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(54) **Title:** FUNCTIONAL MUTANT ALLELES OF EIN5 FOR YIELD INCREASE

(57) **Abstract:** Functional mutant alleles of EIN5 for yield increase The present invention relates to novel functional mutant alleles of EIN5 for increasing biomass in plants. The invention provides mutant plants comprising said alleles and chimeric genes encoding said novel alleles.

FUNCTIONAL MUTANT ALLELES OF EIN5 FOR YIELD INCREASE

Field of the invention

The present invention relates to the field of plant molecular biology. More specifically the present invention provides mutant alleles, chimeric genes comprising said mutant alleles and plants comprising said mutant alleles. Plant comprising said mutant alleles are characterized by having an increase in yield compared to wild type plants.

Introduction to the invention

Leaf growth is a complex process, integrating genetically programmed processes and environmental signals. These environmental signals can trigger inhibition of leaf growth which under drought stress for example, forms a mechanism to limit water evaporation from the leaf surface. During the past decades, extensive research has been performed on *Arabidopsis thaliana* to study plant behavior under extreme environmental conditions such as severe drought (Xiong et al., 2002; Verslues et al., 2006; Seki et al., 2007; Schachtman and Goodger, 2008). These studies mainly scored plant survival or used final rosette area as a measure for plant tolerance to drought (Umezawa et al., 2006). However, it became clear that the often rather harsh conditions used in these studies are not very representative for natural conditions, in which the drought stress is generally milder and does not always threaten survival (Skirycz and Inzé, 2010). Moreover, it was shown that tolerance to severe drought stress and the ability of plants to continue to grow under mild stress conditions are very different traits mediated by different molecular processes (Skirycz et al., 2011a; Claeys and Inzé, 2013). Furthermore, recent studies pointed out the importance of evaluating stress responses at organ or tissue level, as the response to osmotic stress is highly dependent on the organ and its developmental stage (Dinneny, 2008; Harb et al., 2010; Skirycz et al., 2010; Baerenfaller et al., 2012). Thus, in order to understand the specific mechanisms involved in growth inhibition under water deprivation, it is important to study growth response specifically in actively growing leaves.

Leaf growth in *Arabidopsis* is consisting of three major developmental phases (Anastasiou and Lenhard, 2007; Andriankaja et al., 2012; Gonzalez et al., 2012). First, growth of very young leaves emerging from the shoot apical meristem is driven exclusively by cell proliferation. Next, cell division starts to cease at the tip of the leaf and gradually all cells in the leaf exit the cell cycle and start to expand (Donnelly et al.,

1999; Andriankaja et al., 2012; Gonzalez et al., 2012). Finally, cell division in the developing leaves has stopped and further leaf growth mainly occurs by cell expansion (Vlieghe et al., 2005). Under mild drought conditions, studied *in vitro* using osmotic stress as a proxy, both cell proliferation and cell expansion are affected resulting in a final leaf size reduction of about 50% (Skirycz et al., 2010; Skirycz et al., 2011b; Claeys et al., 2012).

When mild stress occurs during the proliferation phase of early leaf development (leaves <0.1mm² in size), cell cycle progression is affected in a two-step process denominated the “Pause-and-Stop” mechanism, involving crosstalk between the plant hormones ethylene and gibberillic acid (GA)(Figure 1a)(Skirycz et al., 2011b; Claeys et al., 2012). In the first step of mild stress response, the ethylene precursor ACC, which accumulates specifically in growing leaves, inactivates the Cyclin Dependent Kinase A (CDKA), thereby transiently blocking further cell cycle progression (“Pause”). In parallel, the accumulation of ACC induces the expression of the downstream ETHYLENE RESPONSE FACTOR 6 (ERF6). This transcription factor occupies a central role in this pathway as on the one hand it activates the stress tolerance mechanisms, while on the other hand it further converts the paused cell cycle into a definitive cell cycle exit (“Stop”) (Dubois et al., 2013). Molecularly, the latter occurs through transcriptional activation of the GA2-OX6 gene, which stimulates breakdown of GAs. Decreased GA levels further result in stabilization of DELLA proteins, which push cells into cell expansion and endoreduplication (Claeys et al., 2012; Achard et al., 2008).

It is however very unlikely that this rather simple linear pathway on its own is the sole regulator of leaf growth under stress. Considering the complexity of regulation of leaf growth under normal conditions (Gonzalez et al., 2012), and the numerous pathways that are responding to changing environments (Skirycz and Inzé, 2010), one can imagine that the integration of environmental signals into leaf growth adaptation is an extremely complex response involving different molecular processes such as signal detection, post-transcriptional regulation, protein complex formation, etc... To identify new genes involved in leaf growth regulation under adverse conditions without restricting our windows towards certain molecular processes or biological functions, we performed a forward genetic screen. As a starting point, we used the easily screenable severe dwarfed phenotype of an inducible ERF6-overexpression line, mutagenized it

with EMS, and screened for suppressor mutants. In the present invention we have identified different alleles from EIN. These alleles encode for mutant, functional proteins which when present in a homozygous state in a plant lead to an increased leaf biomass.

5 Figures

Figure 1: Principle of the EMS-screen and general characteristics of the 7 identified mutants.

(A) ERF6-centered pathway of growth-inhibition and stress tolerance activation under osmotic stress. Upon activation of ERF6, the GA levels are reduced due to induction of
 10 GA2-OX6, resulting in a growth inhibition. (B) As a consequence of the pathway in (A), plants overexpressing *ERF6* are extremely dwarfed. Upon EMS-mutagenesis, the indicated mutant suppresses the ERF6-induced dwarfism. The *in vitro* growth medium contains Kanamycin to select for seedlings containing the ERF6-GR construct, and Dexamethasone to induce the overexpression of *ERF6* and the accompanied
 15 dwarfism. (C) Expression analysis of the mutants upon transfer to Dexamethasone for the overexpression of *ERF6*, the stress-related target gene *MYB51* and the growth-related target gene *GA2-OX6*. Mutants *sgi1* – *sgi4* and *sgi7* show no longer induction of *GA2-OX6* but still induction of *MYB51*, while *sgi5* and *sgi6* mutants do not induce *MYB51* but have reduced levels of *GA2-OX6*. (D) Rosette phenotypes of the 7
 20 selected mutants, grown in soil in the absence of Dexamethasone. Pictures are taken 22 days after stratification.

Figure 2: Structure of the XRN4 protein with location of the identified mutations.

Based on structure predicted by PLAZA (Proost et al., 2009) and on the observations of Nagarajan et al. (2013). Underlined residues indicate key residues for active site
 25 function surrounded by conserved stretches of amino acids. Mutants obtained with the EMS-screen are indicated in red, mutants used in previous studies in green (Potuschak et al., 2006). Small arrows indicate point mutants, large triangles indicate T-DNA insertions.

Figure 3: Rosette size of the EMS-mutants and the previously described *ein5* mutants

30 (A) Rosette size of the EMS-mutants at 22 days after stratification (DAS) upon growth in soil. Values were normalized to the wild type size. (B) Rosette size of the previously

characterized *ein5* mutants at 22 days after stratification (DAS) upon growth in soil. Values were normalized to the wild type size. Error bars represent standard error.

Figure 4: Individual leaf size and cellular measurements of *sgi7.1*

(A) Picture of a representative *sgi7.1* rosette (left) and appropriate WT (ERF6-GR) (right) after 25 days of growth in soil. (B) Size per leaf of the *sgi7.1* mutant and wild type at 22 days after stratification (DAS) upon growth in soil. Error bars represent standard deviation. Four biological repeats were done. (C) Cellular measurements of the abaxial epidermal cell layer of representative third leaves from the plants in (B). Error bars represent standard error.

10 Figure 5 : Rosette size of *sgi7.1* under control and drought stress conditions

Rosette size of the *sgi7.1* mutant at 22 days after stratification (DAS) when grown on the Weighing Imaging and Watering Machine under either well-watered conditions or mild drought stress. Values were normalized to the wild type size.

Detailed description of the invention

15 To facilitate the understanding of this invention a number of terms are defined below. Terms defined herein (unless otherwise specified) have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. As used in this specification and its appended claims, terms such as "a", "an" and "the" are not intended to refer to only a singular entity, but include the general class of which
20 a specific example may be used for illustration, unless the context dictates otherwise. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly
25 directed to Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., *current Protocols in Molecular Biology (Supplement 100)*, John Wiley & Sons, New York (2012), for definitions and terms of the art. The definitions provided herein should not
30 be construed to have a scope less than understood by a person of ordinary skill in the art.

The present invention is derived from the unexpected findings that the homozygous presence of specific EIN5 alleles, encoding mutant, functional EIN5 proteins, in a plant which does not express a wild type EIN5 protein, leads to an increased yield of at least 20% with respect to the wild type plant not comprising the specific EIN5 alleles.

5 Specific EIN5 alleles which are functional and encode for mutant EIN5 proteins are selected from the list consisting of: i) a protein having at least one amino acid change in the sequence DGVAPRAKMNQQRSR (SEQ ID NO: 15) present in SEQ ID NO: 1 or in a corresponding plant orthologous sequence thereof, ii) a protein having at least one amino sequence change in the sequence GLDADLIML (SEQ ID NO: 16) present
10 in SEQ ID NO: 1 or in a corresponding plant orthologous sequence thereof, iii) a protein having an amino acid change at position 526 or in a corresponding plant orthologous sequence thereof. Yet another specific EIN5 allele which is functional has its splice acceptor site mutated at the nucleotide position 1603 in SEQ ID NO: 2 or in a corresponding plant orthologous sequence thereof.

15 The term "at least one amino acid change in a sequence" means that at least one of the amino acids present in the sequence can be changed (e.g. by mutation) towards any possible amino acid.

In a specific embodiment the functional mutant EIN5 allele encodes for a functional protein selected from the list consisting of: i) a protein having an amino acid change of
20 G to E in position 105 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof, ii) a protein having an amino acid change of A to V in position 110 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof, iii) a protein having an amino acid change of Q to STOP in position 115 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence
25 thereof, iv) a protein having an amino acid change of D to N in position 236 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof, v) a protein having an amino acid change of Q to STOP in position 526, or in a corresponding position in a plant orthologous sequence thereof or the functional allele
30 of EIN5 has its splice acceptor site mutated at the nucleotide position 1603 in SEQ ID NO: 2 from G to A, or in a corresponding position of a plant orthologous sequence thereof.

The EIN5 gene is sometimes referred to in the art also as the AIN1 or XRN4 gene. Thus a representative of the plant EIN5 gene in *Arabidopsis thaliana* is AT1G54490

(TAIR accession, www.arabidopsis.org), which genome sequence is depicted in SEQ ID NO: 2 and its protein coding sequence is depicted in SEQ ID NO: 1.

The term "plant yield" or "yield" as used herein generally refers to a measurable product from a plant, particularly a crop. Yield and yield increase (in comparison to a wild-type plant) can be measured in a number of ways, and it is understood that a skilled person will be able to apply the correct meaning in view of the particular embodiments, the particular crop concerned and the specific purpose or application concerned. The terms "improved yield" or "increased yield" can be used interchangeably. As used herein, the term "improved yield" or the term "increased yield" means any improvement in the yield of any measured plant product, such as biomass (e.g. leaf biomass), grain, fruit, leaf, root, or fiber. In accordance with the invention, changes in different phenotypic traits may improve yield. For example, and without limitation, parameters such as floral organ development, root initiation, root biomass, seed number, seed weight, harvest index, leaf formation, phototropism, apical dominance, and fruit development, are suitable measurements of improved yield. Increased yield includes higher fruit yields, higher seed yields, higher fresh matter production, higher leaf biomass and/or higher dry matter production. Any increase in yield is an improved yield in accordance with the invention. For example, the improvement in yield can comprise a 0.1%, 0.5%, 1%, 3%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater increase in any measured parameter. For example, an increase in the bu/acre yield of soybeans derived from a soy bean plant which does not express wild type EIN5 protein but comprises and expresses allelic variants of EIN5 in a homozygous state, as specified herein before, as compared with the bu/acre yield from wild type soybeans cultivated under the same conditions, is an improved yield in accordance with the invention. The increased or improved yield can be achieved in the absence or presence of stress conditions. For example, enhanced or increased "yield" refers to one or more yield parameters selected from the group consisting of biomass yield, dry biomass yield, aerial dry biomass yield, underground dry biomass yield (e.g. root biomass), fresh-weight biomass yield, aerial fresh-weight biomass yield (e.g. leaf biomass), underground fresh-weight biomass yield; enhanced yield of harvestable parts, either dry or fresh-weight or both, either aerial or underground or both; enhanced yield of crop fruit, either dry or fresh-weight or both, either aerial or underground or both; and enhanced yield of

seeds, either dry or fresh-weight or both, either aerial or underground or both. "Crop yield" is defined herein as the number of bushels of relevant agricultural product (such as grain, forage, or seed) harvested per acre. Crop yield is impacted by abiotic stresses, such as drought, heat, salinity, and cold stress, and by the size (biomass) of the plant. The yield of a plant can depend on the specific plant/crop of interest as well as its intended application (such as food production, feed production, processed food production, biofuel, biogas or alcohol production, or the like) of interest in each particular case. Thus, in one embodiment, yield can be calculated as harvest index (expressed as a ratio of the weight of the respective harvestable parts divided by the total biomass), harvestable parts weight per area (acre, square meter, or the like); and the like. The harvest index is the ratio of yield biomass to the total cumulative biomass at harvest. Harvest index is relatively stable under many environmental conditions, and so a robust correlation between plant size and grain yield is possible. Measurements of plant size in early development, under standardized conditions in a growth chamber or greenhouse, are standard practices to measure potential yield advantages conferred by the presence of the EIN5 alleles of the invention in a homozygous state in a background with no wild type EIN5 expression.

There are two major options to produce a plant expressing the mutant, functional EIN5-alleles of the invention: i) introducing (e.g. by breeding or by genetic transformation) the mutant, functional EIN5-alleles in a plant which does not express wild type alleles of EIN5 and ii) modifying the wild type EIN5-alleles in a plant by introducing targeted mutations in the plant so that the mutant, functional EIN5 alleles are present in a homozygous state. The various steps to generate plants expressing a functional, mutant EIN5-allele of the invention are hereunder discussed.

Methods for generating a plant with knock-out alleles of EIN5

As used herein a "an EIN5 knock-out allele" is an EIN5 allele which is mutated as compared to the wild type EIN5 allele (i.e. it is a non-functional EIN5 mutant allele) and which encodes a non-functional EIN5 protein or results in a significantly reduced amount of EIN5 protein (by introducing for example a mutation in a regulatory region such as a promoter), or which encodes a protein with significantly reduced activity. Said "EIN5 knock-out allele" may encode no EIN5 protein, or which may encode a non-functional EIN5 protein or an EIN5 protein with significantly reduced function. Such an

EIN5 allele may also be referred as an inactivated EIN5 allele. A wild type EIN5 allele is the form of the EIN5 allele as it occurs in nature.

In a specific embodiment, the activity of an EIN5 protein may be reduced or eliminated by disrupting the genes encoding EIN5. The disruption inhibits expression or activity of
5 EIN5 protein compared to a corresponding control plant cell lacking the disruption. A specific embodiment, the disruption step comprises insertion of one or more transposons, where the one or more transposons are inserted into the endogenous EIN5 genes. The disruption can be a homozygous disruption in the EIN5 gene. Alternatively, the disruption is a heterozygous disruption in the EIN5 gene. In certain
10 embodiments, when more than one EIN5 gene is involved, there is more than one disruption, which can include homozygous disruptions, heterozygous disruptions or a combination of homozygous disruptions and heterozygous disruptions. Methods for the transposon tagging of specific genes in plants are well known in the art. See, for example, Meissner, *et al* (2000) *Plant J.* 22:265-21. In addition, the TUSC process for
15 selecting Mu insertions in selected genes has been described in US5962764, which is herein incorporated by reference.

In a specific embodiment, a gene encoding for a zinc finger protein that binds to a gene encoding an EIN5 polypeptide is introduced into a plant or plant cell, resulting in a reduced expression of the EIN5 gene. In particular embodiments, the zinc finger
20 protein binds to a regulatory region of an EIN5 gene. In other embodiments, the zinc finger protein binds to a messenger RNA encoding an EIN5 polypeptide and prevents its translation. Methods of selecting sites for targeting by zinc finger proteins have been described, for example, in US6453242, and methods for using zinc finger proteins to inhibit the expression of genes in plants are described, for example, in
25 US2003/0037355, each of which is herein incorporated by reference.

In another specific embodiment, a gene encoding a TALE protein, that binds to a gene encoding an EIN5 polypeptide, is introduced into a plant or plant cell which results in a reduced expression of the EIN5 gene. In particular embodiments, the TALE protein binds to a regulatory region of an EIN5 gene. In other embodiments, the TALE protein
30 binds to a messenger RNA encoding an EIN5 polypeptide and prevents its translation. Methods of selecting sites for targeting by zinc finger proteins have been described in e.g. Moscou MJ, Bogdanove AJ (2009) (A simple cipher governs DNA recognition by

TAL effectors. Science 326:1501) and Morbitzer R, Romer P, Boch J, Lahaye T (2010) (Regulation of selected genome loci using de novo-engineered transcription activator-like effector (TALE)-type transcription factors. Proc Natl Acad Sci USA 107:21617–21622.)

5 Additional methods for decreasing or eliminating the expression of endogenous genes in plants are also known in the art and can be similarly applied to the instant invention. These methods include other forms of mutagenesis, such as ethyl methanesulfonate-induced mutagenesis, deletion mutagenesis and fast neutron deletion mutagenesis used in a reverse genetics sense (with PCR) to identify plant lines in which the
10 endogenous gene has been deleted. For examples of these methods see, Ohshima, et al, (1998) Virology 243:472-481; Okubara, et al, (1994) Genetics 137:867-874 and Quesada, et al, (2000) Genetics 154:421-436, each of which is herein incorporated by reference. In addition, a fast and automatable method for screening for chemically induced mutations, TILLING (Targeting Induced Local Lesions in Genomes), using
15 denaturing HPLC or selective endonuclease digestion of selected PCR products is also applicable to the instant invention. See, McCallum, et al, (2000) Nat. Biotechnol 18:455-457, herein incorporated by reference. Mutations that impact gene expression or that interfere with the function of the encoded protein are well known in the art. Insertional mutations in gene exons usually result in null-mutants. Mutations in
20 conserved residues are particularly effective in inhibiting the activity of the encoded protein. Conserved residues of plant EIN5 polypeptides suitable for mutagenesis with the goal to eliminate EIN5 activity have been described. Such mutants can be isolated according to well-known procedures, and mutations in different EIN5 loci can be stacked by genetic crossing. See, for example, Gruis, et al (2002) Plant Cell 14:2863-
25 2882.

Methods for producing a plant having knock-out alleles of EIN5 further comprising specific EIN5 alleles of the invention

In a specific embodiment a plant in which no EIN5 expression occurs (i.e. a plant having knock-out alleles of EIN5) is transformed with a chimeric gene comprising a
30 plant-expressible promoter operably linked to an EIN5 allele of the invention and a suitable plant terminator sequence.

A chimeric gene, as used herein, refers to a gene that is made up of heterologous elements that are operably linked to enable expression of the gene, whereby that combination is not normally found in nature. As such, the term "heterologous" refers to the relationship between two or more nucleic acid or protein sequences that are derived from different sources. For example, a promoter is heterologous with respect to an operably linked nucleic acid sequence, such as a coding sequence, if such a combination is not normally found in nature. In addition, a particular sequence may be "heterologous" with respect to a cell or organism into which it is inserted (i.e. does not naturally occur in that particular cell or organism).

The expression "operably linked" means that said elements of the chimeric gene are linked to one another in such a way that their function is coordinated and allows expression of the coding sequence, i.e. they are functionally linked. By way of example, a promoter is functionally linked to another nucleotide sequence when it is capable of ensuring transcription and ultimately expression of said other nucleotide sequence. Two proteins encoding nucleotide sequences, e.g. a transit peptide encoding nucleic acid sequence and a nucleic acid sequence encoding a protein according to the invention, are functionally or operably linked to each other if they are connected in such a way that a fusion protein of first and second protein or polypeptide can be formed.

A gene, e.g. a chimeric gene comprising a specific EIN5 allele of the invention, is said to be expressed when it leads to the formation of an expression product. An expression product denotes an intermediate or end product arising from the transcription and optionally translation of the nucleic acid, DNA or RNA, coding for such product. During the transcription process, a DNA sequence under control of regulatory regions, particularly the promoter, is transcribed into an RNA molecule.

As the skilled person will be well aware, various promoters may be used to promote the transcription of the nucleic acids encoding the functional EIN5 alleles of the invention. Such promoters include for example constitutive promoters, inducible promoters (e.g. stress-inducible promoters, drought-inducible promoters, hormone-inducible promoters, chemical-inducible promoters, etc.), tissue-specific promoters, developmentally regulated promoters and the like.

The chimeric gene of the invention may also comprise a 3' end region, i.e. a transcription termination or polyadenylation sequence, operable in plant cells. As a transcription termination or polyadenylation sequence, use may be made of any corresponding sequence of bacterial origin, such as for example the nos terminator of
5 Agrobacterium tumefaciens, of viral origin, such as for example the CaMV 35S terminator, or of plant origin, such as for example a histone terminator as described in published Patent Application EP 0 633 317 A1. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA. The 3' end sequence to be added may be derived from, for example, the nopaline synthase
10 or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene.

Methods for producing plants having functional, mutant EIN5 alleles in plants

In specific embodiments the introduction of the functional mutant EIN5 allele of the invention occurs in a plant having wild type EIN5 alleles.

15 In a specific embodiment the functional mutant EIN5 alleles of the invention can be introduced into said plant e. g. through mutagenesis. "Mutagenesis", as used herein, refers to the process in which plant cells are subjected to a technique which induces mutations in the DNA of the cells, such as contact with a mutagenic agent, such as a chemical substance (such as ethylmethylsulfonate (EMS), ethylnitrosourea (ENU),
20 etc.) or ionizing radiation (neutrons (such as in fast neutron mutagenesis, etc.), alpha rays, gamma rays (such as that supplied by a Cobalt 60 source), X-rays, UV-radiation, etc.), T-DNA insertion mutagenesis (Azpiroz-Leehan et al. (1997) Trends Genet 13:152-156), transposon mutagenesis (McKenzie et al. (2002) Theor Appl Genet 105:23-33), or tissue culture mutagenesis (induction of somaclonal variations), or a
25 combination of two or more of these. Thus, the desired mutagenesis of one or more EIN5 genes or EIN5 alleles may be accomplished by one of the above methods. While mutations created by irradiation are often large deletions or other gross lesions such as translocations or complex rearrangements, mutations created by chemical mutagens are often more discrete lesions such as point mutations. For example, EMS
30 alkylates guanine bases, which results in base mispairing: an alkylated guanine will pair with a thymine base, resulting primarily in G/C to A/T transitions. Following mutagenesis, plants are regenerated from the treated cells using known techniques.

For instance, the resulting seeds may be planted in accordance with conventional growing procedures and following pollination seed is formed on the plants. Additional seed that is formed as a result of such pollination in the present or a subsequent generation may be harvested and screened for the presence of mutant functional EIN5
5 alleles of the invention. Several techniques are known to screen for specific mutant alleles, e.g., Deleteagene™ (Delete-a-gene; Li et al., 2001, Plant J 27: 235-242) uses polymerase chain reaction (PCR) assays to screen for deletion mutants generated by fast neutron mutagenesis, TILLING (targeted induced local lesions in genomes; McCallum et al., 2000, Nat Biotechnol 18:455-457) identifies EMS-induced point
10 mutations, etc.

In yet another embodiment the mutant functional EIN5 alleles can also be introduced via gene targeting techniques. The term “gene targeting” refers herein to directed gene modification that uses mechanisms such as double stranded DNA break repair via non-homologous end-joining, homologous recombination, mismatch repair or site-
15 directed mutagenesis. The method can be used to replace, insert and delete endogenous sequences or sequences previously introduced in plant cells. Methods for gene targeting can be found in, for example, WO 2006/105946 or WO2009/002150. Double stranded DNA breaks can be induced in a targeted manner using custom designed sequence specific nucleases and nuclease systems such as
20 meganucleases/homing endonucleases, zinc finger nucleases, TALENs or CRISPR/CAS (for a review see Gaj et al., 2013, Trends Biotechnol 31: 397-405). In addition to making mutant alleles, such techniques can also be used to delete entire genes encoding proteins having the activity of a protein having any of the above amino acid sequences.

25 A mutant functional EIN5 allele of the invention can also be introduced through introgression of a generated mutant functional EIN5 allele into said plant.

Isolation of orthologous plant sequences of the identified EIN5 alleles

Based on the identified functional EIN5 allelic sequences of the invention, the skilled person can isolate homologous sequences or functional variants of the sequences
30 disclosed herein using methods well known in the art, e.g., alignments, either manually or by using computer programs such as BLAST (Altschul et al. (1990), Journal of Molecular Biology, 215, 403-410), which stands for Basic Local Alignment Search Tool

or ClustalW (Thompson et al. (1994), *Nucleic Acid Res.*, 22, 4673-4680) or any other suitable program which is suitable to generate sequence alignments. Homologous sequences as described above can comprise orthologous or paralogous sequences. Several different methods are known by those of skill in the art for identifying and defining these functionally homologous sequences. Three general methods for defining orthologous and paralogous genes are described; an ortholog, paralog or homolog may be identified by one or more of the methods described below. Orthologs and paralogs are evolutionarily related genes that have similar sequence and similar functions. Orthologs are structurally related genes in different species that are derived by a speciation event. Paralogs are structurally related genes within a single species that are derived by a duplication event. Within a single plant species, gene duplication may result in two copies of a particular gene, giving rise to two or more genes with similar sequence and often similar function known as paralogs. A paralog is therefore a similar gene formed by duplication within the same species. Paralogs typically cluster together or in the same clade (a group of similar genes) when a gene family phylogeny is analyzed using programs such as CLUSTAL (Thompson, et al, (1994) *Nucleic Acids Res.* 22:4673-4680; Higgins, et al, (1996) *Methods Enzymol.* 266:383-402). Groups of similar genes can also be identified with pairwise BLAST analysis (Feng and Doolittle, (1987) *J. Mol. Evol.* 25:351-360). Orthologous sequences can also be identified by a reciprocal BLAST strategy. Once an orthologous sequence has been identified, the function of the ortholog can be deduced from the identified function of the reference sequence. Orthologous genes from different organisms have highly conserved functions, and very often essentially identical functions (Lee, et al, (2002) *Genome Res.* 12:493-502; Remm, et al, (2001) *J. Mol. Biol.* 314:1041-1052). Paralogous genes, which have diverged through gene duplication, may retain similar functions of the encoded proteins. In such cases, paralogs can be used interchangeably with respect to certain embodiments of the instant invention (for example, transgenic expression of a coding sequence).

Functional variants or homologues of the sequences disclosed herein may also be identified and isolated by hybridization under stringent conditions using as probes identified nucleotide sequences or fragments thereof. For example, homologous sequences from other monocot species than the specific sequences disclosed herein are said to be substantially identical or essentially similar if they can be detected by hybridization under stringent, preferably highly stringent conditions. 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
5 sequence hybridizes 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 hybridizations (Northern blots using a probe of e.g. 100nt) are for example those which include at least one wash in
10 0.2X SSC at 63°C for 20min, or equivalent conditions.

“High stringency conditions” can be provided, for example, by hybridization at 65°C in an aqueous solution containing 6x SSC (20x SSC contains 3.0 M NaCl, 0.3 M Na-citrate, pH 7.0), 5x Denhardt's (100X Denhardt's contains 2% Ficoll, 2% Polyvinyl
15 pyrollidone, 2% Bovine Serum Albumin), 0.5% sodium dodecyl sulphate (SDS), and 20 µg/ml denaturated carrier DNA (single-stranded fish sperm DNA, with an average length of 120 - 3000 nucleotides) as non-specific competitor. Following hybridization, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridization temperature in 0.2-0.1× SSC, 0.1% SDS. “Moderate
20 stringency conditions” refers to conditions equivalent to hybridization in the above described solution but at about 60-62°C. Moderate stringency washing may be done at the hybridization temperature in 1x SSC, 0.1% SDS. “Low stringency” refers to conditions equivalent to hybridization in the above described solution at about 50-
25 52°C. Low stringency washing may be done at the hybridization temperature in 2x SSC, 0.1% SDS.

Example 8 depicts plant orthologs and EIN5 alleles.

In a specific embodiment the chimeric genes comprising a functional, mutated EIN5-allele of the invention are incorporated in recombinant vectors. Typically such recombinant vectors are plant transformation vectors further comprising a selectable
30 marker or screenable marker gene.

"Selectable or screenable marker", "selectable or screenable marker gene" or "reporter gene" includes any gene that confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells that are transfected or transformed

with a nucleic acid construct of the invention. These marker genes enable the identification of a successful transfer of the nucleic acid molecules via a series of different principles. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance, that introduce a new metabolic trait or that allow visual selection. Examples of selectable marker genes include genes conferring resistance to antibiotics (such as nptII that phosphorylates neomycin and kanamycin, or hpt, phosphorylating hygromycin, or genes conferring resistance to, for example, bleomycin, streptomycin, tetracyclin, chloramphenicol, ampicillin, gentamycin, geneticin (G418), spectinomycin or blasticidin), to herbicides (for example bar which provides resistance to Basta[®]; aroA or gox providing resistance against glyphosate, or the genes conferring resistance to, for example, imidazolinone, phosphinothricin or sulfonylurea), or genes that provide a metabolic trait (such as manA that allows plants to use mannose as sole carbon source or xylose isomerase for the utilisation of xylose, or antinutritive markers such as the resistance to 2-deoxyglucose). Expression of visual marker genes results in the formation of colour (for example β -glucuronidase, GUS or β -galactosidase with its coloured substrates, for example X-Gal), luminescence (such as the luciferin/luciferase system) or fluorescence (Green Fluorescent Protein, GFP, and derivatives thereof). This list represents only a small number of possible markers. The skilled worker is familiar with such markers. Different markers are preferred, depending on the organism and the selection method.

It is known that upon stable or transient integration of nucleic acids into plant cells, only a minority of the cells takes up the foreign DNA and, if desired, integrates it into its genome, depending on the expression vector used and the transfection technique used. To identify and select these integrants, a gene coding for a selectable marker (such as the ones described above) is usually introduced into the host cells together with the gene of interest. These markers can for example be used in mutants in which these genes are not functional by, for example, deletion by conventional methods. Furthermore, nucleic acid molecules encoding a selectable marker can be introduced into a host cell on the same vector that comprises the sequence encoding the polypeptides of the invention or used in the methods of the invention, or else in a separate vector. Cells which have been stably transfected with the introduced nucleic acid can be identified for example by selection (for example, cells which have integrated the selectable marker survive whereas the other cells die).

Since the marker genes, particularly genes for resistance to antibiotics and herbicides, are no longer required or are undesired in the transgenic host cell once the nucleic acids have been introduced successfully, the process according to the invention for
5 introducing the nucleic acids advantageously employs techniques which enable the removal or excision of these marker genes. One such a method is what is known as co-transformation. The co-transformation method employs two vectors simultaneously for the transformation, one vector bearing the nucleic acid according to the invention and a second bearing the marker gene(s). A large proportion of transformants receives
10 or, in the case of plants, comprises (up to 40% or more of the transformants), both vectors. In case of transformation with *Agrobacteria*, the transformants usually receive only a part of the vector, i.e. the sequence flanked by the T-DNA, which usually represents the expression cassette. The marker genes can subsequently be removed from the transformed plant by performing crosses. In another method, marker genes
15 integrated into a transposon are used for the transformation together with desired nucleic acid (known as the Ac/Ds technology). The transformants can be crossed with a transposase source or the transformants are transformed with a nucleic acid construct conferring expression of a transposase, transiently or stable. In some cases (approx. 10%), the transposon jumps out of the genome of the host cell once
20 transformation has taken place successfully and is lost. In a further number of cases, the transposon jumps to a different location. In these cases the marker gene must be eliminated by performing crosses. In microbiology, techniques were developed which make possible, or facilitate, the detection of such events. A further advantageous method relies on what is known as recombination systems; whose advantage is that
25 elimination by crossing can be dispensed with. The best-known system of this type is what is known as the Cre/lox system. Cre1 is a recombinase that removes the sequences located between the loxP sequences. If the marker gene is integrated between the loxP sequences, it is removed once transformation has taken place successfully, by expression of the recombinase. Further recombination systems are
30 the HIN/HIX, FLP/FRT and REP/STB system (Tribble *et al.*, *J. Biol. Chem.*, 275, 2000: 22255-22267; Velmurugan *et al.*, *J. Cell Biol.*, 149, 2000: 553-566). A site-specific integration into the plant genome of the nucleic acid sequences according to the invention is possible. Similarly, marker genes can be excised using one or more rare-cleaving double strand break inducing enzyme such as meganucleases (naturally

occurring or engineered to recognize a specific DNA sequence), zinc finger nucleases, TALE nucleases and the like, if recognition sites for such enzymes are present in the vicinity of the marker gene. Excision can occur via homologous recombination if homology regions flank the marker gene, or via non-homologous end-joining with two
5 recognition sites flanking the marker gene,

For the purposes of the invention, "transgenic", "transgene" or "recombinant" means with regard to, for example, a nucleic acid sequence, an expression cassette, gene construct or a vector comprising the nucleic acid sequence or an organism
10 transformed with the nucleic acid sequences, expression cassettes or vectors according to the invention. The term "nucleic acid molecule" as used interchangeably with the term "polynucleotide" in accordance with the present invention, includes DNA, such as cDNA or genomic DNA, and RNA.

15 A transgenic plant for the purposes of the invention is thus understood as meaning, as above, that the nucleic acids used in the method of the invention (e.g. the chimeric genes) are not present in, or originating from, the genome of said plant, or are present in the genome of said plant but not at their natural locus in the genome of said plant, it being possible for the nucleic acids to be expressed homologously or heterologously.
20 However, as mentioned, transgenic also means that, while the nucleic acids according to the invention or used in the inventive method are at their natural position in the genome of a plant, the sequence has been modified with regard to the natural sequence, and/or that the regulatory sequences of the natural sequences have been modified. Transgenic is preferably understood as meaning the expression of the
25 nucleic acids according to the invention at an unnatural locus in the genome, i.e. homologous or, heterologous expression of the nucleic acids takes place. Preferred transgenic plants are mentioned herein.

The term "expression" or "gene expression" means the transcription of a specific gene
30 or specific genes or specific genetic construct. The term "expression" or "gene expression" in particular means the transcription of a gene or genes or genetic construct into structural RNA (rRNA, tRNA) or mRNA with or without subsequent translation of the latter into a protein. The process includes transcription of DNA and processing of the resulting mRNA product.

The term "introduction" or "transformation" as referred to herein encompass the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated there from. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, mega-gametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem). The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid. Alternatively, it may be integrated into the host genome. The resulting transformed plant cell may then be used to regenerate a transformed plant in a manner known to persons skilled in the art.

The transfer of foreign genes into the genome of a plant is called transformation. Transformation of plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. The methods described for the transformation and regeneration of plants from plant tissues or plant cells may be utilized for transient or for stable transformation. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., (1982) *Nature* 296, 72-74; Negrutiu I et al. (1987) *Plant Mol Biol* 8: 363- 373); electroporation of protoplasts (Shillito R.D. et al. (1985) *Bio/Technol* 3, 1099-1 102); microinjection into plant material (Crossway A et al., (1986) *Mol. Gen Genet* 202: 179-185); DNA or RNA-coated particle bombardment (Klein TM et al., (1987) *Nature* 327: 70) infection with (non-integrative) viruses and the like. Transgenic plants, including transgenic crop plants, are preferably produced via *Agrobacterium*-mediated transformation. An advantageous transformation method is the transformation in planta. To this end, it is possible, for example, to allow the *agrobacteria* to act on plant seeds or to inoculate the plant meristem with *agrobacteria*.

It has proved particularly expedient in accordance with the invention to allow a suspension of transformed agrobacteria to act on the intact plant or at least on the flower primordia. The plant is subsequently grown on until the seeds of the treated plant are obtained (Clough and Bent, *Plant J.* (1998) 16, 735-743). Methods for

5 Agrobacterium-mediated transformation of rice include well known methods for rice transformation, such as those described in any of the following: European patent application EP1198985, Aldemita and Hodges (*Planta* 199: 612-617, 1996); Chan et al. (*Plant Mol Biol* 22 (3): 491 -506, 1993), Hiei et al. (*Plant J* 6 (2): 271 -282, 1994), which disclosures are incorporated by reference herein as if fully set forth. In the case

10 of corn transformation, the preferred method is as described in either Ishida et al. (*Nat. Biotech.* 14(6): 745-50, 1996) or Frame et al. (*Plant Physiol* 129(1): 13-22, 2002), which disclosures are incorporated by reference herein as if fully set forth. Said methods are further described by way of example in B. Jenes et al., *Techniques for Gene Transfer*, in: *Transgenic Plants, Vol. 1 , Engineering and Utilization*, eds. S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42 (1991) 205-225). The nucleic acids or the construct to be expressed is preferably cloned into a vector, which is suitable for transforming

15 *Agrobacterium tumefaciens*, for example pBin19 (Bevan et al (1984) *Nucl. Acids Res.* 12-8711). Agrobacteria transformed by such a vector can then be used in known

20 manner for the transformation of plants, such as plants used as a model, like *Arabidopsis* or crop plants such as, by way of example, tobacco plants, for example by immersing bruised leaves or chopped leaves in an agrobacterial solution and then culturing them in suitable media. The transformation of plants by means of *Agrobacterium tumefaciens* is described, for example, by Hofgen and Willmitzer in

25 *Nucl. Acid Res.* (1988) 16, 9877 or is known inter alia from F.F. White, *Vectors for Gene Transfer in Higher Plants*; in *Transgenic Plants, Vol. 1 , Engineering and Utilization*, eds. S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38.

In addition to the transformation of somatic cells, which then have to be regenerated

30 into intact plants, it is also possible to transform the cells of plant meristems and in particular those cells which develop into gametes. In this case, the transformed gametes follow the natural plant development, giving rise to transgenic plants. Thus, for example, seeds of *Arabidopsis* are treated with agrobacteria and seeds are obtained from the developing plants of which a certain proportion is transformed and

thus transgenic [Feldman, KA and Marks MD (1987). *Mol Gen Genet* 208:1 -9; Feldmann K (1992). In: C Koncz, N-H Chua and J Shell, eds, *Methods in Arabidopsis Research*. Word Scientific, Singapore, pp. 274-289]. Alternative methods are based on the repeated removal of the inflorescences and incubation of the excision site in the center of the rosette with transformed agrobacteria, whereby transformed seeds can likewise be obtained at a later point in time (Chang (1994). *Plant J.* 5: 551 -558; Katavic (1994). *Mol Gen Genet*, 245: 363-370). However, an especially effective method is the vacuum infiltration method with its modifications such as the "floral dip" method. In the case of vacuum infiltration of Arabidopsis, intact plants under reduced pressure are treated with an agrobacterial suspension [Bechthold, N (1993). *CR Acad Sci Paris Life Sci*, 316: 1 194-1 199], while in the case of the "floral dip" method the developing floral tissue is incubated briefly with a surfactant-treated agrobacterial suspension [Clough, SJ and Bent AF (1998) *The Plant J.* 16, 735-743]. A certain proportion of transgenic seeds are harvested in both cases, and these seeds can be distinguished from non-transgenic seeds by growing under the above-described selective conditions. In addition the stable transformation of plastids is of advantages because plastids are inherited maternally in most crops reducing or eliminating the risk of transgene flow through pollen. The transformation of the chloroplast genome is generally achieved by a process which has been schematically displayed in Klaus et al., 2004 [Nature Biotechnology 22 (2), 225-229]. Briefly the sequences to be transformed are cloned together with a selectable marker gene between flanking sequences homologous to the chloroplast genome. These homologous flanking sequences direct site specific integration into the plastome. Plastidal transformation has been described for many different plant species and an overview is given in Bock (2001) *Transgenic plastids in basic research and plant biotechnology*. *J Mol Biol.* 2001 Sep 21; 312 (3):425-38 or Maliga, P (2003) *Progress towards commercialization of plastid transformation technology*. *Trends Biotechnol.* 21, 20-28. Further biotechnological progress has recently been reported in form of marker free plastid transformants, which can be produced by a transient co-integrated marker gene (Klaus et al., 2004, *Nature Biotechnology* 22(2), 225-229).

The genetically modified plant cells can be regenerated via all methods with which the skilled worker is familiar. Suitable methods can be found in the abovementioned publications by S.D. Kung and R. Wu, Potrykus or Hofgen and Willmitzer.

Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant-expressible genes co-transferred with the gene of interest, following which the transformed material is regenerated into a whole plant. To select transformed plants, the plant material
5 obtained in the transformation is, as a rule, subjected to selective conditions so that transformed plants can be distinguished from untransformed plants. For example, the seeds obtained in the above-described manner can be planted and, after an initial growing period, subjected to a suitable selection by spraying. A further possibility consists in growing the seeds, if appropriate after sterilization, on agar plates using a
10 suitable selection agent so that only the transformed seeds can grow into plants. Alternatively, the transformed plants are screened for the presence of a selectable marker such as the ones described above.

Following DNA transfer and regeneration, putatively transformed plants may also be
15 evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be monitored using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

20 The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed and homozygous second-generation (or T2) transformants selected, and the T2 plants may then further be propagated through
25 classical breeding techniques. The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

30 The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, leaves, roots (including tubers), flowers, and tissues and organs, wherein each of the aforementioned comprise the gene/nucleic acid of interest. The term "plant" also encompasses plant

cells, suspension cultures, callus tissue, embryos, meristematic regions, gametophytes, sporophytes, pollen and microspores, again wherein each of the aforementioned comprises the gene/nucleic acid of interest.

- 5 In some embodiments, the plant cell according to the invention is non-propagating or cannot be regenerated into a plant.

Plants that are particularly useful in the methods of the invention include in particular monocotyledonous and dicotyledonous plants including fodder or forage legumes,
 10 ornamental plants, food crops, trees or shrubs selected from the list comprising *Acer* spp., *Actinidia* spp., *Abelmoschus* spp., *Agave sisalana*, *Agropyron* spp., *Agrostis stolonifera*, *Allium* spp., *Amaranthus* spp., *Ammophila arenaria*, *Ananas comosus*, *Annona* spp., *Apium graveolens*, *Arachis* spp., *Artocarpus* spp., *Asparagus officinalis*, *Avena* spp. (e.g. *Avena sativa*, *Avena fatua*, *Avena byzantina*, *Avena fatua* var. *sativa*,
 15 *Avena hybrida*), *Averrhoa carambola*, *Bambusa* sp., *Benincasa hispida*, *Bertholletia excelsea*, *Beta vulgaris*, *Brassica* spp. (e.g. *Brassica napus*, *Brassica rapa* ssp. [canola, oilseed rape, turnip rape]), *Cadaba farinosa*, *Camellia sinensis*, *Canna indica*, *Cannabis sativa*, *Capsicum* spp., *Carex elata*, *Carica papaya*, *Carissa macrocarpa*, *Carya* spp., *Carthamus tinctorius*, *Castanea* spp., *Ceiba pentandra*, *Cichorium endivia*,
 20 *Cinnamomum* spp., *Citrullus lanatus*, *Citrus* spp., *Cocos* spp., *Coffea* spp., *Colocasia esculenta*, *Cola* spp., *Corchorus* sp., *Coriandrum sativum*, *Corylus* spp., *Crataegus* spp., *Crocus sativus*, *Cucurbita* spp., *Cucumis* spp., *Cynara* spp., *Daucus carota*, *Desmodium* spp., *Dimocarpus longan*, *Dioscorea* spp., *Diospyros* spp., *Echinochloa* spp., *Elaeis* (e.g. *Elaeis guineensis*, *Elaeis oleifera*), *Eleusine coracana*, *Eragrostis tef*,
 25 *Erianthus* sp., *Eriobotrya japonica*, *Eucalyptus* sp., *Eugenia uniflora*, *Fagopyrum* spp., *Fagus* spp., *Festuca arundinacea*, *Ficus carica*, *Fortunella* spp., *Fragaria* spp., *Ginkgo biloba*, *Glycine* spp. (e.g. *Glycine max*, *Soja hispida* or *Soja max*), *Gossypium hirsutum*, *Helianthus* spp. (e.g. *Helianthus annuus*), *Hemerocallis fulva*, *Hibiscus* spp., *Hordeum* spp. (e.g. *Hordeum vulgare*), *Ipomoea batatas*, *Juglans* spp., *Lactuca sativa*,
 30 *Lathyrus* spp., *Lens culinaris*, *Linum usitatissimum*, *Litchi chinensis*, *Lotus* spp., *Luffa acutangula*, *Lupinus* spp., *Luzula sylvatica*, *Lycopersicon* spp. (e.g. *Lycopersicon esculentum*, *Lycopersicon lycopersicum*, *Lycopersicon pyriforme*), *Macrotyloma* spp., *Malus* spp., *Malpighia emarginata*, *Mammea americana*, *Mangifera indica*, *Manihot* spp., *Manilkara zapota*, *Medicago sativa*, *Melilotus* spp., *Mentha* spp., *Miscanthus*

sinensis, Momordica spp., Morus nigra, Musa spp., Nicotiana spp., Olea spp., Opuntia spp., Ornithopus spp., Oryza spp. (e.g. Oryza sativa, Oryza latifolia), Panicum miliaceum, Panicum virgatum, Passiflora edulis, Pastinaca sativa, Pennisetum sp., Persea spp., Petroselinum crispum, Phalaris arundinacea, Phaseolus spp., Phleum
5 pratense, Phoenix spp., Phragmites australis, Physalis spp., Pinus spp., Pistacia vera, Pisum spp., Poa spp., Populus spp., Prosopis spp., Prunus spp., Psidium spp., Punica granatum, Pyrus communis, Quercus spp., Raphanus sativus, Rheum rhabarbarum, Ribes spp., Ricinus communis, Rubus spp., Saccharum spp., Salix sp., Sambucus spp., Secale cereale, Sesamum spp., Sinapis sp., Solanum spp. (e.g. Solanum
10 tuberosum, Solanum integrifolium or Solanum lycopersicum), Sorghum bicolor, Spinacia spp., Syzygium spp., Tagetes spp., Tamarindus indica, Theobroma cacao, Trifolium spp., Tripsacum dactyloides, Triticosecale rimpai, Triticum spp. (e.g. Triticum aestivum, Triticum durum, Triticum turgidum, Triticum hybernum, Triticum macha, Triticum sativum, Triticum monococcum or Triticum vulgare), Tropaeolum
15 minus, Tropaeolum majus, Vaccinium spp., Vicia spp., Vigna spp., Viola odorata, Vitis spp., Zea mays, Zizania palustris, Ziziphus spp., amongst others.

The choice of suitable control plants is a routine part of an experimental setup and may include corresponding wild type plants or corresponding plants without the gene of interest. The control plant is typically of the same plant species or even of the same
20 variety as the plant to be assessed. The control plant may also be a nullizygote of the plant to be assessed. Nullizygotes are individuals missing the transgene by segregation. A "control plant" as used herein refers not only to whole plants, but also to plant parts, including seeds and seed parts.

The term "expression cassette" refers to any recombinant expression system for the purpose of expressing a nucleic acid sequence of the invention in vitro or in vivo,
25 constitutively or inducibly, in any cell, including, in addition to plant cells, prokaryotic, yeast, fungal, insect or mammalian cells. The term includes linear and circular expression systems. The term includes all vectors. The cassettes can remain episomal or integrate into the host cell genome. The expression cassettes can have the ability to
30 self-replicate or not (i.e., drive only transient expression in a cell). The term includes recombinant expression cassettes that contain only the minimum elements needed for transcription of the recombinant nucleic acid.

The following non-limiting Examples describe methods and means according to the invention. Unless stated otherwise in the Examples, all techniques are carried out according to protocols standard in the art. The following examples are included to illustrate embodiments of the invention. Those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific
5 embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results
10 would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

Examples and materials and methods

15 1. Screen for mutants suppressing ERF6-induced dwarfism

It has been shown in the art that leaf growth under stress is regulated by the ERF6-centered pathway in a GA/DELLA-dependent way (Dubois et al., 2013; Claeys et al., 2012)(Figure 1a). Consistently, plants highly overexpressing the transcription factor *ERF6* are extremely dwarfed, and show dark green, down curling leaves. We used this
20 easily detectable phenotype as a starting point for a forward genetics screen and subjected 10000 seeds of an ERF6-GR line (M1) to treatment with the mutagen 1-Methylsulfonyloxyethane (EMS). This commonly used mutagen introduces random and stable point mutations in the genome (Weigel and Glazebrook, 2006). Mutagenized seeds were selfed to obtain M2 mutants homozygous for both the ERF6-GR construct
25 and for the introduced mutations, enabling the identification of recessive mutations. To screen for mutants suppressing the ERF6-induced phenotype 20 000 Arabidopsis (M2) seeds were grown *in vitro* on medium containing dexamethasone (DEX) to induce the ERF6-overexpression, and on Kanamycin (Km). As the ERF6-GR construct contains a Kanamycin resistance marker, this step enabled removal of wild type seeds that
30 possibly contaminated the seed stock. In the presence of the inducing compound (DEX), the non-mutated plants and those that were mutated in a gene unrelated to growth were extremely dwarfed (Figure 1b). In contrast, 12 plants able to repress the

ERF6-phenotype because they were mutated in a gene related to ERF6-mediated growth regulation, grew normally. They were selected and selfed to obtain large amounts of non-segregating M3 seeds. The mutants were further crossed with each other to enable identification of allelic groups. In total, the 12 mutants were classified in 7 different allelic groups: 6 groups containing each only 1 mutant, and 1 group containing 6 allelic mutants. For this last group of 6 mutants, a representative mutant was chosen for further analysis. The 7 non-allelic mutants were named *sgi1* – *sgi7* (Suppressor of ERF6-mediated Growth Inhibition).

To validate the mutants, we first checked by PCR and sequencing for the presence of an intact ERF6-GR construct. Two mutants, named *sgi1* and *sgi2* were found to be mutated in the ERF6-domain of the ERF6-GR construct. This provoked missense mutations, respectively A270T and R285Q which are situated in the DNA-binding AP2-domain of the ERF6 protein. However, because upon crossing with a wild type the progeny of both mutants segregated according to the 2nd Mendelian law for 2 independent alleles, we concluded that the mutation in *ERF6* itself is not responsible for the suppression of the ERF6-induced dwarfism.

Secondly, we investigated whether the suppression of the ERF6-induced dwarfism is a specific suppression of the growth-regulatory pathway controlled by ERF6, or a more general abolishment of all functions of ERF6. As we reported previously, ERF6 plays a dual role under stress with, next to the activation of the growth-inhibitory pathway, the induction of several stress tolerance-related transcription factors such as *MYB51*, *STZ* and *WRKY33* (Dubois et al., 2013; Sakamoto et al., 2000, 2004; Gigolashvili et al., 2007; Jiang and Deyholos, 2009; Birkenbihl et al., 2012; Li et al., 2012; Niu et al., 2012; Dubois et al., 2013). We thus performed expression analysis by qRT-PCR to measure the capacity of ERF6 to induce the stress tolerance-related targets, and to confirm the inability to activate the growth-related target gene *GA2-OX6*. To induce the overexpression of ERF6, 15-days old seedlings were exposed to dexamethasone-containing medium and RNA was extracted from the actively expanding third true leaf. We observed that in 5 of the 7 mutants (*sgi1-sgi4* and *sgi7*) the induction of *GA2-OX6* by ERF6 does not longer occur, while the stress tolerance genes were still induced (Figure 1c). Amongst these 5 mutants are the 2 mutants *sgi1* and *sgi2* with mutated ERF6, confirming that the functionality of ERF6 was not affected by the *in cis* mutation. The other 2 mutants, *sgi5* and *sgi6* were also affected in the induction of the tolerance-

related genes, but showed a remarkable down-regulation of the growth-inhibitory GA2-OX6 gene and were for that reason kept in the screen (Figure 1c).

Interestingly, when growing the 7 different mutants in soil, under non-DEX conditions, we observed that several mutants show additional phenotypes, on top of the capacity to suppress the ERF6-GR phenotype (Figure 1d). For example, the *sgi3* mutant exhibits a clear shade avoidance phenotype accompanied by early flowering. The *sgi4* mutant has a very striking epinastic phenotype and shows trichomes at both sides of the rosette leaves, while these are generally only observed on the adaxial leaf size. This mutant also shows defective formation of siliques, which often remain very small and contain only few seeds. Importantly, this *sgi7* mutant showed visible increase in rosette size. Larger rosettes were sometimes also observed for *sgi1* and *sgi6*, although this was not reproducible. We thus concluded that 7 non-allelic mutants are able to suppress ERF6-induced dwarfism through different molecular mechanisms and that some of these mutants show interesting rosette phenotypes on top of the capacity to suppress the ERF6-phenotype.

2. Identification of the causal genes

To identify the gene mutated in each of the 7 mutants, each mutant (in Col-0 background) was outcrossed with the Arabidopsis Ler-1 accession. About 3000 F2 seeds were screened *in vitro* on growth medium containing both Km and DEX. Upon segregation, 400 mutants showing a normal growth phenotype, and thus containing at least a single copy of the ERF6-GR and being homozygous for the causal mutation were selected and pooled for bulk segregant analysis using Shoremap (Schneeberger et al., 2009). Following genes were found to carry the causal mutation in the respective mutants (see Table 1).

The mutants *sgi1* and *sgi2* were mutated in, respectively, AT1G54820 and AT2G36350. Both genes encode putative kinases without apparent homology, but both have previously been associated with the response in Arabidopsis leaves upon infection by the Gemini virus (Ascencio-Ibanez et al., 2008). Because ERF6 C-terminally possesses a phosphorylation site necessary for proper ERF6 function (Nakano et al., 2006; Meng et al., 2013; Wang et al., 2013), the fact that the 2 mutants mutated in the ERF6-domain of the ERF6-GR both show a causal mutation in a kinase enzyme raises the question whether an impaired post-translational modification of

ERF6 by these mutants kinases and/or by the mutant ERF6 could explain the suppression of the ERF6-induced dwarfism. This is rather unlikely as the mutations are not situated in the putative phosphorylation site, but the exact molecular mechanism and putative connection between ERF6 and these 2 kinases certainly deserves further
5 consideration.

The mutation in *sgi3* still remains to be identified. The clear shade avoidance phenotype and the early flowering could suggest a mutation in a gene involved in GA-biosynthesis or signalling, as for example overexpression the *GA20-OX1* GA-biosynthetic enzyme shows a comparable phenotype. The same hold for mutant *sgi5*,
10 in which the causal mutation still remains unidentified.

The mutant *sgi4* appeared to be mutated in AT3G05040, a gene encoding the HASTY protein. A G -> A mutation in this gene generates a premature stop codon and probably a truncated protein. The HASTY protein is a nuclear membrane-located protein from the importin/exportin family which imports/exports different substrates
15 such as mRNA or small proteins to or out of the nucleus (Telfer and Poethig, 1998; Bollman et al., 2003). Importantly, the HASTY gene has already been identified in multiple EMS-screens including some based on GR-lines (Allen et al., 2013; REFs). As the functionality of GR lines is relying on proper transport of the constitutively overexpressed fusion protein to the nucleus, it can be speculated that HASTY is
20 involved in this translocation and that truncated HASTY proteins fail in this process. However, it then remains elusive how induction of the stress-related ERF6 target genes still occurs in *sgi4*. We might speculate that few ERF6-GR proteins sporadically reach the nucleus through other transporters, which can be sufficient for the activation of some but not all ERF6 targets. Another hypothesis might involve putative protein-
25 interaction partners of ERF6, which could be necessary to activate a subset of the ERF6-targets (such as the *GA2-OX6*) and of which the transport to the nucleus is HASTY-dependent. This is however completely speculative. Mutants in the HASTY gene have previously been reported to exhibit the same phenotype as observed for *sgi4*, including the highly pronounced epinasty and the trichomes on the lower side of
30 the leaf, indicating a faster maturation of the shoot (Bollman et al., 2003).

In the mutant *sgi6*, the mutated gene AT3G27670 encodes the transmembrane receptor-like protein *RESURRECTION1* (*RST1*). This gene has previously been reported to negatively affect the cuticular wax biosynthesis, as mutants in *RST1* have

increased levels of cuticular wax with an altered fatty acid composition (Chen et al., 2005). As mutants produce 70% of shrunken and unviable seeds (which was also observed for the *sgi6* mutant), the *RST1* gene is also thought to play a role in embryo development (Chen et al., 2005). Finally, the *RST1* protein is a positive regulator of
5 plant defence against biotrophic pathogens, and a negative regulator of defence against necrotrophes (Mang et al., 2009). In *sgi6*, the mutation in the splice acceptor site of the first intron generates a postponed splicing engendering a frameshift and thus likely results in loss-of-function of *RST1*.

Finally, the *sgi7* mutant carried a mutation in the gene encoding EIN5, also named
10 XRN4. EIN5 is a 5' -> 3' exoribonuclease and degrades transcripts carrying preferential EIN5 binding sites (Souret et al., 2004; Rymarquis et al., 2011). The EIN5 enzyme has been involved in the ethylene signalling pathways as reverse genetic mutants of EIN5 are ethylene insensitive, and do not longer show the typical triple response of ethylene-treated seedlings grown in the dark (Van der Straeten et al.,
15 1993; Roman et al., 1995; Olmedo et al., 2006; Potuschak et al., 2006). Consistently with this identification, the *sgi7* mutant did not show triple response upon germination in the dark on medium containing the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The *sgi7* mutant looked particularly interesting as it showed a visible increase in leaf size and as it was the representative mutant of the group of 6
20 allelic mutants, further called *sgi7.1* – *sgi7.6*. We therefore chose to characterize these mutants in more detail.

Mutant	Mutated gene	Gene name	Predicted effect on protein	Additional phenotype
<i>sgi1</i>	AT1G54820 + mutation in ERF6	Putative kinase		/
<i>sgi2</i>	AT2G36350 + mutation in ERF6	Putative kinase		/
<i>sgi3</i>				Shade avoidance
<i>sgi4</i>	AT3G05040	<i>HASTY</i>	Truncated protein	Epinasty
<i>sgi5</i>				/
<i>sgi6</i>	AT3G27670	<i>RST1</i>	Frameshift	/
<i>sgi7</i>	AT1G54490	<i>EIN5</i>	Multiple, See Table 2	Larger, serrated rosette leaves

Table 1: Identification of the mutations in the 7 non-allelic EMS-mutants.

3. Multiple mutations identified in conserved domains of *EIN5*

- 5 As the promising *sgi7* mutant (from now on named *sgi7.1*) was the representative of an allelic group of 6 mutants, we also identified the exact mutation in each of the 5 other mutants (see Table 2). The mutations mapped on 6 different sites on the coding sequence of *EIN5* and had predicted different effects on the protein sequence: in *sgi7.1*, *sgi7.2* and *sgi7.4*, the point mutation could result in an amino acid substitution, while in *sgi7.3* and *sgi7.5*, the mutation is likely to generate a nonsense mutation. Finally, in *sgi7.6*, the mutation was situated in the splice acceptor site of the 14th intron, which could generate incorrect splicing and finally result in a frameshift.

The *EIN5/XRN4* protein is a rather huge protein of 947 amino acids with relatively well-conserved orthologs in *Drosophila* and yeast (Nagarajan et al., 2013). The protein structure of the yeast *XRN1* has recently been described, and the N-terminal half of the protein, containing the most important functional domains, is thought to be

conserved between the orthologs. At its N-terminal, the exoribonuclease EIN5 contains several small well-conserved domains, such as a steric barrier to prevent penetrance of dsRNA, a basic pocket for substrate stabilisation and a RNA binding motif (Figure 2). Next to this, the N-terminal side contains several extremely well-conserved amino acids which are thought to be crucial for proper functionality of the active site and which are situated in short stretches of very well-conserved amino acids. Interestingly, several of the identified mutations are localized within these very conserved and thus potentially important motifs (Figure 2).

Mutant	Nt position	Mutation Nt	AA position	Mutation AA
<i>sgi7.1</i>	314	G -> A	105	G -> E
<i>sgi7.2</i>	329	C -> T	110	A -> V
<i>sgi7.3</i>	343	C -> T	115	Q -> STOP
<i>sgi7.4</i>	706	G -> A	236	D -> N
<i>sgi7.5</i>	1476	C -> T	526	Q -> STOP
<i>sgi7.6</i>	1603	G -> A	Splice mutated	acceptor site

Table 2. Position of the mutations in the EMS-mutants for EIN5 and their effect.

Six mutations were identified on different positions of the EIN5 coding sequence. Based on the validated mutations, the effect on protein level was predicted: three of them generate a missense mutation, two a nonsense mutation and one generates an incorrect splicing variant probably resulting in a frameshift. Nt = nucleotide, AA = amino acid.

4. Different alleles with different effects on leaf growth

All identified mutants are able to suppress ERF6-induced dwarfism but interestingly, some of them show additional phenotypes under non-dex conditions (Figure 1d). To investigate whether all identified mutations in *EIN5* had a similar or different effect on Arabidopsis leaf growth, all mutants were simultaneously grown in soil for 22 days and rosette size was measured by making leaf series. Interestingly, the six *sgi7* alleles showed different levels of increase in leaf size (Figure 3a). The most pronounced increase in leaf size was observed for the *sgi7.1* mutant, with an average increase of

49% ($p = 2E-9$). The mutants *sgi7.5* and *sgi7.2* also showed a significant increase in final rosette area of respectively 26% and 15%. In contrast, the mutants *sgi7.3* and *sgi7.6* were not larger than the WT (ERF6-GR). To further evaluate these growth phenotypes, 3 independent previously used mutants of *EIN5* (*ein5.1*, *ein5.6* and *xrn4*) were phenotyped in a similar way. These three mutants are confirmed loss-of-function mutants as they carry a 1-bp deletion causing a frameshift, a T-DNA insertion in the fifth exon, and a T-DNA insertion in an intron, respectively (Figure 2) (Olmedo et al., 2006). Although these mutants have been extensively studied, their leaf growth has never been characterized in detail. At 22 days after stratification (DAS), the final rosette area was unaltered as compared to wild-type rosette size (Figure 3b). The observation that in these loss-of-function mutants the leaf size is not increased reinforces our hypothesis that the mutations in *sgi7.1*, *sgi7.2* and *sgi7.5* generate *EIN5* alleles likely to alter the activity of this protein.

5. The *EIN5*^{G105E} allele of *sgi7.1* enhances leaf growth

To unravel the mechanisms behind the stimulation of leaf growth in the *sgi7* mutants, we further focussed on the mutant with the largest rosette area, *sgi7.1*. Detailed analysis showed that this mutant is on average 53% larger resulting from an increased size of all leaves (Figure 4a and 4b). To uncover the cellular mechanism behind it, cellular drawings of the abaxial epidermal layer were made to determine whether increased leaf growth results from more and/or larger cells. We observed a pronounced increase in cell area, with an average increase of 36% (Figure 4c). The increase in cell area was however not sufficient to explain the increased leaf size (51% for the third leaf), but the cell number was only slightly and not significantly increased in all biological repeats. We thus conclude that the leaf size increase in the *sgi7.1* mutant mainly results from an increase in cell area and that the *EIN5*^{G105E} allele mainly affects the expansion phase of leaf development, although small effects on cell division cannot entirely be excluded.

6. The *sgi7.1* mutant is more tolerant to mild drought stress

Because the growth-inhibitory pathway on which the forward genetics screen is based is mainly active under adverse environmental conditions reducing rosette growth, we exposed the *sgi7.1* mutant to mild drought stress. Mutant and control were grown on the Weighing Imaging and Watering Machine (WIWAM) under a well-watered regime

for 11 days and then exposed to a mild drought stress regime reducing wild-type rosette size by about 30-40% (Skirycz et al., 2011). Rosette size was measured at 22 DAS. In three independent biological repeats, the drought-induced growth inhibition was less pronounced in the *sgi7.1* mutant (-23% in *sgi7.1* compared to -33% in WT) (Figure 5). As this mutant already shows a growth advantage under normal conditions, this resulted in *sgi7.1* mutants being under mild drought equally large as wild type under control conditions. We thus conclude that the EIN5^{G105E} allele not only positively affects growth under control conditions, but also under mild drought stress.

7. Introduction of an EIN5 mutant functional allele in *Zea mays*

The maize homolog of EIN5 is GRMZM2G099630 and the nucleotide sequence is depicted in SEQ ID NO: 14 (see example 8). In order to introduce mutations in the DNA coding for the conserved sequence (GDVAP) (SEQ ID NO: 17) we use the CRISPR technology by stably transforming plants with a construct containing UBI::CAS9 and U6::gRNA, with gRNA containing the sequence targeting the conserved box (gatggtgttgctccaagg) (SEQ ID NO: 18). The gRNA will guide the CAS9 to the target sequence which will be cleaved. NHEJ will repair the nick while causing insertions, deletions, substitutions. The resulting lines will be genotyped and the lines with no frame shift will be further analyzed and phenotyped.

8. Plant orthologues of EIN5 genes

SEQ ID NO: 1: wild type EIN5 protein sequence of *Arabidopsis thaliana*

Conserved sequences between different plant orthologs of EIN5 are underlined. The positions of the *sgi7.1*-*sgi7.5* allelic variants in the sequence are marked as [].

MGVPAFYRWLADRYPKSISDVVEEPTDGGRGDLIPVDITRPNPNGFEFDNLYLDMN
 GIIHPCFHPEGKPAPATYDDVFKSMFEYIDHLFTLVRPRKILYLAIDGVAPRAKMNQQR
 25 SRRFRAAKDAEAEAEAEERLRKDFEMEGQILSAKEKAETCDSNVITPGTPFMAILSVA
 LQYYIQSRLNHNPGWRYVKVILSDSNVPGEGEHKIMSYIRLQRNLPGFDPNTRHCLY
GLDADLIMLSLATHEVHFSILREVITYPGQQEKCFVCGQTGHFASDCPGKSGSNNA
 DIPIHKKKYQFLNIWVLREYLQYELAIPDPPFMINFERIIDDFVFLCFFVGNDFLPHMPTL
 EIREGAINLLMHVYRKEFTAMGGYLTDSGEVLLDRVEHFIQAVAVNEDKIFQKRTRIKQ
 30 SMDNNEEMKQRSRRDPSEVPPEPIDDKIKLGEPGYKERYA EK FSTTNPEETE QIKQ
 DMVLKYVEGLCWVCRYYYQGVCSWQWFYPYHYAPFASDLKNLPDLEITFFIGEPFKP

FDQLMGTLPAASSNALPGEYRKLMTDPSSPILKFYPADFELDMNGKRFAWQGIAKLP
 FIEEKL⁵LLAATR⁵KLEETLTV⁵EEQQRNSV⁵MLDLLYVHPA⁵HPLGQRILQYYHFYQH⁵MPPH
 ECLP⁵WMIDPNSSQGMNGFLWFSERNGFQTRV⁵DS⁵PV⁵NGLPCIEQNRALNV⁵TYLCPAK
 HSHISEPPRGAIIPDKILTSVDIKPF⁵PPLWHEDNSNRRRQAR⁵DRPQVV⁵GAIAGPSLGEA
 AHRLIKNTLN⁵MKSSTGAASGLIDP⁵NGYYRN⁵VPGNYSYGGVNR⁵PRAPG⁵PSPYRKAYD
 DDSSYYYGKYNNSTQ⁵GTFNNGPRYP⁵PSNGSQDYN⁵RNYNSKIVAEQHNRGGLGAG
 MSGLSIEDNGRSKQLYSSYTEANANLN⁵PLPSPPTQWIGTQP⁵GGNFVGGYYRDG⁵VG
 YSETNGKSVKKVIYQAKTQPSHRGANL

The position of the identified functional mutant alleles of EIN5 in *A. thaliana* is shown in Table 3

Mutant	Nt position	Mutation Nt	AA position	Mutation AA
<i>sgi7.1</i>	314	G -> A	105	G -> E
<i>sgi7.2</i>	329	C -> T	110	A -> V
<i>sgi7.3</i>	343	C -> T	115	Q -> STOP
<i>sgi7.4</i>	706	G -> A	236	D -> N
<i>sgi7.5</i>	1476	C -> T	526	Q -> STOP
<i>sgi7.6</i>	1603	G -> A	Splice mutated	acceptor site

Table 3: position of mutated amino acids in SEQ ID NO: 1 for the different functional allelic mutants of EIN5

- 15 SEQ ID NO: 2 (nucleotide sequence of the *Arabidopsis thaliana* wild type EIN5 gene)
- SEQ ID NO: 3 (Glycine max) amino acid sequence of EIN5 protein
- SEQ ID NO: 4 (Glycine max) nucleotide sequence of EIN5 gene
- SEQ ID NO: 5 *Malus domestica* – amino acid sequence of EIN5 protein
- SEQ ID NO: 6 *Malus domestica* – nucleotide sequence of EIN5 gene
- 20 SEQ ID NO: 7 *Oryza sativa japonica* amino acid sequence of EIN5 protein
- SEQ ID NO: 8 *Oryza sativa japonica* nucleotide sequence of the EIN5 gene

SEQ ID NO: 9 *Oryza sativa indica* amino acid sequence of EIN5 protein

SEQ ID NO: 10 *Oryza sativa indica* nucleotide sequence of EIN5 gene

SEQ ID NO: 11 *Populus trichocarpa* protein sequence of EIN5

SEQ ID NO: 12 *Populus trichocarpa* nucleotide sequence of EIN5 gene

5 SEQ ID NO: 13 amino acid sequence of *Zea mays* EIN5 protein

SEQ ID NO: 14 nucleotide sequence of *Zea mays* EIN5 gene

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Claims

1. A functional allele of EIN5 encoding for a mutant protein selected from the list consisting of:

- 5
- i. a protein having at least one amino acid change in the sequence DGVAPRAKMNQQRSR present in SEQ ID NO: 1 or in a corresponding plant orthologous sequence thereof,
 - ii. a protein having at least one amino sequence change in the sequence GLDADLIML present in SEQ ID NO: 1 or in a corresponding plant orthologous sequence thereof,
 - 10 iii. a protein having an amino acid change at position 526 in SEQ ID NO: 1 or in a corresponding plant orthologous sequence thereof,

or a functional allele of EIN5 having its splice acceptor site mutated at the nucleotide position 1603 in SEQ ID NO: 2 or in a corresponding plant orthologous sequence thereof.

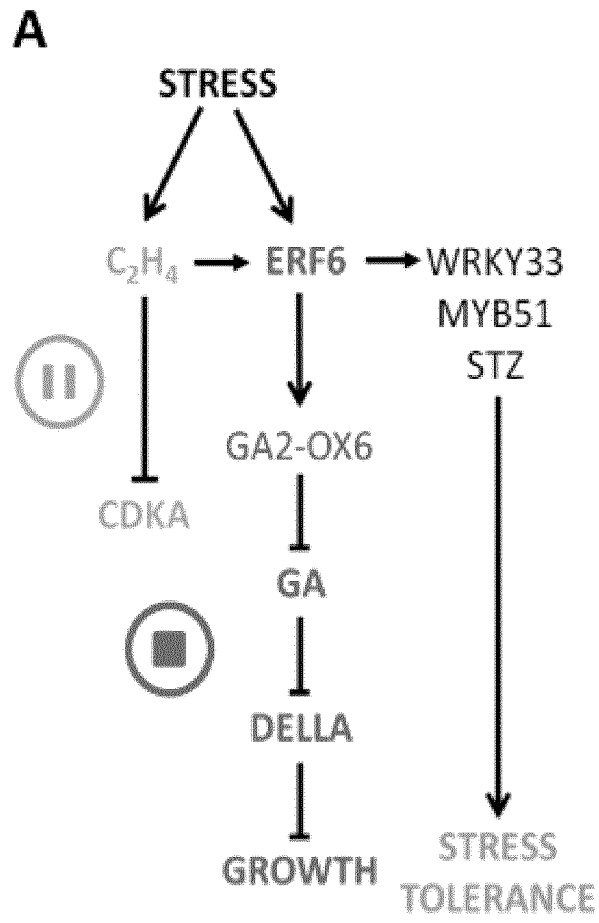
15 2. The functional allele according claim 1 encoding for a functional protein selected from the list consisting of:

- 20
- i. a protein having an amino acid change of G to E in position 105 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof,
 - ii. a protein having an amino acid change of A to V in position 110 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof,
 - 25 iii. a protein having an amino acid change of Q to STOP in position 115 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof,
 - iv. a protein having an amino acid change of D to N in position 236 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof,
 - 30 v. a protein having an amino acid change of Q to a STOP-codon in position 526 of SEQ ID NO: 1, or in a corresponding position in a plant orthologous sequence thereof,

or a functional allele of EIN5 having its splice acceptor site mutated at the nucleotide position 1603 in SEQ ID NO: 2 from G to A, or in a corresponding position of a plant orthologous sequence thereof.

3. A chimeric gene comprising a plant expressible promoter, a mutant, functional EIN5 allele according to claims 1 or 2 and a functional terminator sequence.
4. A mutant plant, or a cell, part, seed or progeny thereof, having knock-out EIN5 genes, comprising a chimeric gene according to claim 3.
5. A mutant plant, or a cell, part, seed or progeny thereof, having its wild type EIN5 genes replaced by mutant, functional EIN5 alleles in a homozygous state wherein said alleles are defined according to claims 1 or 2.
6. The plant according to claims 4 or 5 wherein the yield is increased by at least 20% compared to the plant having wild type EIN5 alleles.
7. The plant according to claims 4 or 5 wherein the drought tolerance is increased compared to the plant having wild type EIN5 alleles.

Figure 1



B

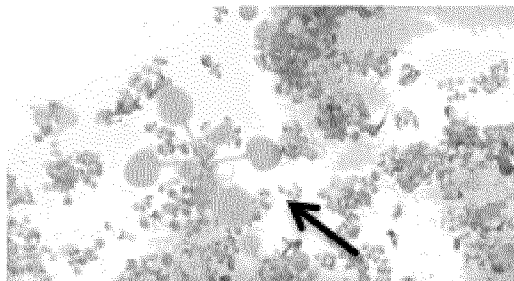
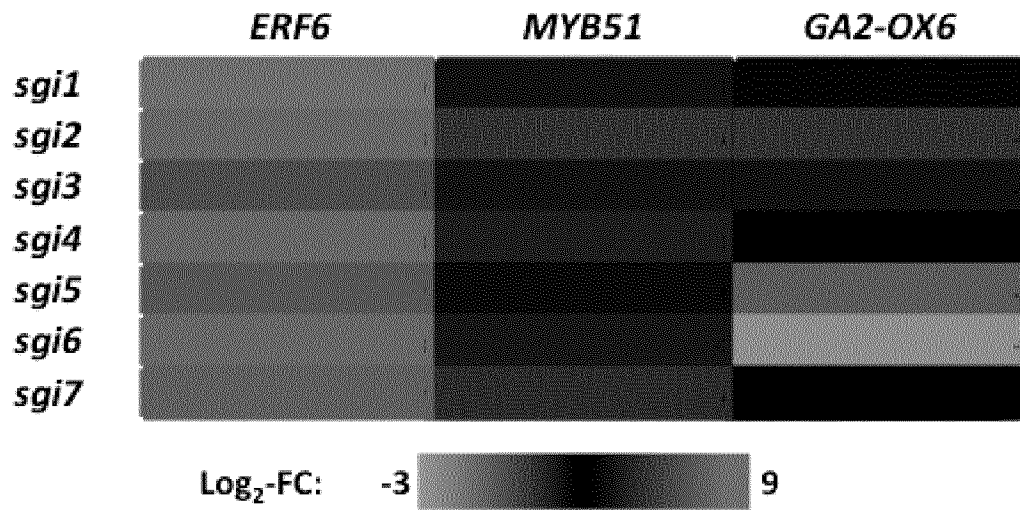


Figure 1 continued

C



D

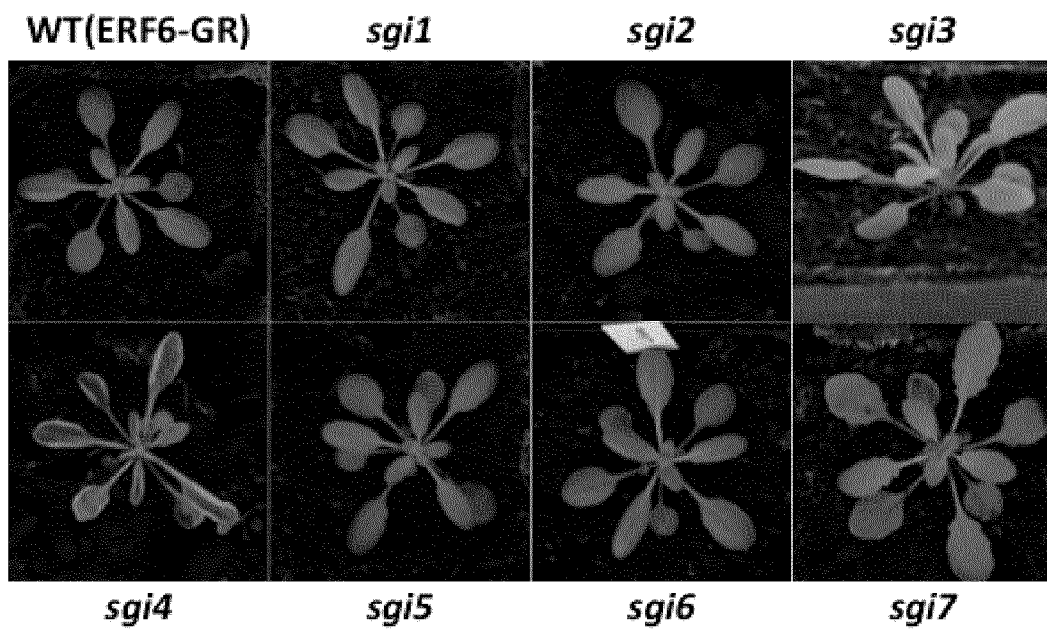


Figure 2

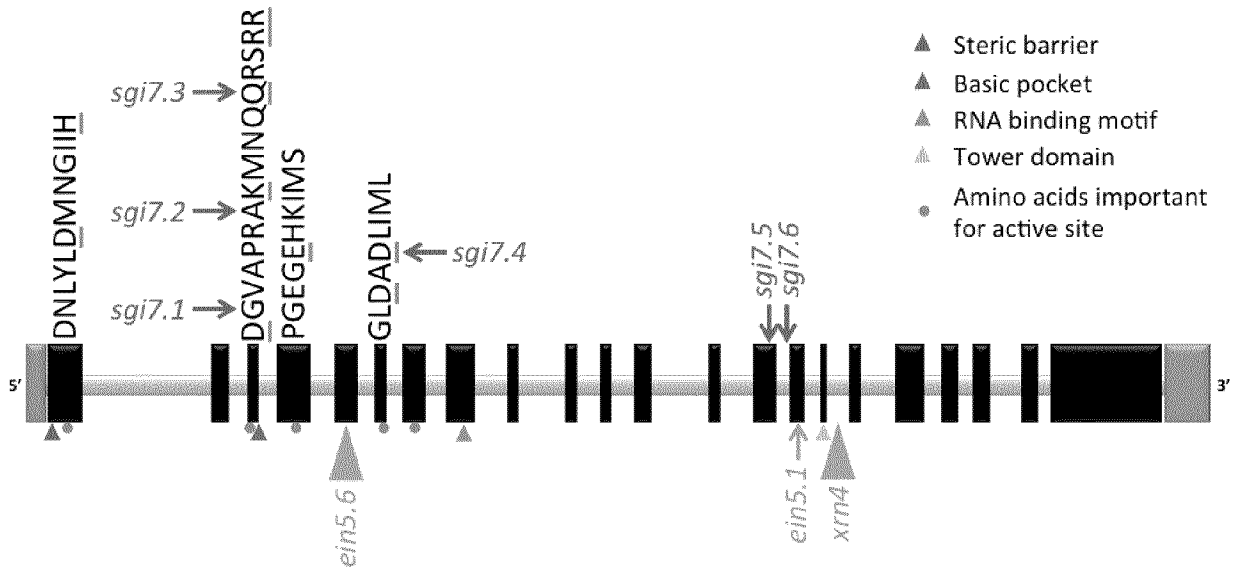


Figure 3

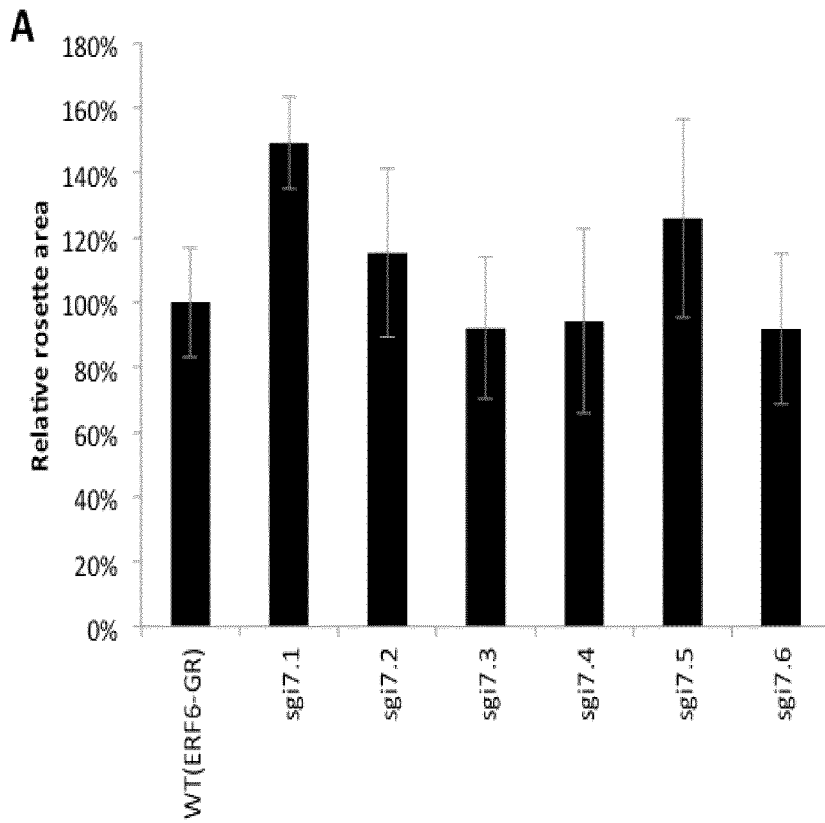


Figure 3 continued

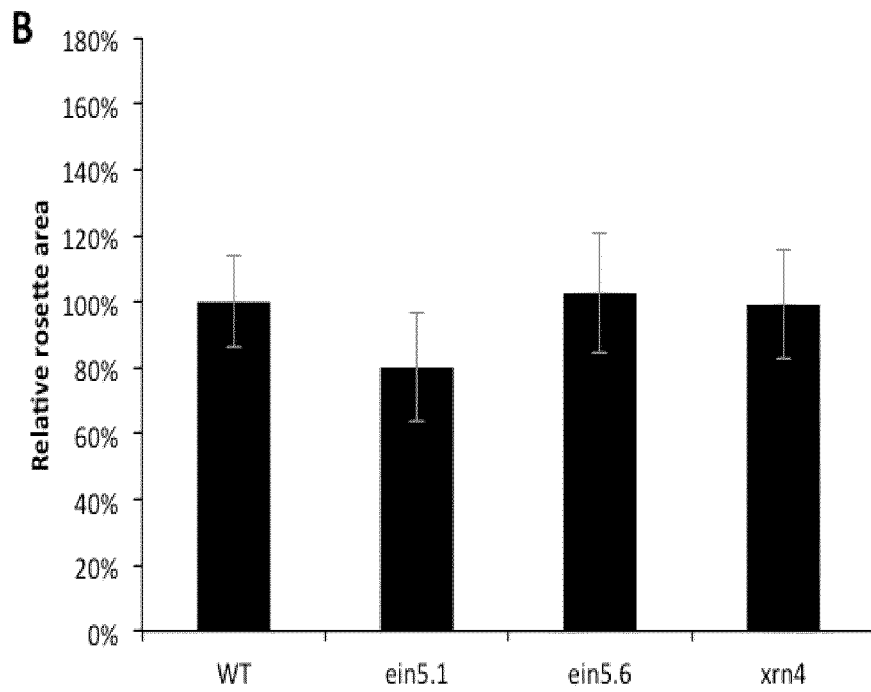


Figure 4

A

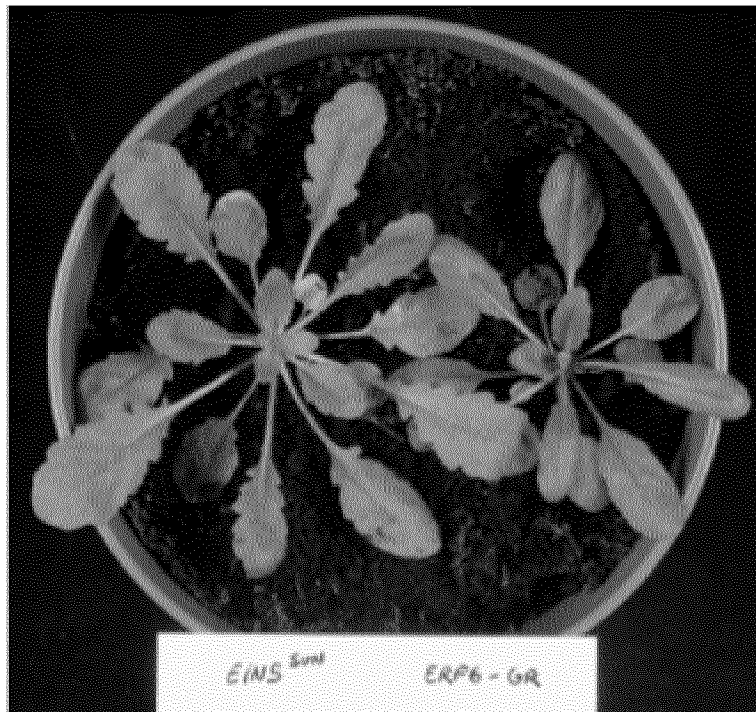


Figure 4 continued

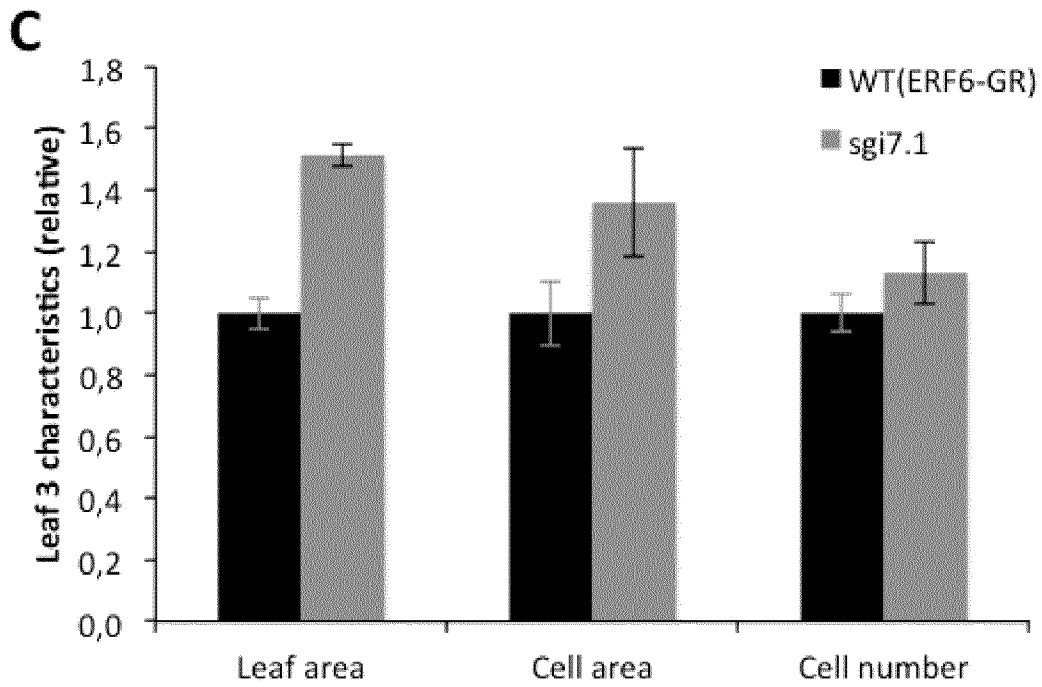
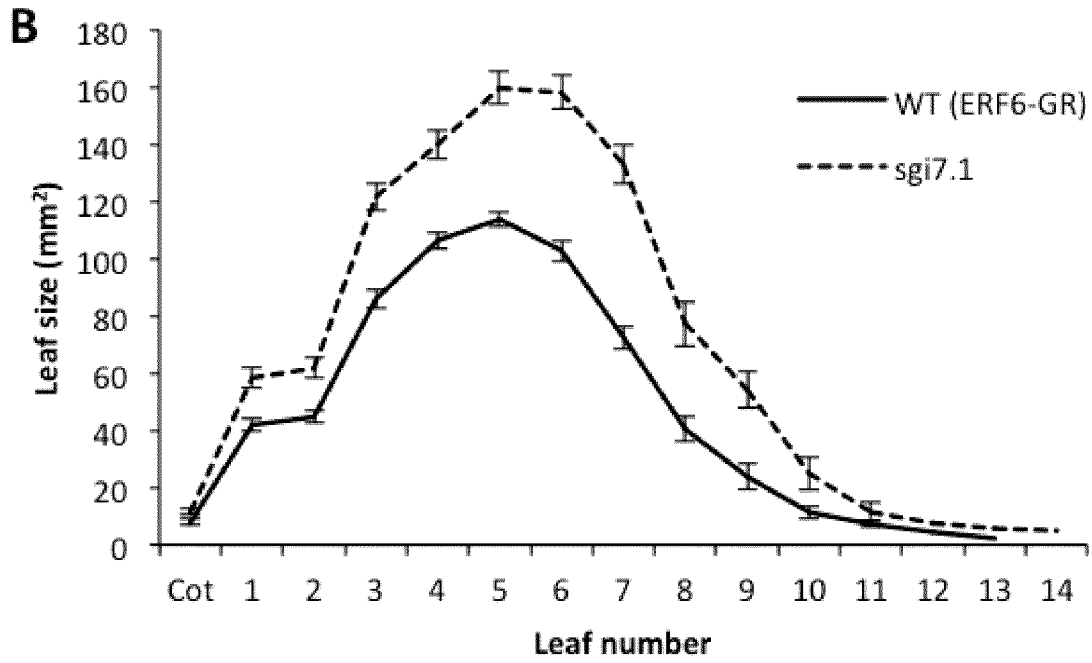
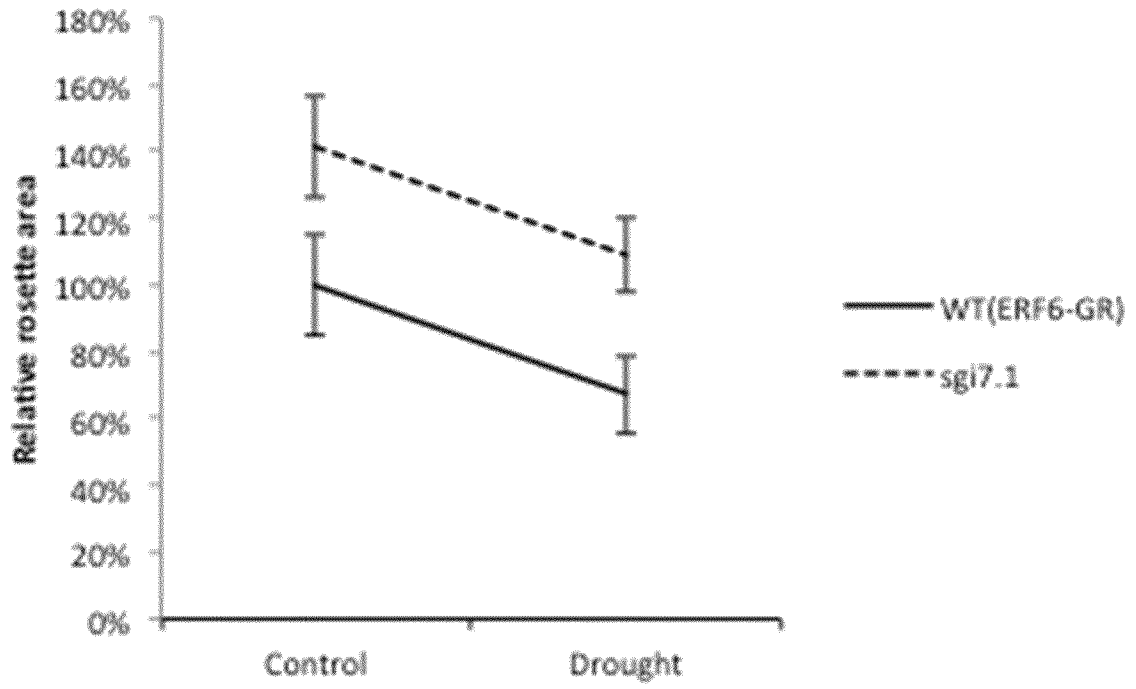


Figure 5



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/072924

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/22 C12N15/82
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, MEDLINE, WPI Data, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2012/143545 A1 (VIB VZW [BE]; UNIV GENT [BE]; INZE DIRK GUSTAAF [BE]; SKIRYCZ ALEKSAND) 26 October 2012 (2012-10-26) page 3, lines 1-28 page 15, lines 7-32 page 18, lines 5-7 page 29, line 27 - page 34, line 33 -----	1-7
A	WO 2009/067580 A2 (PIONEER HI BRED INT [US]; SIVASANKAR SHOBA [US]; REIMANN KELLIE [US]) 28 May 2009 (2009-05-28) page 1, line 33 - page 3, line 29 page 19, lines 11-13 page 51, line 1 - page 60, line 14 ----- -/--	1-7

Further documents are listed in the continuation of Box C.

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* Special categories of cited documents :

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Date of the actual completion of the international search 1 December 2015	Date of mailing of the international search report 16/12/2015
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Bilang, Jürg

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/072924

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>A. SKIRYCZ ET AL: "Pause-and-Stop: The Effects of Osmotic Stress on Cell Proliferation during Early Leaf Development in Arabidopsis and a Role for Ethylene Signaling in Cell Cycle Arrest", THE PLANT CELL ONLINE, vol. 23, no. 5, 1 May 2011 (2011-05-01), pages 1876-1888, XP055045867, ISSN: 1040-4651, DOI: 10.1105/tpc.111.084160 page 1881, left-hand column, paragraph 2 - right-hand column, paragraph 2 page 1884, left-hand column figure 2</p> <p style="text-align: center;">-----</p>	1-7
A	<p>GABRIELA OLMEDO ET AL: "ETHYLENE-INSENSITIVE5 encodes a 5' -> 3' exonuclease required for regulation of the EIN3-targeting F-box proteins EBF1&#x3E;2", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 103, no. 36, 5 September 2006 (2006-09-05), pages 13286-13293, XP055232358, cited in the application the whole document</p> <p style="text-align: center;">-----</p>	1-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/072924

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		CA 2833800 A1	26-10-2012
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		WO 2009067580 A2	28-05-2009
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