimal silencing and drought tolerance).

[0094] In one embodiment of the invention it is desired to enhance water loss, i.e. to decrease drought tolerance of specific tissues and/or to decrease ABA sensitivity of specific tissues, for example fruits, by overexpressing *SlPP2C1* in at least such tissues. For example, to achieve fruits (e.g. tomatoes) with an increased water loss and therefore a more solid fruit flesh and/or enhanced taste, a fruit specific or fruit preferred promoter is suitable, operably linked to a nucleic acid sequence encoding a functional S1PP2C protein (SEQ ID NO: 2 or a variant thereof). To confer expression to fruits, a tomato fruit and peel specific promoter e.g. beta-galactosidase II (Smith et al., 1998, Plant Physiol 117: 417-23) or tomato epicuticular wax promoter LeCER6 (Vogg *et al,* 2004, supra) can be used. Or a fruit skin or epidermal promoter can be identified and isolated by one skilled in the art, using microarrays and confirmation by transformation of promoter reporter gene fusions. Such promoters can also be used for *SlPP2C1* silencing in specific tissues, such as fruits.

[0095] The transgenic plants, or parts thereof, in which *SlPP2C1* is silenced, have significantly enhanced drought tolerance. Significantly enhanced drought tolerance (as described above) is used herein to refer to an enhanced ability of transformants (compared to wild type or control transformants) to tolerate one or more periods of drought (water deprivation/depletion leading to e.g. visible leaf wilting symptoms in control plants), as described above. Preferably, the plants are able to recover subsequently, thereby leading to a reduced overall yield loss, as more plants per m^2 survive and/or the yield of the surviving plants is not significantly reduced.

[0096] Significantly enhanced drought tolerance can be assessed in controlled environments (green house or growth chambers) as described above or using equivalent methods. For example, an alternative method is the following: at least about 10 transformants per transformation event and at least 10 control plants are placed for various time periods (ranging from a few hours to 1-4 weeks or more) into the environment without watering them, until leaf wilting or loss of turgor is caused on control plants, and subsequently watering the plants again for e.g. at least 1 week, 2 weeks or longer, while their recovery phenotype is analyzed. Transformants with enhanced drought tolerance survive at least 2, 3, 4, 5, 6, 7 days, preferably at least 2-5 days longer without water than control-transformants (e.g. transformed with an empty vector) or wild type plants do under the same conditions, and which show irreversible tissue damage. Alternatively, % survival can be calculated at a certain timepoint, whereby the drought tolerant plants have a %survival which is at least 10%, 20%, 30%, or more, higher than that of the control. This alternative method may also be used for determining whether mutant plants (i.e. non-transgenic plants comprising one or more mutant *SlPP2C1* alleles) have significantly enhanced drought tolerance. It is understood that when mutant plants are analyzed for their phenotype, the control plants are preferably near isogenic lines of the mutant, which comprise the wild type allele(s). The period of water deprivation / stress and the period of recovery may vary depending on the plant species. For example, in rice 9.5 hours water stress followed by 10 days of recovery (watering) are suitable for determining whether a plant line or transformation event has enhanced drought tolerance compared to the control, as the percentage survival is significantly increased in the drought tolerant line (see e.g. Zheng et al. Biochemical and Biophysical Research Communications 2009, 985-989).

[0097] Ultimately, field trials are used to show that transformants (or mutant plants described further down) have significantly enhanced drought tolerance compared to wild type plants.

[0098] As already mentioned, transformants having an optimal silencing level can be selected by e.g. analysing copy number (Southern blot analysis), mRNA transcript levels (e.g. RT-PCR using *SlPP2C1* primer pairs) or by analysing the presence and/or level of S1PP2C1 protein in various tissues (e.g. SDS-PAGE; ELISA assays, etc). Optimal transgenic events are then used for further crossing / backcrossing / selfing until a high performing elite event with a stable transgene is obtained. In one embodiment especially the transgenic seeds derived from such plants are provided, which may be sold as being "drought tolerant".

[0099] Transformants expressing one or more *SlPP2C1* genes according to the invention may also comprise other trans genes, such as other genes conferring drought tolerance or conferring tolerance to other biotic or abiotic stresses. To obtain such plants with "stacked" transgenes, other transgenes may either be introgressed into the *SlPP2C1* transformants, or the transformants may be transformed subsequently with one or more other genes, or alternatively several chimeric genes may be used to transform a plant line or variety. For example, several chimeric genes may be present on a single vector, or may be present on different vectors which are co-transformed.

[0100] In one embodiment the following genes are combined with *SlPP2C1* silencing according to the invention: Genes encoding other AP2/EREBP type transcription factors, preferably ones which have a role in the plant's response to environmental stresses, such as for example the CBF1, CBF2, CBF3 and/or CBF4 encoding genes from *Arabidopsis* (Jaglo-Ottosen et al 1998, Science 280, 104-106, 1998; Kasuga et al 1999 Nat. Biotechnol. 17, 287-291) or orthologs thereof from other species (Dubouzet et al 2003, Plant J. 33: 751), with insect resistance genes such as *Bacillus thuringiensis* toxin genes (encoding insecticidal proteins, such as cry genes, vip genes, etc. see http://www.biols.susx.ac.uk/home/ for a list of available genes), fungal resistance genes, herbicide resistance genes, or other genes.

[0101] The stacked transformants may thus have an even broader environmental stress tolerance, to for example salinity, cold stress, insect resistance, pathogen resistance, heat stress, water stress, etc.

[0102] Whole plants, seeds, cells, tissues and progeny (such as F1, F2 seeds/plants, etc) of any of the transformed plants described above are encompassed herein and can be identified by the presence of the transgene in the DNA, for example by PCR analysis. Also "event specific" PCR diagnostic methods can be developed, where the PCR primers are based on the plant DNA flanking the inserted chimeric gene, see US6563026. Similarly, event specific AFLP fingerprints or RFLP fingerprints may be developed which identify the transgenic plant or any plant, seed, tissue or cells derived there from. It is understood that the transgenic plants according to the invention preferably do not show non-desired phenotypes, such as yield reduction, less fruits per plant, enhanced susceptibility to diseases or undesired architectural changes (dwarfing, deformations) etc. and that, if such phenotypes are seen in the primary transformants, these can be removed by normal breeding and selection methods (crossing / backcrossing / selfing, etc.). Any of the transgenic plants described herein may be homozygous or hemizygous for the transgene.

Non-transgenic drought tolerant plants and methods for making these

[0103] It is also an embodiment of the invention to use non-transgenic methods, e.g. target mutant generation and identification 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, Henikoff et al. 2004, Plant Physiol. 135: 630. 636) and selection to generate plant lines which comprise at least one mutation in an endogenous *SlPP2C1* allele and whereby the plants comprising the mutant *SlPP2C1* allele in heterozygous or homozygous form have significantly enhanced drought tolerance and/or a significantly enhanced sensitivity to ABA compared to plants lacking the mutant allele (having wild type allele(s) at the *SlPP2C1 l*ocus). Thus, in one embodiment of the invention plants comprising one or more mutant *SlPP2C1* alleles in the genome and having significantly enhanced drought tolerance compared to plants lacking said mutant allele(s), but comprising wild type alleles instead, are provided herein, as well as plant parts (e.g. harvested fruit, harvested leaves, etc.), seeds, clonal propagations of such plants, progeny of such plants comprising the mutant allele.

[0104] A "significantly reduced sensitivity to water stress" or "significantly enhanced drought tolerance" refers to a (statistically significant) reduced leaf wilting symptoms of a plurality of plants comprising the mutant allele(s) by at least 10% compared to control levels (e.g. same plants lacking the mutant allele, such as the non-mutated plants) and can for example be tested as described herein for transgenic plants or using equivalent alternative methods. In short, a plurality of mutant plants (preferably at least 10, 15, 20 or more plants of a line comprising a particular mutation in the *SlPP2C1* allele) and controls of the same age are saturated with water at the start of the experiment and are then not watered for an extended period of time, e.g. 7, 8, 9, 10, 11, 12, 13, 14, 15 days or more. When the controls start to show wilting of the leaves ("slight wilting" or "moderate wilting"), all plants are assessed for signs of wilting of the leaves using e.g. visual assessment. Wilting symptoms of leaves can be scored on a scale of 1 to 4, as "highly wilted" (4), "moderate wilting" (3), "slightly wilted"(2) or "no wilting" (1), for example. Mutant plants are said to have a significantly enhanced drought tolerance if average wilting is reduced by at least 10%, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to the wild type control plants. For example, if 10 plants are scored for wilting symptoms (w) per line, the control may have an average score of w = 40/10 = 4.0, while the drought tolerant line may have an average score of w = 36/10 = 3.6 or less, e.g. w = 3.4, 3.2, 2.0, 1,4, or 1.0.

[0105] Alternatively or in addition field trials can be used to determine whether a significantly enhanced drought tolerance is conferred on the line by the mutant *SlPP2C1* allele. For example, a plurality of plants comprising a particular mutant *SlPP2C1* allele (preferably at least 10, 20, 30 or more plants of a line) are planted in the field together with control plants and not watered for an extended period of time, such as 1, 2, 3, 4 weeks or more. When the control plants show wilting, wilting symptoms may be assessed as described above. Alternatively %recovery and/or %survival after being supplied with water (recovery) may be assessed. When leaf wilting or loss of turgor is caused on control plants, plants are watered again (e.g. for 1 week, 2 weeks or more) while their recovery phenotype is analyzed. Mutants with enhanced drought tolerance survive at least 2, 3, 4, 5, 6, 7 days, preferably at least 2-5 days longer without water than control-plants (e.g. wild type or near isogenic lines) under the same conditions, and which show irreversible tissue damage. Similarly, % survival can be calculated, with the drought tolerant mutant plant line having a (average) % survival which is increased by at least 10%, 20%, 30%, or more, compared to that of the control.

[0106] As mentioned earlier, other assays for drought tolerance may be used. The most suitable assay may differ for different crop species, i.e. for tomato plants a different assay may be suitable than for lettuce or rice. For example a statistically significant increase in plants (comprising the mutant allele) recovering from a period of drought may be measured and/or a significantly reduced plant mortality after a period of drought may be measured in plants comprising the mutant allele compared to plants lacking it. See e.g. the methods described in Zheng *et al.* (2009, supra), Yu *et al.* (2008, supra) or Xiong *et al.* 2006 (supra).

[0107] "Significantly enhanced ABA sensitivity" can also be tested as described in the Examples for transgenic plants. In summary, average seed germination on medium comprising ABA is at least 10%, 20%, 30%, 40%, 50% or more lower for the plant comprising the *SlPP2C1* mutant allele than for the control on the same ABA concentration. For example, on 1 or 3 μM ABA, 50% of wild type seeds germinate, while less than 40%, 35%, 30%, or less, of mutant seeds germinate. Thus, seed germination of plants comprising the mutant allele is inhibited more by ABA. Root growth of plants comprising the mutant allele is also inhibited more by ABA.

[0108] Preferably the plants phenotyped for drought tolerance and/or ABA sensitivity as described above are homozygous for the mutant *SlPP2C1* allele, although heterozygous plants may also be phenotyped and may also show enhanced drought tolerance and/or enhanced ABA sensitivity. To generate plants comprising the mutant allele in homozygous form, selfing can be used, optionally combined with genotyping (detecting the presence of the mutant allele e.g. by PCR using allele specific primers and/or sequencing). If TILLING populations are used the mutant plants (M1) are preferably selfed one or more times to generate for example M2 populations or preferably M3 or M4 populations for phenotyping. In M2 populations the mutant allele is present in a ratio of 1 (homozygous for mutant allele) : 2 (heterozygous for mutant allele) : 1 (homozygous for wild type allele). Segregation of drought tolerance should correlate with segregation

of the mutant allele.

[0109] The plant comprising the mutant *SlPP2C1* allele, or a variant thereof, and having enhanced drought tolerance may be of any species, as the tomato sequences provided herein can be used to generate and identify plants comprising mutations in homologs and orthologs of the gene, as described further below. The endogenous *SlPP2C1* variant nucleic acid sequence in the plant can be identified, which can then be used as target gene in the generation and/or identification of plants comprising a mutant allele of the *SlPP2C1* variant. Thus, the mutant drought tolerant plant may be a dicotyledonous or monocotyledonous species. Preferably the plant is a cultivated plant, although it is also an embodiment herein to identify mutant alleles in wild plants or non-cultivated plants and transfer these by breeding techniques into cultivated plants.

[0110] In one disclosure, the plant comprising at least one mutant *SlPP2C1* allele (in homozygous or heterozygous form) and having significantly enhanced drought tolerance (and/or significantly increased sensitivity to ABA), is of the family *Solanaceae,* i.e. encompassing the genera *Solanum, Capsicum, Nicotiana* and others. In another disclosure the plant is of the genus *Solanum,* e.g. encompassing cultivated tomato, potato, eggplant, and others.

[0111] In one disclosure, a drought tolerant potato plant comprises a mutant *SlPP2C1* allele (e.g. *StPP2C1* of SEQ ID NO: 14 or a variant thereof), encoding a reduced-function or loss of function protein (e.g. a reduced function or loss of function StPP2C1 protein of SEQ ID NO: 15 or a variant thereof).

[0112] In the present invention the plant is of the species *S. lycopersicum.* Any *S. lycopersicum* may be generated and/or identified having at least one mutant *SlPP2C1* allele in its genome and being drought tolerant. The tomato plant may, thus, be any cultivated tomato, any commercial variety, any breeding line or other, it may be determinate or indeterminate, open pollinated or hybrid, producing fruits of any shape and size. The mutant allele generated and/or identified in a particular tomato plant, or in a sexually compatible relative, may be easily transferred into any other tomato plant by breeding (crossing with a plant comprising the mutant allele and then selecting progeny comprising the mutant allele).

[0113] The plant of the present invention is of the species *Solanum lycopersicum,* which species is either mutagenized itself to generate the mutant allele (e.g. by TILLING) or in which one or more natural or spontaneous mutations in the *SlPP2C1* gene (or variant) is/are identified, e.g. by Ecotilling.

[0114] The mutant allele is in one embodiment generated or identified in a cultivated plant, but may also be generated and/or identified in a wild plant or non-cultivated plant and then transferred into an cultivated plant using e.g. crossing and selection (optionally using interspecific crosses with e.g. embryo rescue to transfer the mutant allele). Thus, a mutant *SlPP2C1* allele may be generated (human induced mutation using mutagenesis techniques to mutagenize the target *SlPP2C1* gene or variant thereof) and/or identified (spontaneous or natural allelic variation) in other *Solanum* species including for example S. *cheesmanii,* S. *chilense, S. habrochaites, S. chmielewskii, S. lycopersicum x S. peruvianum,* S. *glandulosum,* S. *hirsutum, S. minutum, S. parviflorum, S. pennellii,* S. *peruvianum,* S. *peruvianum* var. *humifusum* and *S. pimpinellifolium,* and then transferred into a cultivated *Solanum* plant, e.g. *Solanum lycopersicum* by traditional breeding techniques. The term "traditional breeding techniques" encompasses herein crossing, selfing, selection, double haploid production, embryo rescue, protoplast fusion, etc. as known to the breeder, i.e. methods other than genetic modification by which alleles can be transferred.

[0115] The mutation(s) in the *SlPP2C1* allele cause(s) the plant to have a significantly enhanced drought tolerance and/or significantly enhanced ABA sensitivity compared to plants lacking the mutant allele(s) (i.e. comprising wild type *SlPP2C1* alleles), as described above.

[0116] Without limiting the invention, the mutation in *SlPP2C1* (SEQ ID NO: 1, or variants thereof, or in the corresponding genomic sequence, e.g. SEQ ID NO: 11 from nucleotides 2676 to 4975, or variants thereof), result in reduced functionality or loss-of function of the S1PP2C1 protein, for example through single base transition(s), mis-sense or non-sense mutations, or insertion or deletion of one or more amino acids or a frame-shift in the coding sequence, which in turn results in the changed phenotype. The presence and type of mutation(s) can be analyzed by sequencing the gene, using *SlPP2C1* specific primers. A "significant reduction" of the S1PP2C1 protein's functionality is preferably determined indirectly *in vivo* by the phenotype (i.e. significantly enhanced drought tolerance) in plants heterozygous or, preferably, homozygous for the mutant allele. The drought tolerant phenotype co-segregates with the mutant allele. However, a "significant reduction" of the protein's functionality can also be determined *in vitro* by protein phosphatase assays, whereby the mutant S1PP2C1 protein phosphatase activity is reduced by at least 10, 20, 30, 40, 50, 60, 70, 80, 90 (reduced-function) or 100% (loss of function mutation). To do this, the mutant allele is cloned and expressed e.g. in *E. coli,* followed by an *in vitro* phosphatase assay as for example described by Gosti et al. 1999, Plant Cell 11: 1897-1910, Material and Methods - PP2C Activities, page 1907.

[0117] In one embodiment of the invention a plant of the species *Solanum lycopersicum* is provided, which comprises one or more mutations in SEQ ID NO:1 or SEQ ID NO: 11 (from nucleotides 2676 to 4975), or in the corresponding genomic sequence of any one of these, whereby the mutation results in the encoded S1PP2C1 protein (or variant) having reduced activity (compared to the wild type functional protein) or no activity *in vivo* and/or *in vitro* and wherein said plant comprises significantly enhanced drought tolerance compared to a plant (preferably tomato) comprising a

nucleic acid sequence encoding a wild type S1PP2C 1 protein (or variant).

[0118] In one embodiment a plant of the species *Solanum lycopersicum* is provided, which comprises one or more mutations in the nucleotide sequence encoding the protein of SEQ ID NO: 2, or a protein comprising at least 90%, 95%, 98%, 99% or more sequence identity to SEQ ID NO: 2 (as defined), and wherein the (tomato) plant comprises significantly enhanced drought tolerance compared to a (tomato) plant lacking said one or more mutations.

[0119] In one embodiment a drought tolerant plant of the species *Solanum lycopersicum* comprising a mutant *SlPP2C1* allele is provided, characterized in that the mutation is a loss-of-function or reduced-function mutation of the encoded S1PP2C1 protein, said protein being a protein comprising at least 90%, 95%, 98%, 99% or more amino acid sequence identity to SEQ ID NO: 2.

[0120] The plant (e.g. the tomato plant) is preferably homozygous for the mutant *SlPP2C1* allele.

[0121] Mutant plants can be distinguished from non-mutants by molecular methods, such as the mutation(s) present in the *SlPP2C1* genomic DNA or mRNA (cDNA), S1PP2C1 protein levels and/or protein activity, etc., and by the modified phenotypic characteristics compared to the wild type. The mutant allele can be transferred into other plants which are sexually compatible with the mutant plant using traditional crossing and selection. Thus, the mutant allele can be used to generate drought tolerant tomato varieties of any type, e.g. open pollinate varieties, hybrid varieties, F1 hybrids, Roma type, cherry type, determinate or indeterminate types, etc. In one embodiment the plant (*S. lycopersicum*) comprising the mutant *SlPP2C1* allele and having enhanced drought tolerance is an F1 hybrid plant or a F1 seed, from which an F1 hybrid plant is grown. The inbred parents used to make the F hybrid preferably both comprise the same mutant *SlPP2C1* allele in their genome in homozygous form.

[0122] In another embodiment, the tomato plant comprising the mutant *SlPP2C1* allele is crossed with another plant of the same species or of a closely related species, to generate a hybrid plant (hybrid seed) comprising the mutant *SlPP2C1* allele. Such a hybrid plant is also an embodiment of the invention. Also a method for transferring a mutant *SlPP2C1* allele to another plant is provided, comprising providing a plant comprising a mutant *SlPP2C1* allele in its genome, crossing said plant with another plant and obtaining the seeds of said cross. Optionally plants obtained from these seeds may be further selfed and/or crossed and progeny selected comprising the mutant allele and having enhanced drought tolerance.

[0123] In one embodiment, the parents used to make the F1 hybrid comprise different mutant *SlPP2Cl* alleles in homozygous form, so that the hybrid comprises two different mutant *SlPP2C1* alleles. For example, parent 1 may comprise loss-of-function mutant while parent 2 comprises a reduced-function mutant. The F1 hybrid then comprises one allele from each parent. Thus, also tomato plants comprising two different mutant *SlPP2Cl* alleles at the S1PP2C1 locus and having enhanced drought tolerance are provided herein.

[0124] Plants comprising a mutant *SlPP2C1* allele, encoding a loss-of-function or reduced-function protein (e.g. a truncated protein as a result of a non-sense mutation, a protein having a modified amino acid sequence, resulting e.g. in a modified catalytic site, a modified folding, etc., for example due to a mis-sense mutation, frame-shift mutation and/or a splice site mutation), can be generated and/or identified by using mutagenesis methods or by screening natural populations for natural variants in the *SlPP2C1* allele. In one embodiment of the invention TILLING is used to generate such plants and/or to identify such mutagenesis induced mutations and/or EcoTILLING is used to identify plants, such as wild plants or non-cultivated plants, comprising natural (spontaneous) mutations in the *SlPP2C1* gene, which can then be transferred into cultivated plants by traditional breeding techniques. However, any other mutagenesis method may be used and it is understood that both human induced mutants, UV or X-ray mutagenesis, chemical mutagens etc. and spontaneous mutants of the *SlPP2C1* gene generated in or transferred into cultivated plants or crop plants by traditional breeding are encompassed herein.

[0125] In one specific disclosure the drought tolerant mutant plant is a plant of a different species than tomato, e.g. a monocotyledonous cultivated plant, preferably a rice, maize, wheat or barley plant comprising a mutant *SlPP2C1* allele in its genome. When using methods such as TILLING, the amplification of the target gene fragment may be based on SEQ ID NO: 1, or fragments thereof (e.g. using specific or degenerate primers, for example designed based on one or more of the conserved domains of S1PP2C1), or one may first isolate the *SlPP2C1* ortholog and base primer design on the orthologous sequence.

[0126] TILLING (Targeting Induced Local Lesions IN Genomes) is a general reverse genetic technique that uses traditional chemical mutagenesis methods to create libraries of mutagenized individuals that are later subjected to high throughput screens for the discovery of mutations. TILLING combines chemical mutagenesis with mutation screens of pooled PCR products, resulting in the isolation of mis-sense and non-sense mutant alleles of the targeted genes. Thus, TILLING uses traditional chemical mutagenesis (e.g. EMS or MNU mutagenesis) or other mutagenesis methods (e.g. radiation such as UV) followed by high-throughput screening for mutations in specific target genes, such as *SlPP2C1* according to the invention. S1 nucleases, such as CEL1 or ENDO1, are used to cleave heteroduplexes of mutant and wildtype target DNA and detection of cleavage products using e.g. electrophoresis such as a LI-COR gel analyzer system, see e.g. Henikoff et al. Plant Physiology 2004, 135: 630-636. TILLING has been applied in many plant species, such as tomato (see http://tilling.ucdavis.edu/index.php/Tomato_Tilling), rice (Till et al. 2007, BMC Plant Biol 7: 19),

Arabidopsis (Till et al. 2006, Methods Mol Biol 323: 127-35), Brassica, maize (Till et al. 2004, BMC Plant Biol 4: 12), etc. Also EcoTILLING, whereby mutants in natural populations are detected, has been widely used, see Till et al. 2006 (Nat Protoc 1: 2465-77) and Comai et al. 2004 (Plant J 37: 778-86). In one embodiment herein, classical TILLING is modified and instead of using enzyme based mutant detection (enzymatic digestion with a single-strand specific nuclease and high resolution polyacrylamide gel electrophoresis), two different high throughput detection systems can be used which have previously obly been used in humans. These detection protocols are adaptations of CSCE (Conformation Sensitive Capillary Electrophoresis, see Rozycka et al. 2000, Genomics 70, 34-40) or of HRM (High Resolution Melting, see Clin Chem 49, 853-860). See also the Examples.

[0127] Thus, non-transgenic plants, seeds and tissues comprising a mutant *SlPP2Cl* allele in one or more tissues and comprising one or more of the phenotypes conferred by a reduced-function or loss-of-function S1PP2C1 protein according to the invention (e.g. enhanced drought tolerance as described above) and methods for generating and identifying such plants is encompassed herein.

[0128] Also a method for generating and/or identifying a mutant *SlPP2Cl* allele suitable for generating drought tolerant plants and/or a method for generating a plant comprising enhanced drought tolerance is provided, comprising the steps of:

(a) mutagenizing plant seeds (e.g. by EMS mutagenesis) to generate an M1 population or providing mutagenized plant seeds or providing plants comprising natural variation,
(b) optionally selfing the plants of (a) one or more times to generate an M2, M3 or M4 families,
(c) preparing DNA of the plants of (a) or (b) and pooling DNA of individuals,
(d) PCR amplification of all or part of the *SlPP2Cl* target gene (genomic or cDNA), or a variant thereof, from the DNA pools,
(e) detecting the presence of mutated *SlPP2C1* allele(s) in the PCR amplification products and thereby also in the DNA pools,
(f) selecting the corresponding individual plants comprising the mutant *SlPP2C1* allele(s),
(g) optionally sequencing the mutant *SlPP2C1* allele of the plant;
(h) phenotyping the plants of (f), or progeny thereof, for drought tolerance and/or ABA sensitivity, and
(i) selecting drought tolerant plants, and optionally
(j) breeding with the plant of (i) to generate cultivated a drought tolerant plant having good agronomic characteristics.

[0129] Step (a) may also be simply providing such plants.

[0130] In step (c) alternatively plant tissue may be pooled and DNA isolated from the pooled tissue samples, to provide a DNA pool of different individuals.

[0131] In step (d) primers which amplify all or part of the target gene, *SlPP2Cl* (SEQ ID NO: 1) or a variant thereof, are designed using standard methods, such as CODDLE (http://www.proweb.org/doddle). Primers may be designed to amplify e.g. at least about 100, 200, 250, 300, 400, 500, 600, 800 bp or at least about 1000 bp or more of the target gene , i.e. of SEQ ID NO: 1, or of a variant of SEQ ID NO: 1, or of the genomic sequence of SEQ ID NO: 1 (i.e. further comprising introns, e.g. for tomato SEQ ID NO:11 from nucleotides 2676-4975). The genomic sequence can be easily isolated and its sequence determined as described in the Examples. Preferably a fragment comprising all or part of a conserved domain of the S1PP2C1 protein is amplified by the primer, e.g. the C-terminal domain or the putative magnesium binding domain described.

[0132] For plant species other than tomato, it may be desirable to first identify the sequence of the endogenous *SlPP2Cl* gene in order to be able to design good primer sequences. The sequence may be identified *in silico* or by, for example, designing degenerate PCR primers and amplifying all or part of the *SlPP2Cl* gene variant (ortholog of the tomato *SlPP2C1* gene) from the genome of the plant species. The sequence of the endogenous *SlPP2C1* gene is then preferably used to design suitable primers for TILLING.

[0133] Step (e) may make use of Sl nucleases, such as CEL1, to detect mismatches between the PCR amplification product, i.e. between the wild type *SlPP2Cl* PCR product and the mutant *SlPP2Cl* PCR product which form heteroduplexes. Alternatively, step (e) may use CSCE or HRM for detection. In CSCE homoduplexes (WT/WT or mutant/mutant fragments) are formed and heteroduplexes (mutan/WT fragments). Becuase of the mismatch formed, heteroduplexes migrate at a different speed than the homoduplexes through capillaries, thus allowing the identification of pools containing a mutation within the target fragment. HRM is also a non-enzymatic technique. During the PCR amplification of the target gene fragments LCgreen Plus+TM molecules are incorporated between each annealed base pair of the double stranded DNA molecule, which - when captured in the molecule - will emit fluorescence. A LightScanner records the fluorescence intensity while the plate is progressively heated. At a certain temperature the PCR products start to melt and release the LCgreen Plus+TM, whereby fluorescence decreases. DNA pools containing a mutation (heteroduplexes) are identified because their melting temperature is lower than that of homoduplexes.

[0134] Step (j) may involve traditional breeding methods and phenotypic and/or marker assisted selection methods. Many different drought tolerant varieties can be generated this way.

[0135] Extensive protocols for carrying out TILLING have been published, see e.g. http://blocks.fh-crc.org/%7Esteveh/TILLING_publications.html and Till et al. (2006) Nature Protocols 1:2465-2477; Till et al. (2006) Methods Mol Biol. 323:127-135 and Till et al. (2003) Methods Mol Biol. 236:205 -220.

[0136] Once a plant comprising a mutant allele which confers the desired phenotype has been identified, this allele can be transferred to other plants by traditional breeding techniques, e.g. by crossing the plant with another plant and collecting progeny of the cross. In step (j) the allele may thus be used to generate plants which are drought tolerant and which provide good agronomic characteristics.

[0137] As mentioned, it is understood that other mutagenesis and/or selection methods may equally be used to generate mutant plants according to the invention. Seeds may for example be radiated or chemically treated to generate mutant populations. Also direct gene sequencing of *SlPP2Cl* may be used to screen mutagenized plant populations for mutant alleles. For example KeyPoint screening is a sequence based method which can be used to identify plants comprising mutant *SlPP2Cl* alleles (Rigola et al. PloS One, March 2009, Vol 4(3):e4761).

[0138] Thus, non-transgenic mutant plants which produce lower levels of (functional) wild type S1PP2C1 protein in one or more tissues (particularly at least in leaf tissue) are provided, or which completely lack functional S1PP2C1 protein in specific tissues or which produce a non-functional S1PP2C1 protein in certain tissues, e.g. due to mutations in one or more endogenous *SlPP2Cl* alleles. These mutants may be generated by mutagenesis methods, such as TILLING or variants thereof, or they may be identified by EcoTILLING or by any other method. *SlPP2Cl* alleles encoding non-functional or reduced-functional S1PP2C1 protein may be isolated and sequenced or may be transferred to other plants by traditional breeding methods.

[0139] In one embodiment a plant having enhanced drought tolerance due to the mutant allele of tomato plants 2, 3, 5, 7 or 10 of Example 4 being present in the genome is provided herein, and progeny and parts thereof comprising the allele.

[0140] Any part of the plant, or of drought tolerant progeny thereof, is provided, including harvested fruit, harvested tissues or organs, seeds, pollen, flowers, etc.

[0141] Also provided are kits for detecting whether or not a plant comprises a mutant *SlPP2C1* allele according to the invention. Such a kit may comprise PCR primers or probes detection of the allele in a tissue sample.

[0142] Plant (and corresponding seed) comprising one or more mutant *SlPP2C1* alleles according to the invention may be marketed and/or labelled as having (enhanced) "drought tolerance" or as being "drought resistant".

[0143] Preferably, the drought tolerant mutant plants also have good other agronomic characteristics, i.e. they do not have reduced fruit numbers and/or reduced yield compared to wild type plants and/or product quality is not reduced. Preferably yield and/or survival rate of such plants is higher under both long term and/or short term drought stress. In the present invention the plant is a tomato plant and the fruit is a tomato fruit, such as a processing tomato, fresh market tomato of any shape or size or colour. Thus, also harvested products of plants or plant parts comprising one or two mutant *SlPP2Cl* alleles are provided. This includes downstream processed products, such as tomato paste, ketchup, tomato juice, cut tomato fruit, canned fruit, dried fruit, peeled fruit, etc. The same applies for other plant species.

[0144] Plants, or plant parts, comprising one or more mutant *SlPP2Cl* alleles and being drought tolerant may be vegetable crops (e.g. tomato).

[0145] Different *in vivo* expression of wild type *SlPP2C1* alleles (or variants, including orthologs) may also lead to plants having significantly enhanced drought tolerance. For example wild type *SlPP2C1* alleles comprising promoters having a different expression pattern, especially reduced expression, than the alleles found in cultivated tomato plants may be identified (e.g. in wild relatives of tomato) and introgressed (transferred through crossing) into cultivated tomato. Or alleles having mutations in the *SlPP2C1* promoter may be identified or generated and used to generate plants having enhanced drought tolerance.

SEQUENCES

[0146]

SEQ ID NO 1: cDNA sequence of the wild type *SlPP2Cl* allele from tomato
SEQ ID NO 2: protein sequence of the S1PP2C1 protein encoded by SEQ ID NO: 1
SEQ ID NO 3 and 4: primer 1 and 2 for amplifying *SlPP2Cl* cDNA (Q PCR)
SEQ ID NO 5 and 6: primer pair for amplifying actin cDNA
SEQ ID NO 7 and 8: primer pair for amplifying ubiquitin cDNA
SEQ ID NO 9 and 10: primer pair for amplifying full length *SlPP2Cl* cDNA
SEQ ID NO 11: genomic sequence of the wild type *SlPP2Cl* allele from tomato; transcription regulatory elements (e.g. promoter elements) are comprised in nucleotides 1-2675; protein encoding sequences (exons) consist of nucleotides 2676 - 3419 (exon 1), 4247-4351 (exon 2) and 4632-4975 (exon 3). The primary RNA is depicted in nucleotides 2591-5050.
SEQ ID NO 12 and 13: primers used to detect *SlPP2Cl* mutations in mutagenized tomato plants (see Example 4)

SEQ ID NO 14: putative potato *SlPP2C1* cDNA (termed *StPP2C1* herein)
SEQ ID NO 15: putative potato S1PP2C1 protein (termed StPP2C1 herein)

FIGURE LEGENDS

[0147]

Fig. 1: A) Relative mRNA levels of *SlPP2C1* in leaf (dark grey bars) of transgenic lines ($T6_{OE}$, $T34_{OE}$, $T55_{OE}$) are 25-fold higher as compared to wild type (Wt). B) Relative mRNA levels of *SlPP2C1* in pollinated ovaries (light grey bars) in $T55_{OE}$ are similar to wild type, while the levels in $T6_{OE}$ and $T34_{OE}$ are lower as compared to wild type. C) Relative mRNA levels in transgenic lines $T12_{CS}$ and T35cs are lower in both leaves (dark grey bars) and unpollinated ovaries (light grey bars) as compared to wild type and T18. Mean values of biological replicas are shown with SE, wild type levels were set to one.

Fig. 2: A) Seed germination is inhibited less by ABA in the over-expression lines $T6_{OE}$ and $T55_{OE}$ and more in the co-suppression lines T12cs and T35cs as compared to wild type (Wt). Line $T34_{OE}$ behaves atypically. Dark grey lines represent over-expression lines, light grey lines represent co-suppression lines and black line represents wild type. B) Root growth is inhibited more by ABA in the co-suppression lines $T12_{CS}$ (white) and T35cs (light grey) as compared to T18 (dark grey) and wild type (black). C) Root growth is inhibited less by ABA in the over-expression line $T34_{OE}$ (dark grey), but more in $T6_{OE}$ (white) and $T55_{OE}$ (light grey), as compared to wild type (black). Mean root growth percentage is depicted with SD. D) Nine days after the start of water withholding co-suppression lines are not wilted at all, while wild type shows moderate wilting and over-expression lines show severe wilting.

[0148] The following non-limiting Examples describe the use of *SlPP2C1* genes for modifying plant phenotypes. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, and Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY; and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

EXAMPLES

EXAMPLE 1 - isolation and characterization of S1PP2C1

1.1 Materials and methods

1.1.1 Plant material

[0149] Tomato plants (Solanum lycopersicum L. cv. Moneymaker) were grown under greenhouse conditions from March to October under 16/8h day-night rhythm. Supplementary lights (600 Watt high pressure sodium lights, Philips, Eindhoven, The Netherlands) turned on below 200 W/m2 and turned off above 300 W/m2 . Temperature was kept above 20°C during the light period and 17°C during the dark period with the PRIVA Integro versie 724 system. Plants were watered daily and given fertilizer weekly. Leaf and ovary tissues were dissected form adult tomato plants and root and hypocotyls from 10-day-old seedlings. Tissues were harvested between 11.00 hrs and 13.00 hrs and directly frozen in liquid nitrogen.

1.1.2 *SlPP2C1* gene-expression analysis and Q-PCR

[0150] cDNA-AFLP was preformed as described in Vriezen et al. (2008, New Phytologist, 177:60-76, see page 61 and 62), comparing pollinated ovaries with gibberellic acid (GA_3) treated ovaries. A differentially expressed fragment of 326 bp was cut out from the gel and the eluted DNA was re-amplified under the same conditions as used for the selective amplification. The fragment was ligated in a T-tailed EcoRV digested phagemid pBluescriptII SK(+) (Stratagene, La Jolla, CA, USA) and sequenced using the CEQ DTCS Quick Start Kit and CEQ2000 DNA Analysis System (Beckman Coulter, Fullerton, CA, USA).

[0151] For quantitative PCR analysis RNA was isolated with the Trizol (Invitrogen, Carlsbad, CA, USA). Photometric RNA measurements were done to equilibrate the RNA concentrations of different samples. Equal amounts of RNA were

DNAse treated with RNAse free DNAse (RQ1, Promega, Madison, USA). RNA (0.5 μg) was reverse transcribed (RT) using a cDNA synthesis kit (iScript^{tm}, Bio-rad Laboratories, Hercules, CA, USA) following protocol.

[0152] Real-time-quantitative PCR (Q-PCR) primers for quantifying *SlPP2C1* mRNA transcript levels were designed using a computer program (Beacon Designer Software, Premier Biosoft International, CA, USA), and checked for cross-homology with other PP2C sequences.

Primer 1:	5'-TCGGAAGGAGAAGATTACG-3'	(SEQ ID NO: 3)
Primer 2:	5'-TCCACAATTCGCAACAAC-3'	(SEQ ID NO: 4)

[0153] Primer pair 1 and 2 amplifies the following fragment of the *SlPP2C1* transcript (174 bp):

5'TCGGAAGGAGAAGATTACGATGGGAAGAGTATTAACTGGGAGAAAGTTATGACGGA GAGTTTCCGTAAAATGGACGAAAAGGTGAACAAGGAAGGGGCGGAGATGGCGACGATA GGATCAACGGCGGTGGTAGCGGTGGTGGGAGTGGAGGAATTT**GTTGTTGCGAATTGTG GA 3'**

[0154] PCR reactions were performed in a 96-well thermocycler (Bio-Rad iCycler, Bio-rad laboratories) using SYBR green mix (iB-SYBR Green supermix, Bio-rad laboratories). The PCR program started with 3 minutes at 95°C then 40 cycles consisting of 15 second at 95°C and 45 seconds at 57°C and finally the melting temperature of the amplified product was determined to verify the presence of a specific product. Five microliter of 25-fold diluted cDNA was used per sample. Technical and biological replicates were always preformed.

[0155] Both actin mRNA and ubiquitin were used as internal control genes.

Actin primer 1: 5' -GGACTCTGGTGATGGTGTTAG-3'	(SEQ ID NO: 5)
Actin primer 2: 5' -CCGTTCAGCAGTAGTGGTG-3'	(SEQ ID NO: 6)
Ubiquitin primer 1: 5' -CCCTGGCTGATTACAACATTC-3'	(SEQ ID NO: 7)
Ubiquitin primer 2: 5' -TGGTGTCAGTGGGTTCAATG-3'	(SEQ ID NO: 8)

[0156] Diluted DNase treated RNA was also included in the Q-PCR as a control for genomic DNA contamination.

1.1.3 Plant transformation

[0157] To generate transgenic *SlPP2C1*- lines, the coding region of *SlPP2C1* (SEQ ID NO: 1) was PCR amplified and cloned in pDONR vector. PCR primers used for amplifying the complete S1PP2C1 cDNA were designed as follows:

Primer 1 (forward): 5' CACCTGCAGTCACCGTCTTCACATTAAAAT 3' (SEQ ID NO: 9)

Primer 2 (reverse): 5' ATTTGTATGGGAAGCTTAACTATCA 3' (SEQ ID NO: 10) Using Gateway cloning the *SlPP2C1* was cloned behind the *Cauliflower Mosaic Virus 35S* promoter in the pAD625 vector (de Folter et al. 2006, The Plant Journal 47: 934-946) which also contains a nopaline synthase terminator (3'nos). Transgenic tomato plants were generated by *Agrobacterium tumefaciens*-mediated transformation and tissue culture as described in De Jong *et al.* (2008, supra).

1.1.4 Water stress experiment

[0158] The pots of wild type and *SlPP2C1* transgenic lines of the same age and size, were saturated with water at the start of the experiment. Several plants were used per line. Plants were withheld from water for ten days. Wilting of leaves was assessed visually when the first signs of wilting (slight wilting or moderate wilting) was seen in the wild type control at day 8 and 9, respectively. A scale of 1-4 was used, with 4 being severely wilted, 3 being moderately wilted, 2 being slightly wilted and 1 showing no signs of leaf wilting.

[0159] Photographs depicted in Figure 2D were taken nine days after start of the experiment.

1.1.5 Seed germination assay and root growth assay

Seed germination:

[0160] Seeds of the transgenic lines were harvested, dried and stored at 4 degrees Celsius. Seeds were sterilized in diluted bleach (4% (w/v) hypochlorite) containing 0.1% (v/v) Tween-20, washed and sown on 1/2MS media (2.25 mg MS basal salts supplied with 1% (w/v) sucrose, and Nitsch vitamins, Duchefa, Haarlem, the Netherlands) containing, 0, 1, 3, 10, or 30 μM ABA (Acros, Geel, Belgium). At least 40 seeds per ABA concentration were used.

[0161] The plates were placed in a growth chamber at 25°C under 16/8h day-night rhythm. Seed germination (radicle protrusion) was scored after ten days and seed germination percentages were calculated.

Root growth:

[0162] Seeds were sterilized as described above and sown in water containing 1μM GA_3 . After radicle protrusion ten seeds of each line were placed on 1/2MS media containing either 0, 5, 10, or 40 μM ABA. The roots were measured and the plates were placed in vertical position and grown under conditions described above. Seven days after transfer the length of the root was measured again and root growth was calculated as a percentage of root growth compared to 1/2MS media without ABA (100%). Experiments were carried out three times and mean values plus SD are shown. Student's t-tests were performed to test for significance ($p<0.05$).

1.2 Results

1.2.1 Expression of *SlPP2C1* during fruit set (data not shown)

[0163] The *SlPP2C1* gene is differentially expressed during fruit set (Vriezen *et al.* 2008, supra). *SlPP2C1* is expressed highest in the pericarp of unpollinated ovaries and lower after pollination and GA_3-treatment. The expression in ovules/placenta does not seem to change. We confirmed the expression of *SlPP2C1* within the tissues of the tomato ovary by quantitative PCR. Again it was observed that the mRNA levels of *SlPP2C1* are higher in the pericarp than in the ovules or placenta in control tissue. In the pericarp, but also in the placenta, a lower mRNA level of *SlPP2C1* was found three days after pollination. In ovules mRNA levels did not change. The *SlPP2C1* gene is expressed in mature unpollinated ovaries at anthesis at a relatively high level compared to vegetative tissues such as leaf, root and hypocotyl. In flower buds and in ovaries three days before anthesis the *SlPP2C1* mRNA level is comparable to leaf, and it is much lower than in ovaries at anthesis (control, Ct). Three days after pollination *SlPP2C1* mRNA levels in the ovary were reduced to approximately 50% of the level in unpollinated ones.

1.2.2 Functional analysis of *SlPP2C1*

Overexpression of SlPP2C1

[0164] The over-expression (OE) approach resulted in three transgenic tomato lines with on average 25-fold higher mRNA levels of *SlPP2C1* in leaves (overexpressing lines $T6_{OE}$, $T34_{OE}$ and $T55_{OE}$, Fig. 1A). In pollinated ovaries the *SlPP2C1* mRNA levels of these lines were not higher as in wild type (Fig. 1B). On the contrary, $T6_{OE}$ and $T34_{OE}$ displayed a strong reduction in *SlPP2C1* mRNA levels as compared to wild type. $T55_{OE}$ had mRNA levels similar to wild type in pollinated ovaries. The *SlPP2C1* mRNA levels in unpollinated ovaries of these transgenic lines were comparable to the mRNA levels in pollinated ovaries and are not depicted here.

Silencing of SlPP2C1

[0165] Additionally, two lines (co-suppression lines $T12_{CS}$ and $T35_{CS}$) were obtained that had lower *SlPP2C1* mRNA levels in leaves and unpollinated ovaries, as compared to wild type (Fig 1C), indicating that the overexpression construct led to co-suppression of *SlPP2C1* in these lines. Line T18 has mRNA levels that are comparable to wild type in both tissues.

Sensitivity to ABA

[0166] Phenotypic characterization of the transgenic lines revealed changed sensitivity for ABA. Figure 2A shows the percentage of seed germination on media containing different concentrations of ABA. The co-suppression lines $T12_{CS}$ and $T35_{CS}$ have lower germination percentages on 1μM and 3 μM ABA, while the overexpressing lines $T6_{OE}$ and $T55_{OE}$ have slightly higher germination percentages than wild type. Line $T34_{OE}$, which is an over-expression line in leaves,

behaved differently from the other over-expression lines and showed lower seed germination on ABA as compared to wild type.

[0167] Root growth after germination is reduced by ABA in wild type. In Figure 2B it can be seen that root growth in co-suppression lines T12 $_{CS}$ and T35$_{CS}$ was inhibited more strongly than in wild type or T18. Overexpression line T34$_{OE}$ was less sensitive to root growth inhibition by ABA than wild type (Fig. 2C). Root growth of lines T6$_{OE}$ and T55$_{OE}$ was more sensitive to ABA.

[0168] Thus, co-suppression of *SlPP2C1* leads to plants having a significantly enhanced ABA sensitivity than wild type (seed germination is inhibited more and root growth is inhibited more than in wild type), while overexpression leads to plants having a reduced ABA sensitivity, indicating that *SlPP2C1* encodes a negative regulator of ABA.

Drought tolerance

[0169] Figure 2 D shows that at 9 days wild type plants having moderate wilting, co-suppressing lines showing no wilting and over expressing lines showing severe wilting. At 9 days, the over-expressing lines were severely wilted (average wilting score = 3.6), while co-suppressing lines showed little wilting (score 1.25) and controls showed moderate wilting (score = 3.0). The experiment was repeated once (data not shown). Thus, wilting was reduced in the co-suppressing lines by 58% compared to wild type control.

[0170] These data indicate that down-regulation of *SlPP2C1* leads to plants having significantly enhanced drought tolerance compared to wild type.

Discussion

[0171] Two of the transgenic lines harbouring an over-expression construct had lower levels of *SlPP2C1* mRNA in both leaves and ovaries. RNA silencing by co-suppression is a well accepted phenomenon although the mechanism is not fully understood. It has been suggested that co-suppression is induced by hairpin-RNA transcripts from inverted-repeat transgene copies, resulting in siRNAs that are incorporated into the RNAi pathway (Tomita et al. 2004, FEBS Lett. 2004 Aug 27;573(1-3):117-20; Wang and Metzlaff 2005, Curr Opin Plant Biol. 8(2): 216-22). Southern blot analysis (data not shown) revealed that in the two co-suppression lines of *SlPP2C1* multiple insertions were present, which might have resulted in a hairpin-like structure that could silence the endogenous *SlPP2C1* gene.

[0172] The three lines with a 25-fold higher mRNA level of *SlPP2C1* in leaf did not have higher *SlPP2C* mRNA levels in ovaries. This might be partly explained by the relatively high mRNA level of the endogenous gene in ovaries, which is seven fold higher than in leaf. The contribution of the transgene to the total expression levels of *SlPP2C1* in ovaries might therefore have been very small. Remarkable is that in line T6$_{OE}$ and T34$_{OE}$ the mRNA level of *SlPP2C1* in ovary is even lower (10-50%) than in wild type. Tissue specific control of endogenous mRNA levels by a transgene has however been reported before. Tomita *et al.* (2004, *supra)* showed that in the same plant the *NtFAD3* gene was co-suppressed in leaf but over-expressed in root, resulting in the equivalent phenotypes in leaves and roots. The mechanisms by which tissue specific regulation of co-suppression occurs are unknown, but the level of endogenous transcript seems to be important (Tomita *et al.* 2004, *supra).*

[0173] The two tomato transgenic lines with reduced mRNA levels in leaf and ovary also displayed ABA-hypersensitive responses during seed germination and root growth and enhanced tolerance to water stress. This indicates that the *SlPP2C1* gene is a negative regulator of the ABA signalling cascade in tomato. Moreover, the three transgenic lines with relative high mRNA levels of *SlPP2C1* in leaf also wilted stronger.

EXAMPLE 2 - TILLING mutants comprising enhanced drought tolerance and comprising mutant *SlPP2C1* alleles

2.1 Tomato TILLING population

[0174] A highly homozygous inbred line used in commercial processing tomato breeding was used for mutagenesis treatment with the following protocol. After seed germination on damp Whatman® paper for 24h, ~20,000 seeds, divided in 8 batches of 2500 respectively, were soaked in 100 ml of ultra pure water and ethyl methanesulfonate (EMS) at a concentration of 1% in conical flasks. The flasks were gently shaken for 16h at room temperature. Finally, EMS was rinsed out under flowing water. Following EMS treatment, seeds were directly sown in the greenhouse. Out of the 60% of the seeds that germinated, 10600 plantlets were transplanted in the field. From the 8810 M1 lines that gave fruits, two fruits per plant were harvested. DNA was isolated from seeds coming from the first fruit, constituting the M2 population DNA stock. These were selfed and M3 seeds were isolated from the fruits and the seeds were used for DNA isolation and constitute the M3 population DNA bank.

2.2 Target S1PP2C1 gene for PCR amplification from TILLING population

[0175] Genomic sequence containing the complete transcribed region including coding region (COD), untranslated 5'end region (5'UTR) and 3'end region (3'UTR) was determined using PCR with genomic tomato DNA as template and primers designed on the flanks of the tomato S1PP2C1 sequence. By comparison of the genomic gene sequence with the *SlPP2C1* cDNA sequence the location, number and size of introns and exons in this gene was determined.

[0176] The genomic sequence is depicted in SEQ ID NO: 11. The primary RNA (transcribed region) is from nucleotide 2591-5050, comprising a 5'UTR (2591-2675), exon 1 (2676-3419), intron 1 (3420 - 4246), exon 2 (4247-4351), intron 2 (4352 - 4631), exon 3 (4632 - 4975) and the 3'UTR (4976 - 5050).

[0177] DNA of the tomato TILLING population described above was then screened for single nucleotide polymorphisms in the *SlPP2C1* target gene. For this purpose PCR primer pairs were designed to amplify overlapping fragments of about 400-500bp from the coding (exon) sequences of the *SlPP2C1* gene (SEQ ID NO: 1) or a sequence comprising all or part of the nucleic acid which encodes the C-terminus of the S1PP2C1 protein (as mutations in the catalytic domain are likely to result in reduced function or loss of function of the protein), i.e. encoding amino acids 84-391 of SEQ ID NO: 2. See Example 4 below, where mutations in amino acids 101-192 were identified.

[0178] The primer pairs were used to amplify target sequences from the M2 or M3 DNA of the TILLING population and heteroduplexes between mutant and wild type target sequences were detected using CSCE or HRM as described below. The ID number of the DNA samples is linked to seed batches of plants carrying the wild type allele or the mutated allele either in heterozygous or in homozygous form.

[0179] Seeds were germinated and the presence of the particular mutation in individual plants was confirmed by PCR using primers flanking the mutated site and genomic DNA of these plants as templates. DNA sequencing of the fragments identified mutants homozygous and heterozygous for the expected mutation. Homozygous mutants are selected or obtained after selfing and subsequent selection and the effect of the mutation on the corresponding protein and phenotype of the plant is determined.

[0180] Plants comprising one or more mutations in the target sequences are screened phenotypically for drought tolerance using e.g. the assay described above and/or field assessment.

2.3 Conformation Sensitive Capillary Electrophoresis (CSCE)

[0181] Multiplex PCR reactions are performed in 10 μl volume with 0.15 ng, 4 times pooled genomic DNA. Labeled primers are added to the PCR master mix to a concentration 5 times lower (1 μM) than that of the unlabeled primers. Post PCR, samples are diluted 10 times. Before the CSCE run, 2 μl of the diluted products are added to 38 μl of MQ water.

[0182] The samples are loaded on 50 cm capillaries (injection time and voltage: 16 seconds, 10 KVolts; Run voltage: 15KVolts) from the ABI 3130x1 apparatus filled with semi-denaturating polymers of the following composition: 5 g Conformation Analysis Polymer (CAP) (Applied Biosystems, 434037, 9%), 2,16 g Ureum, 0,45 g 20xTTE (national diagnostics, EC-871), completed with MQ water up to 9 g. The running buffer is prepared with 1x diluted TTE and 10% glycerol. The oven temperature is set to 18°C.)

[0183] Raw data is analysed with the HeteroDuplex Analysis (HDA) software from BioNumerics, The program differentiates peak patterns of hetero-duplexes (mutant) and homo-duplex molecules (wild type) thus providing the possibility of selecting DNA-pools containing an individual line mutated in the target gene.

2.4 High Resolution Melt curve analysis (HRM)

[0184] The LCgreen PCRs are performed on 8x flat pools in FramStar 96-wells plates (4titude, UK). 2μl (15 ng) of pooled DNA is mixed with 2μl of F-524 Phire™ 5x reaction buffer (FINNZYMES, Finland), 0.1μl Phire™ Hot Start DNA Polymerase (FINNZYMES, Finland), 1 μl LCGreen™ Plus+ (BioChem, USA), 0.25μl of 5mM primers, and completed to 10μl with MQ water) according to manufacturer recommendations. Pools containing a mutation are screened using a LightScanner® System (Idaho Technology Inc., USA). Positive pools are selected by analyzing the melting temperature profiles; when the pool contains a mutation it will show a lower melting temperature.

EXAMPLE 3 - transfer of mutant *SlPP2C1* alleles into tomato cultivars

[0185] TILLING mutants comprising a mutant *SlPP2C1* allele are crossed with different tomato lines in order to transfer the mutant allele into these lines, generating tomato plants with good agronomic characteristics and significantly enhanced drought tolerance.

[0186] A TaqMan® SNP Genotyping Assays (Applied Biosystems) marker is developed to identify the presence of the modified nucleotide. This assay is used for Marker-assisted foreground selection which is effective for the transfer of recessive genes to a required background, for example commercial tomato parent lines, since their classical transfer

requires additional recurrent selfing generations (Ribaut et al. Plant Molecular Biology Reporter 15:154-162).

EXAMPLE 4

[0187] DNA of the M2 TILLING population described in Example 2.1 was screened for single nucleotide polymorphisms in the *SlPP2C1* target gene (as described in 2.2), in particular for mutations in the sequence encoding amino acids 101 to 192.

[0188] For this purpose the following PCR primer pair was designed to amplify a 277 bp fragment of nucleotides 301-577 of SEQ ID NO: 1:

Forward primer: "3863" 5'- GTGACGTGCTGTTCACATGGATC-3' (SEQ ID NO: 12)

Reverse primer: "3861" 5' -TACGGAAACTCTCCGTCATAAC-3' (SEQ ID NO: 13)

[0189] The primer pair was used to amplify target sequences from the M2 DNA of the Tilling population. The amplified target sequence comprises nucleotide 301 to 577 of SEQ ID NO: 1, i.e. the region encoding amino acids 101 to 192 of SEQ ID NO: 2. Heteroduplexes between mutant and wild type target sequence were identified using HRM as described in Example 2.3 (or 2.4).

[0190] Ten plants comprising a SNP in the target region were identified and the PCR product of the target sequence was sequenced in order to determine the nature and position of the SNP. The results are shown in Table 2 below.

Table 2

Plant number (M2, heterozygous)	SNP in target sequence	Mutation effect on protein sequence (SEQ ID NO: 2)	SIFT prediction on protein function
1	T → A at nucleotide 372 of SEQ ID NO: 1 (atT → atA)	Ile 124 → Ile	silent
2	G → A at nucleotide 442 of SEQ ID NO: 1 (Ggg → Agg)	Gly 148 → Arg	deleterious
3	C → T at nucleotide 512 of SEQ ID NO: 1 (tCg → tTg)	Ser 171 → Leu	tolerated
4	G → A at nucleotide 504 of SEQ ID NO: 1 (caG → caA)	Gln 168 → Gln 168	silent
5	G → A at nucleotide 463 of SEQ ID NO: 1 (Gcg → Acg)	Alal55 → Thr	deleterious
6	G → A at nucleotide 465 of SEQ ID NO: 1 (gcG → gcA)	Alal55 → Ala	silent
7	G → A at nucleotide 394 of SEQ ID NO: 1 (Ggc → Age)	Gly 132 → Ser	tolerated
8	To be determined	To be determined	To be determined
9	To be determined	To be determined	To be determined
10	G → A at nucleotide 463 of SEQ ID NO: 1 (Gcg → Acg)	Ala 155 → Thr	deleterious

[0191] Based on SIFT analysis (Pauline C. Ng and Henikoff 2003, Nucleic Acid Research Vol. 31, pp 3812-3814) the effect on protein function was predicted, see Table 2.

[0192] Three plants (plants number 1, 4 and 6) contained a silent mutation in the *SlPP2C1* gene, while five plants (number 2, 3, 5, 7 and 10) contained SNPs that lead to amino acid substitutions. Plants 5 and 10 contained identical mutations. Based on SIFT analysis, it is predicted that plants number 2, 5 and 10 comprise mutations in the *SlPP2C1* allele which reduce or abolish PP2C1 protein function and therefore confer enhanced drought tolerance. It is noted that the mutant *SlPP2C1* allele found in plant 2 is in the Asp-Gly-His (DGH) domain.

[0193] Plants number 3 and 7 may also comprise a mutant allele which may confer enhanced drought tolerance.

[0194] To test the response of plants 2, 3, 5, 7 and 10 to drought and/or other abiotic stresses, 14-d-old seedlings are subjected to various forms of stress treatments selected from one or more of the following treatments.

1. Drought stress can be imposed as described in the general description, the Examples above and/or by growing seedlings for 21 days *in vitro* on MS (to obtain homogenous populations) after which they are transferred to pots containing sandy soil. Two weeks after transfer water is withheld for 6 days after which 50 ml water/plant is added once. Results are scored when the azygous and control plants turn yellow (modified from The Plant Journal, 2004, 41, 95-106).
2. Salinity stress is imposed by growing seedlings on MS medium containing 250 mM NaCl for 0 to 48 h.
3. Osmotic stress can be imposed by growing seedlings on MS medium with added mannitol at 75 mM or ABA at 0.1 or 1.0 mM. Total root length and the amount of lateral roots developing during plant growth is a measure for osmotic stress tolerance and ABA sensitivity, respectively (modified from Xion et al., Plant Physiology, 2006, 142, 1065-1074).

[0195] Plants comprising significantly enhanced stress tolerance, especially drought tolerance, compared to controls are used to generate tomato cultivars with good agronomic characteristics as e.g. described in Example 3. Plants with significantly enhanced drought tolerance may also in addition or alternatively comprise significantly enhanced salinity and/or osmotic stress tolerance compared to controls.

SEQUENCE LISTING

[0196]

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<151> 2009-06-08

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Ser Thr Arg Arg Arg Arg Leu Asp Glu Arg Leu Leu Lys Ser Thr Thr
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Lys Arg Lys Lys Ser Thr Val Thr Asp Thr Asp Asp His Arg Val Gln
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Glu Asp Ala Val Ala Ile Tyr Pro Cys Phe Phe Ser Glu Gly Gly Gly
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Gly Gly Ser Arg Arg Tyr Asp Tyr Phe Gly Val Tyr Asp Gly His Gly
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Trp Glu Lys Val Met Thr Glu Ser Phe Arg Lys Met Asp Glu Lys Val
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aac aag gaa ggg gcg gag atg gcg acg ata gga tca acg gcg gtg gta      3317
Asn Lys Glu Gly Ala Glu Met Ala Thr Ile Gly Ser Thr Ala Val Val
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tgtgaccaac aaaccaaatc ccataagaga gatgatcagt caagtgaagg tgtcaaagaa 1080

agtctcgcag cacgagcagc ttccttcctt gcagagttgg caattgctcg gggtagtagg 1140

gataacatca gtgtaattgt cgtcgatttg aatagatctg tatgttcatc cactgactga 1200

<210> 15
<211> 399
<212> PRT
<213> Solanum tuberosum

<400> 15

```
Met Ile Asp Asn Val Lys Gly Leu Pro Pro Val Ala Glu Lys Gly Cys
1               5                  10                  15

Arg Leu Thr Ala Leu Val Asp Ser Gly Gly Leu Ala Glu Val Asp Leu
            20                  25                  30

Ser Glu Lys Glu Pro Asn Phe Thr Arg Arg Arg Arg Leu Asn Glu Arg
        35                  40                  45

Arg Leu Lys Ser Ser Pro Asp Leu Pro Glu Asn Phe Asn Val Phe Ala
    50                  55                  60

Ala Asp Tyr Arg His Tyr Lys Lys Lys Lys Pro Glu Asn Ser Thr Val
65                  70                  75                  80

Thr Asp Thr Asp Asp Gln Val Gln Leu Ala Thr Ser Ser Glu Val Lys
                85                  90                  95

Lys Val Arg Glu Ser Leu Val Thr Cys Cys Ser His Gly Ser Ile Ser
            100                 105                 110

Leu Ile Gly Arg Arg Arg Glu Met Glu Asp Ala Val Ala Ile Tyr Pro
        115                 120                 125

Ser Phe Phe Ser Glu Gly Ser Ser Arg Lys Tyr Asp Tyr Phe Gly Val
    130                 135                 140

Tyr Asp Gly His Gly Gly Ser Arg Val Ala His Ala Cys Arg Asp Phe
145                 150                 155                 160

Leu His Arg Leu Val Ile Gln Gln Val Ser Glu Gly Glu Asp Tyr Asp
                165                 170                 175

Gly Lys Ser Ile Asn Trp Glu Asn Val Met Met Glu Ser Phe Arg Lys
            180                 185                 190

Met Asp Glu Lys Val Asn Lys Glu Gly Ala Glu Met Ala Thr Ile Gly
        195                 200                 205

Ser Thr Ala Val Val Ala Val Val Gly Glu Glu Glu Phe Val Val Ala
```

```
    210                 215                 220

Asn Cys Gly Asp Ser Arg Ala Val Leu Ser Arg Ala Gly Val Ala Val
225                 230                 235                 240

Pro Leu Ser Ile Asp His Lys Pro Asp Arg Pro Asp Glu Leu Asp Arg
                245                 250                 255

Ile Glu Asn Ser Gly Gly Lys Val Ile Asn Trp Asn Gly Gln Arg Val
            260                 265                 270

Leu Gly Val Leu Ala Thr Ser Arg Ser Ile Gly Asp Met Tyr Leu Lys
        275                 280                 285

Pro Tyr Val Ile Pro Asp Pro Glu Val Leu Val Ser Gln Arg Ser Asp
    290                 295                 300

Glu Asp Glu Phe Leu Leu Leu Ala Ser Asp Gly Leu Trp Asp Val Ile
305                 310                 315                 320

Pro Asn Asp Val Ala Cys Asp Val Thr Arg Arg Cys Leu Asn Gly Gln
                325                 330                 335

Thr Phe Arg Arg Cys Asp Gln Gln Thr Lys Ser His Lys Arg Asp Asp
            340                 345                 350

Gln Ser Ser Glu Gly Val Lys Glu Ser Leu Ala Ala Arg Ala Ala Ser
        355                 360                 365

Phe Leu Ala Glu Leu Ala Ile Ala Arg Gly Ser Arg Asp Asn Ile Ser
    370                 375                 380

Val Ile Val Val Asp Leu Asn Arg Ser Val Cys Ser Ser Thr Asp
385                 390                 395
```

Claims

1. A non-transgenic plant of the species *Solanum lycopersicum* comprising a *SlPP2C1 (Solanum lycopersicum* serine/threonine protein phosphatase 2C) allele in its genome, whereby an *SlPP2C1* allele is an allele which encodes a protein comprising at least 90% amino acid sequence identity to SEQ ID NO: 2, **characterized in that** said *SlPP2C1* allele comprises one or more mutations in its nucleotide sequence and whereby as a result of said one or more mutations the plant comprising said mutant allele in its genome has significantly enhanced drought tolerance compared to the plant comprising a wild type *SlPP2C1* allele in its genome.

2. The plant according to claim 1, **characterized in that** said one or more mutations confer loss-of-function or reduced-function onto the encoded SlPP2C1 protein.

3. The plant according to claim 1 or 2, wherein said mutant *SlPP2C1* allele is present in homozygous form.

4. The plant according to any one of the preceding claims, wherein leaf wilting symptoms of said plant are reduced by at least 10% compared to leaf wilting symptoms of a plant comprising a wild type *SlPP2C1* allele in its genome when plants are exposed to the same drought stress.

5. The plant according to any one of the preceding claims, wherein the plant is a hybrid plant.

6. A fruit or seed or a part of a plant according to claim 3.

7. Use of a nucleic acid sequence encoding an SIPP2C1 protein for the generation of transgenic or non-transgenic plants of the species *Solanum lycopersicum* with enhanced drought tolerance, **characterized in that** the SIPP2C1 protein comprises at least 90% amino acid sequence identity to SEQ ID NO: 2.

8. A transgenic *Solanum lycopersicum* plant comprising integrated in its genome a chimeric gene, wherein said chimeric gene comprises a promoter active in plant cells operably linked to a nucleic acid sequence comprising a sense and/or antisense sequence of SEQ ID NO: 1 which upon transcription silences endogenous *SlPP2C1* gene expression and wherein said plant comprises significantly enhanced drought tolerance compared to a plant lacking said chimeric gene, wherein said endogenous *SlPP2C1* gene encodes a protein comprising at least 90% amino acid sequence identity to SEQ ID NO: 2.

9. The plant according to claim 8, wherein said transcription regulatory sequence is selected from the group consisting of: a constitutive promoter, an inducible promoter, a tissue-specific promoter and a developmentally regulated promoter.

10. A seed of a plant according to any one of claims 8-9 comprising said chimeric gene.

11. The plant according to claim 1, or seeds, progeny or tomato fruits thereof, said plant being obtainable by TILLING, wherein the plant, seeds, progeny or tomato fruits comprise a mutant *SlPP2C1* allele in their genome, **characterized in that** said mutant allele encodes an SIPP2C1 protein having reduced-function or loss-of-function compared to the wild type SIPP2C1 protein depicted in SEQ ID NO: 2.

12. The plant according to claim 11, wherein said SIPP2C1 protein comprises an amino acid substitution at Gly148, Ser171, Ala155 or Gly132.

13. The plant according to claim 11 or 12 wherein said SIPP2C1 protein comprises an amino acid substitution at Gly148 into Arginine, Ser171 into Leucine, Ala155 into Threonine or Gly132 into Serine.

14. The plant according to claim 1, which comprises one or more mutations in SEQ ID NO:1
whereby the mutation results in the encoded SIPP2C1 protein having reduced activity compared to the wild type functional protein or no activity in vivo and/or in vitro.

15. The plant according to claim 1, which comprises one or more mutations in SEQ ID NO: 11 from nucleotides 2676 to 4975,
whereby the mutation results in the encoded SIPP2C1 protein having reduced activity compared to the wild type functional protein or no activity in vivo and/or in vitro.

Patentansprüche

1. Nicht transgene Pflanze der Spezies *Solanum lycopersicum,* umfassend ein *SlPP2C1 (Solanum lycopersicum* Serine/Threonine Protein Phosphatase 2C) -Allel in ihrem Genom, wobei es sich bei einem *SlPP2C1*-Allel um ein Allel handelt, das ein Protein codiert, welches eine Aminosäuresequenzidentität von wenigstens 90% mit SEQ ID NO: 2 umfasst, **dadurch gekennzeichnet, dass** das *SlPP2C1*-Allel eine oder mehrere Mutationen in seiner Nukleotidsequenz umfasst, und wobei als ein Ergebnis der einen oder mehreren Mutationen die Pflanze, die das mutierte Allel in ihrem Genom umfasst, eine deutlich verbesserte Dürretoleranz verglichen mit der Pflanze, die ein Wildtyp-*SlPP2C1*-Allel in ihrem Genom umfasst, aufweist.

2. Pflanze nach Anspruch 1, **dadurch gekennzeichnet, dass** die eine oder mehreren Mutationen Funktionsverlust (Loss-of-function) oder Funktionsminderung (Reduced-function) auf das codierte SIPP2C1-Protein übertragen.

3. Pflanze nach Anspruch 1 oder 2, bei der das mutierte *SlPP2C1*-Allel in homozygoter Form vorliegt.

4. Pflanze nach einem der vorhergehenden Ansprüche, bei der Blattwelke-Symptome der Pflanze um wenigstens 10%

verglichen mit Blattwelke-Symptomen einer Pflanze, die ein Wildtyp-*SlPP2C1*-Allel in ihrem Genom umfasst, reduziert sind, wenn Pflanzen dem gleichen Dürrestress ausgesetzt sind.

5. Pflanze nach einem der vorhergehenden Ansprüche, bei der es sich um eine Hybridpflanze handelt.

6. Frucht oder Samen oder Teil einer Pflanze nach Anspruch 3.

7. Verwendung einer Nukleinsäuresequenz, die ein SIPP2C1-Protein codiert, zur Erzeugung transgener oder nicht transgener Pflanzen der Spezies *Solanum lycopersicum* mit verbesserter Dürretoleranz, **dadurch gekennzeichnet, dass** das SIPP2C1-Protein eine Aminosäuresequenzidentität von wenigstens 90% mit SEQ ID NO: 2 umfasst.

8. Transgene *Solanum lycopersicum-Pflanze,* umfassend integriert in ihrem Genom ein chimäres Gen, bei der das chimäre Gen einen in Pflanzenzellen aktiven Promotor in operativer Verknüpfung mit einer Nukleinsäuresequenz umfasst, die eine Sense- und/oder Antisense-Sequenz von SEQ ID NO: 1 umfasst, durch die nach Transkription die endogene *SlPP2C1*-Genexpression stillgelegt wird, und bei der die Pflanze eine deutlich verbesserte Dürretoleranz verglichen mit einer Pflanze, der das chimäre Gen fehlt, umfasst, bei der das endogene *SlPP2C1*-Gen ein Protein codiert, welches eine Aminosäuresequenzidentität von wenigstens 90% mit SEQ ID NO: 2 umfasst.

9. Pflanze nach Anspruch 8, bei der die Transkription-Regulatorsequenz ausgewählt ist aus der Gruppe bestehend aus: einem konstitutiven Promotor, einem induzierbaren Promotor, einem gewebespezifischen Promotor und einem entwicklungsgesteuerten Promotor.

10. Samen einer Pflanze nach einem der Ansprüche 8-9, umfassend das chimäre Gen.

11. Pflanze nach Anspruch 1, oder Samen, Nachkommen oder Tomatenfrüchte davon, wobei die Pflanze durch TILLING erhältlich ist, bei der die Pflanze, Samen, Nachkommen oder Tomatenfrüchte ein mutiertes *SlPP2C1*-Allel in ihrem Genom umfassen, **dadurch gekennzeichnet, dass** das mutierte Allel ein SIPP2C1-Protein mit Funktionsminderung (Reduced-function) oder Funktionsverlust (Loss-of-function) verglichen mit dem unter SEQ ID NO: 2 dargestellten Wildtyp-SIPP2C1-Protein codiert.

12. Pflanze nach Anspruch 11, bei der das SIPP2C1-Protein eine Aminosäuresubstitution an Gly148, Ser171, Ala155 oder Gly132 umfasst.

13. Pflanze nach Anspruch 11 oder 12, bei der das SIPP2C1-Protein eine Aminosäuresubstitution an Gly148 zu Arginin, Ser171 zu Leucin, Ala155 zu Threonin oder Gly132 zu Serin umfasst.

14. Pflanze nach Anspruch 1, die eine oder mehrere Mutationen in SEQ ID NO:1 umfasst,
wobei die Mutation ergibt, dass das codierte SIPP2C1-Protein verminderte Aktivität verglichen mit dem funktionsfähigen Wildtypprotein oder keine Aktivität in vivo und/oder in vitro aufweist.

15. Pflanze nach Anspruch 1, die eine oder mehrere Mutationen in SEQ ID NO: 11 von Nukleotid 2676 bis 4975 umfasst, wobei die Mutation ergibt, dass das codierte SIPP2C1-Protein verminderte Aktivität verglichen mit dem funktionsfähigen Wildtypprotein oder keine Aktivität in vivo und/oder in vitro aufweist.

Revendications

1. Plante non transgénique de l'espèce *Solanum lycopersicum* comprenant un allèle de *SlPP2C1* (sérine/thréonine protéine phosphatase 2C de *Solanum lycopersicum)* dans son génome, moyennant quoi un allèle de *SlPP2C1* est un allèle qui code pour une protéine comprenant une identité de séquence d'acides aminés d'au moins 90% avec la SEQ ID n° : 2,
caractérisée en ce que ledit allèle de *SlPP2C1* comprend une ou plusieurs mutation(s) dans sa séquence nucléotidique et moyennant quoi, comme résultat de ladite ou desdites mutation(s), la plante comprenant ledit allèle mutant dans son génome a une tolérance significativement améliorée à la sécheresse en comparaison avec la plante comprenant un allèle de *SlPP2C1* de type sauvage dans son génome.

2. Plante selon la revendication 1, **caractérisée en ce que** ladite ou lesdites mutation(s) confère/confèrent une perte de fonction ou une fonction réduite à la protéine SIPP2C1 codée.

3. Plante selon la revendication 1 ou 2, dans laquelle ledit allèle de *SlPP2C1* mutant est présent sous forme homozygote.

4. Plante selon l'une quelconque des revendications précédentes, dans laquelle les symptômes de flétrissement foliaire de ladite plante sont réduits d'au moins 10% en comparaison avec les symptômes de flétrissement foliaire d'une plante comprenant un allèle de *SlPP2C1* de type sauvage dans son génome quand les plantes sont exposées au même stress de sécheresse.

5. Plante selon l'une quelconque des revendications précédentes, la plante étant une plante hybride.

6. Fruit ou graine ou partie d'une plante selon la revendication 3.

7. Utilisation d'une séquence d'acides nucléiques codant pour une protéine SlPP2C1 destinée à la génération de plantes transgéniques ou non transgéniques de l'espèce *Solanum lycopersicum* avec une tolérance améliorée à la sécheresse, **caractérisée en ce que** la protéine SlPP2C1 comprend une identité de séquence d'acides aminés d'au moins 90% avec la SEQ ID n° : 2.

8. Plante *Solanum lycopersicum* transgénique comprenant un gène chimère intégré dans son génome, dans laquelle ledit gène chimère comprend un promoteur actif dans des cellules de plante lié, de manière opérationnelle, à une séquence d'acides nucléiques comprenant une séquence sens et/ou antisens de SEQ ID n° : 1 qui, lors de la transcription, rend silencieuse l'expression du gène *SlPP2C1* endogène et ladite plante comprenant une tolérance significativement améliorée à la sécheresse en comparaison avec une plante dépourvue dudit gène chimère, dans laquelle ledit gène *SlPP2C1* endogène code pour une protéine comprenant une identité de séquence d'acides aminés d'au moins 90% avec la SEQ ID n° : 2.

9. Plante selon la revendication 8, dans laquelle ladite séquence de régulation de la transcription est choisie dans le groupe constitué par : un promoteur constitutif, un promoteur inductible, un promoteur spécifique d'un tissu et un promoteur régulé au cours du développement.

10. Graine d'une plante, selon l'une quelconque des revendications 8-9, comprenant ledit gène chimère.

11. Plante, selon la revendication 1, ou graines, ou descendance ou tomates-fruits de celle-ci, ladite plante pouvant être obtenue par TILLING, la plante, les graines, la descendance ou les tomates-fruits comprenant un allèle de *SlPP2C1* mutant dans leur génome, **caractérisée**(s) en ce que ledit allèle mutant code pour une protéine SlPP2C1 ayant une fonction réduite ou une perte de fonction en comparaison avec la protéine SlPP2C1 de type sauvage illustrée dans la SEQ ID n° : 2.

12. Plante selon la revendication 11, dans laquelle ladite protéine SlPP2C1 comprend un remplacement d'acide aminé au niveau de la Gly148, de la Ser171, de l'Ala155 ou de la Gly132.

13. Plante selon la revendication 11 ou 12, dans laquelle ladite protéine SlPP2C1 comprend un remplacement d'acide aminé au niveau de la Gly148 en une Arginine, de la Ser171 en une Leucine, de l'Ala155 en une Thréonine ou de la Gly132 en une Sérine.

14. Plante, selon la revendication 1, qui comprend une ou plusieurs mutation (s) dans la SEQ ID n° : 1, moyennant quoi la mutation conduit à la protéine SlPP2C1 codée qui a une activité réduite en comparaison avec la protéine fonctionnelle de type sauvage ou qui n'a aucune activité in vivo et/ou in vitro.

15. Plante, selon la revendication 1, qui comprend une ou plusieurs mutation (s) dans la SEQ ID n° : 11 des nucléotides 2676 à 4975,
moyennant quoi la mutation conduit à la protéine SlPP2C1 codée qui a une activité réduite en comparaison avec la protéine fonctionnelle de type sauvage ou qui n'a aucune activité in vivo et/ou in vitro.

Figure 1

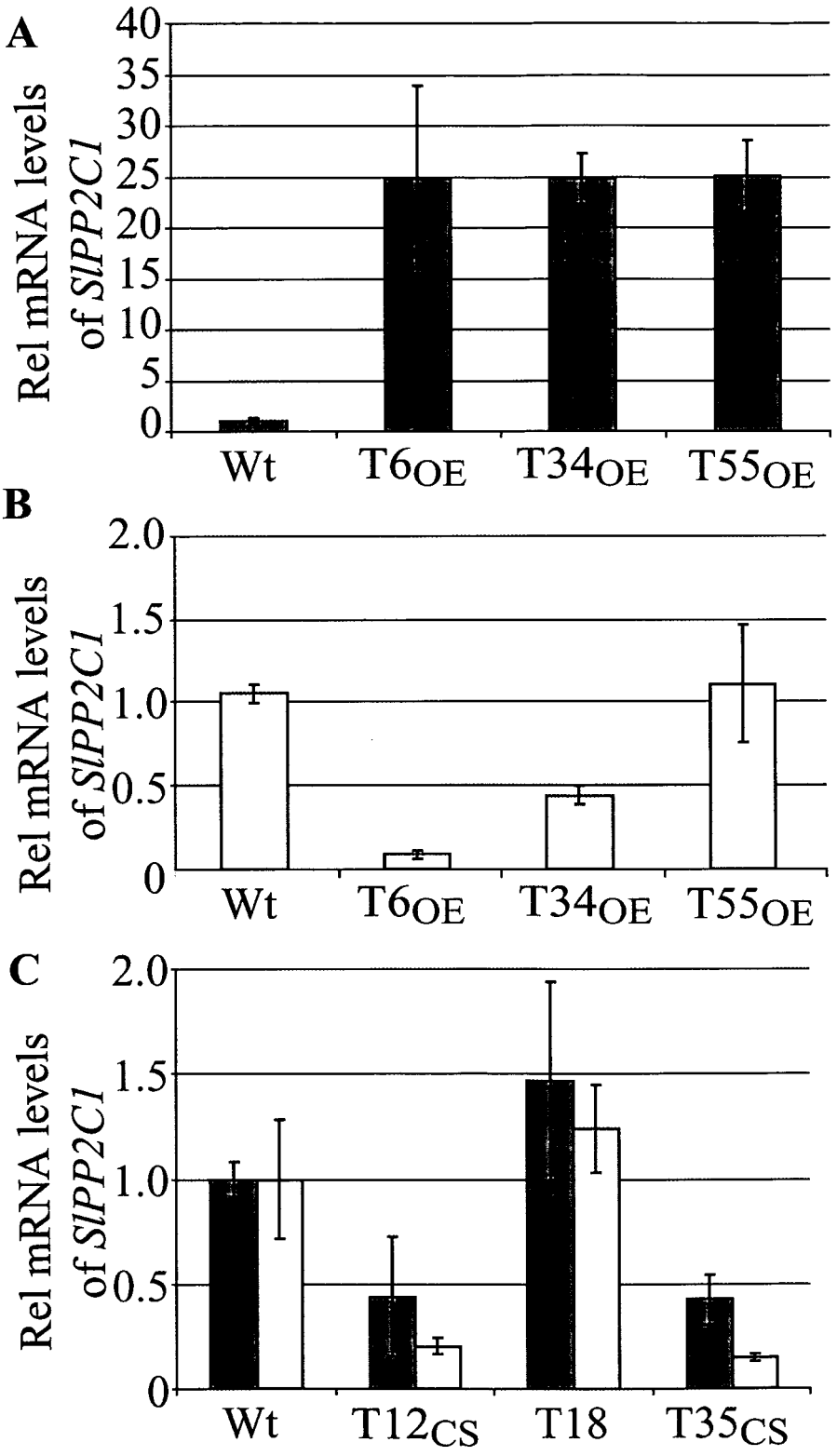
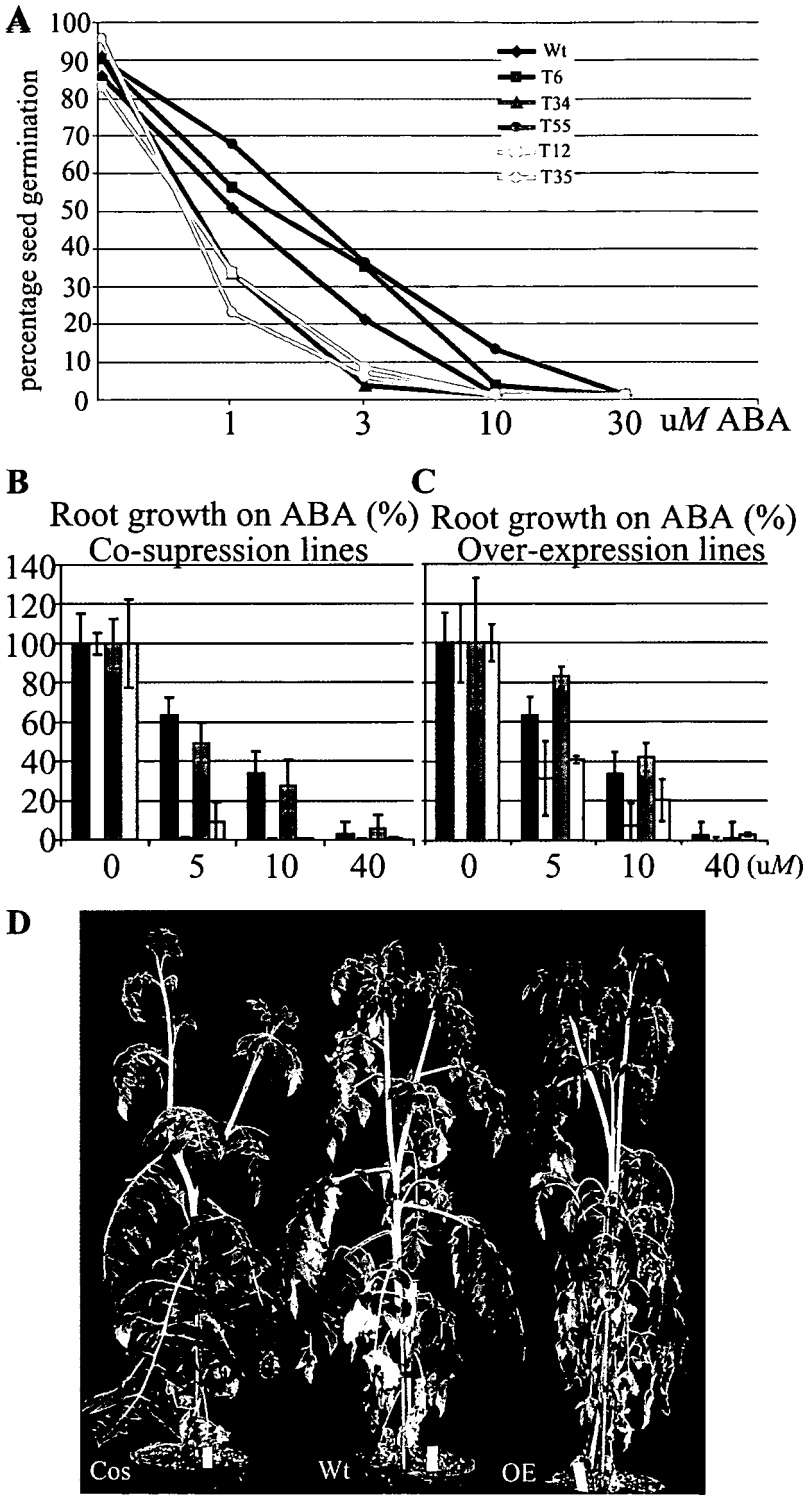

A
Rel mRNA levels of SlPP2C1
40
35
30
25
20
15
10
5
0
Wt
T6OE
T34OE
T55OE
B
Rel mRNA levels of SlPP2C1
2.0
1.5
1.0
0.5
0
Wt
T6OE
T34OE
T55OE
C
Rel mRNA levels of SlPP2C1
2.0
1.5
1.0
0.5
0
Wt
T12CS
T18
T35CS

Figure 2


A
percentage seed germination
100
90
80
70
60
50
40
30
20
10
0
Wt
T6
T34
T55
T12
T35
1
3
10
30 uM ABA
B
Root growth on ABA (%)
Co-supression lines
140
120
100
80
60
40
20
0
0
5
10
40
C
Root growth on ABA (%)
Over-expression lines
0
5
10
40 (uM)
D
Cos
Wt
OE

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