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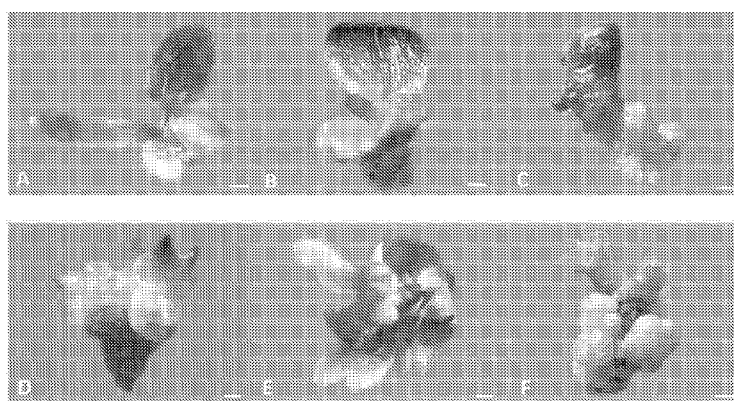


Figure 7

(57) Abstract: A method of cloning plants involves a reprogramming of somatic plant cells of a selected parent plant into undiffer-  
entiated initials by expressing RKD4, BBM, LEC2 or FUS3 transcription factors using a two-component inducible expression sys-  
tem. New plants are then recovered by growing the transformed cells in the absence of the inducer. Regenerated plants are fertile and  
retain epigenetic characteristics of the somatic cells prior to the reprogramming. No changes in gene sequence take place. Regener-  
ated plants are selected so they have at least one epigenetic characteristic of the parent plant. This approach allows a plant, for ex-  
ample *Elaeis guineensis* or *Phalaenopsis sp.* with desirable epigenetic character to be cloned.

## EPIGENETICALLY STABLE CLONED PLANTS

The present invention relates to plants and the fields of agriculture and horticulture, and including the fields of silviculture and viticulture. More particularly the invention concerns  
5 cloning of plants, including micropropagation methods, used to multiply existing desirable plant material, and as used in plant improvement/breeding programmes.

### BACKGROUND

10 Classical plant breeding is a long established and well known field. Individual parent plants, selected for the various characteristics, including growth habit, productivity, resistance to disease or pests, or tolerance of e.g. drought, are crossed and the phenotypes and characteristics of the resulting progeny are assessed. Plants are crossbred to combine the traits from one line or variety with another. For example, a more  
15 rust resistant barley might be crossed with a less rust resistant but higher yielding variety. The desired outcome is a more rust resistant, yet still higher yielding barley variety. Progeny from the cross may then be backcrossed with the higher yielding parent to ensure the high yielding characteristic is not diluted. Inbreeding of progeny may be used to create varieties for further breeding purposes. Classical breeding therefore relies on the naturally  
20 occurring process of homologous recombination and the cross breeding ability of plants.

Breeding techniques have been developed which allow the crossing of plants that would not naturally interbreed. Plant tissue culture can be used to rescue embryos from crosses that would otherwise not develop. Protoplasts may be fused together in an electric field,  
25 and viable recombinant cells regenerated in tissue culture. In another technique, embryonic cells from an interspecific cross that would be otherwise sterile are treated with colchicine, and plants are regenerated from this treated plant material.

More recently, molecular biology has added to the range of plant breeding techniques.  
30 Molecular markers or DNA fingerprinting are able to map thousands of genes simultaneously. Large populations of plants can therefore be screened more efficiently and without necessarily having to grow plants to maturity or to rely on visual identification of traits.

Another process involves making homozygous plants from a heterozygous parent that has all of the desirable traits. A problem with the diploid condition is that the process of homologous recombination during meiosis disrupts what may be desirable linkage between alleles/traits on the same chromosome. Conventional breeding takes about six  
5 generations of inbreeding to create homozygosity. By creating and crossing double haploids of chosen parental plants, homozygosity is achieved in one step, and the process of homologous recombination, which would otherwise be disruptive of trait linkages, is avoided; the outcome, however, is unpredictable and genotype-dependent.

- 10 There is also use of genetic modification, though there are restrictions in some parts of the world applying to commercial research and/or exploitation of this technology. A specific gene DNA sequence from one organism or plant can be introduced and expressed in the desired plant species or variety. For example, insect resistance is achieved by introducing a gene from *Bacillus thuringiensis* (Bt) encoding a protein that is toxic to some insects.  
15 Another example is herbicide resistance, e.g resistance to glyphosate, which can be achieved by introducing a glyphosate-resistant variant gene for 5-enolpyruvoyl-shikimate-3-phosphate synthetase (EPSPS).

In plants, most phenotypic variations, especially those of agronomic value such as drought  
20 resistance or growth rate, are continuously distributed and are often considered as quantitative traits. The genetic control of quantitative traits is often complex because of the large number of genes that are involved. Such traits can be very sensitive to environmental conditions. Because crop yield is known to be a quantitative trait, high accuracy and speed of identifying loci, genes and markers associated with quantitative  
25 traits are mandatory. Relevant loci of quantitative traits are localised by two basic approaches, linkage mapping and association mapping, based on the use of genetic maps and statistical analysis. Regions of the genome identified by linkage mapping are relatively narrow, but can still contain several hundred genes. Identification of genes underlying the quantitative trait loci requires positional cloning or direct tests of promising  
30 candidates. In contrast, association mapping checks directly the relationship between each polymorphism and the phenotypic trait variation in wild populations; here physical linkage and population structure are potential sources of false positives. Finally, one needs to confirm that an individual gene is responsible for the quantitative trait by using genetic or functional complementation.

35

Whole genome sequencing, targeting either the entire genome or genome-wide markers distributed at high density, is now being used in an acceleration of plant breeding programmes. For example, genotyping by sequencing (GBS) allows high throughput genotyping of a large number of SNP markers in a large number of individual plants. This is exemplified by genotyping of maize plants as described by Glaubitz, J. C., *et al* (2014) *"TASSEL\_GBS: A High Capacity Genotyping by Sequencing Analysis Pipeline"* PLoS One 9(2): e90346.

Importantly, plants exhibit not only genetic sequence variability, but also a non-sequence variability known as epigenetic variation. Historically, the phenomenon was observed and studied in cell lineages within organisms. Meiotically or mitotically heritable changes in gene expression were found to occur independently of any changes in DNA sequence. Proposed mechanisms of epigenetic inheritance were mainly derived from mitotic cell studies. An emerging area of study relates to potentially meiotically inherited epigenetic changes in plants, whereby environmental triggers may result in a phenotypic response and an associated epigenetic change that may get fixed and passed on to subsequent generations. Hirsch, S. *et al.* (2012) *"Epigenetic Variation, Inheritance, and Selection in Plant Populations"* Cold Spring Harbor Symposia on Quantitative Biology, Volume LXXVII: 97 – 104 is a review article which collates and summarises research work in this area, yet concludes that there is still controversy about the existence of environmentally induced transgenerational epigenetic inheritance.

Gutzat R., *et al.* (2012) *"Epigenetic responses to stress: triple defense?"* Current Opinion in Plant Biology 15: 568 – 573 is another review article that identifies the connection between stress factors for plants and epigenetic responses to those stresses, but is guarded about whether epigenetic responses to stress are adaptive traits. Also, Boyko, A. & Kovalchuk, I. (2011) *"Genome instability and epigenetic modification – heritable responses to environmental stress"* Current Opinion in Plant Biology 14: 260 – 266 discusses how changes in genome stability and epigenetically mediated changes in gene expression may contribute to plant adaptation to the environment. This review lists, across a range of plant species, examples of environmentally induced transgenerational epigenetic effects that include the appearance of new phenotypes in successive generations of stressed plants. Similarly to the articles mentioned above, the authors' conclusion is that more studies are needed.

35

Roux, F., *et al* (2011) "*Genome-Wide Epigenetic Perturbation Jump-Starts Patterns of Heritable Variation Found in Nature*" *Genetics* Vol 188, 1015-1017. This scientific paper reports on a study of *Arabidopsis* plants looking at DNA methylomes and comparing populations experimentally perturbed and those of natural populations. Alterations in DNA methylation were found to be heritable and there was surprising similarity in heritability patterns between experimental and natural populations of plants.

Stroud, H., *et al* (2013) "*Plants regenerated from tissue culture contain stable epigenome changes in rice*" *eLIFE* (2013) 2:e00354. DOI: 10.7554/eLife.00354. This scientific paper reports on how DNA methylation in rice is lost when subjecting plant material to tissue culture. Rice plants regenerated from tissue culture were found to have lost epigenetic character.

Cortijo, S., *et al* (2014) "*Mapping the epigenetic basis of complex traits*" *Science* Vol 343 1145 – 8 describes the analysis of a population of isogenic *Arabidopsis* lines that segregate experimentally induced DNA methylation changes at hundreds of regions across the genome. Several differentially methylated regions (DMRs) were found to be epigenetic quantitative trait loci (QTL(epi)), and were reproducible and susceptible to artificial selection.

Kooke, R., *et al* (2015) "*Epigenetic Basis of Morphological Variation and Phenotypic Plasticity in Arabidopsis thaliana*" Published online before print February 2015, doi: <http://dx.doi.org/10.1105/tpc.114.133025>. This study on *Arabidopsis* finds extensive heritable epigenetic variation in plant growth and morphology in neutral and saline conditions. The variation in plasticity is associated significantly with certain genomic regions in which the *ddm1-2* inherited epigenotypes caused an increased sensitivity to environmental changes. Many QTLs for morphology and plasticity overlap. The authors suggest epigenetics contributes substantially to variation in plant growth, morphology, and plasticity, especially under stress conditions.

Heterosis is a most important aspect of plant breeding. It circumscribes the often observed phenotypic superiority of a hybrid plant compared to its genetically distinct parents with respect to traits such as biomass, growth rate and yield. The phenomenon has been exploited successfully for many years for many plant species, but the molecular basis of heterosis remains elusive. He, G *et al* (2013) "*Epigenetic Variations in Plant*

*Hybrids and Their Potential Roles in Heterosis*" Journal of Genetics and Genomics 40: 205 – 210 is a review article which postulates relationships between DNA methylation, microRNAs (miRNAs), small interfering RNAs (siRNAs) and histone modifications on the one side and heterosis on the other. Comparative analyses of parents and hybrids suggest that there may be some associations between changed chromatin states and gene activity in hybrids, and that this may equate with heterosis. However, the authors clearly state that the available evidence is well short of that needed to show epigenetic variation as the source of heterosis, and that no particular molecular mechanism is proven.

Heterosis was studied at the single-base-pair resolution level of DNA methylomes in *Arabidopsis thaliana*, reported in Shen, H. *et al.*, (2012) "*Genome-Wide Analysis of DNA Methylation and Gene Expression Changes in Two Arabidopsis Ecotypes and Their Reciprocal Hybrids*" The Plant Cell 24: 875 – 892. DNA methylation of parental lines was compared to that in the respective hybrids. Both hybrids displayed increased methylation compared to the parents, especially in transposon regions, and 77 different genes were associated with methylation changes. The growth vigour of the F1 hybrids was compromised by treatment with a DNA-demethylating agent, suggesting that genome-wide remodelling of DNA methylation may play a role in heterosis.

Plant improvement through breeding programmes can involve microropagation of particular hybrids, genetically modified plants or elite plants using explant, embryo, tissue or callus culture from which plants are regenerated and to provide a multiplicity of clones. All kinds of plant tissues can be used as explants in micropropagation, including stem tips, anthers, petals, pollen, for example. A problem with plant regeneration from cultured explant, tissues, callus or cells is that somaclonal variation may occur amongst the regenerated plants. This can be unpredictable and uncontrollable and can lead to loss of desired traits in regenerated plants.

More generally, in agriculture and horticulture, micropropagation is often used to generate multiplicities of new plants which are clones of a single plant. Advantageously, micropropagation can be used to multiply plants which are sterile or do not produce economically viable amounts of seed. There are some plant species where seed is recalcitrant and cannot be stored successfully; again micropropagation is a practical alternative. Some plant species respond well to micropropagation in that the vegetative progeny are more robust compared to equivalent seeds or cuttings. Cloned plants

produced via micropropagation, despite being genetically identical to the parent plant, are still susceptible to loss of desired traits and this is attributed to what is called somaclonal variation.

- 5 One of the most striking examples of plant tissue culture-induced variation is the ‘mantled’ somaclonal variation of oil palm. This variation affects the formation of floral organs in both male and female flowers in ~5% of the regenerants obtained through somatic embryogenesis, but its occurrence and severity are highly variable between and among clonal progenies. Ong-Abdullah M., et al. (2015) *Nature* vol 525: 533 – 537 describes the  
10 specific epigenetic basis of the “mantled” variant which is the loss of *Karma* transposon methylation. The *Karma* transposon was previously found in rice to be involved in epigenetic change due to its hypomethylation arising in cultured cells and regenerated plants (see Komatsu *et al.* (2003) *Cell* vol 15 1934 – 1944). However, the scientific correlation of *Karma* transposon methylation and somaclonal variation offers no new  
15 practical methods of cloning oil palm or any other plant, such that somaclonal variation is reduced or eliminated in the resulting cloned plants.

Somaclonal variation arising in plants produced via micropropagation is a therefore significant problem in many plant species where cloned plants must be genetically and  
20 phenotypically identical to the originating parent plant.

At least three research groups have described and studied certain plant embryonic transcription factors, their roles in plant embryo growth and development, and how these can be used to induce somatic embryogenesis. Kőszegi, D. *et al.* (2011) “*Members of*  
25 *the RKD transcription factor family induce an egg cell-like gene expression program*” *The Plant Journal* 67: 280 – 291 isolated two RWP-RK domain-containing (RKD) factors from wheat, where they are preferentially expressed in egg cells. The *Arabidopsis* genome has five of these *RKD* genes and these were studied in a more detailed functional analysis. Two of these, *AtRKD1* and *AtRKD2*, were preferentially expressed in the egg cell. The  
30 *AtRKD4* gene was expressed mainly in tissues containing reproductive organs, but ectopic expression of this gene produced no discernible phenotype. Transient expression of an *AtRKD4*-GFP fusion in protoplasts showed localisation of the protein in the nucleus.

In Jeong, S., *et al.* (2011) “*The RWP-RK Factor GROUNDED Promotes Embryonic*  
35 *Polarity by Facilitating YODA MAP Kinase Signalling*” *Current Biology* 21: 1 – 9, the

function of the *GROUNDED* gene (*GRD*), also known as *RKD4* (see above), is explained as being that of a transcription factor which promotes the elongation of the zygote and the development of its basal daughter cell into the suspensor.

- 5 Waki T *et al* (2011) *Current Biology* 21, 1277 - 1281 reports on observational experiments that describe and then explain how the gene *RKD4* controls and affects gene expression in early plant development. The gene is one of the *RWP-RK* genes in *Arabidopsis*. Two mutant alleles of the *RKD4* gene, *rkd4-1* and *rkd4-2* were initially found to be associated with germination defects, whereby the seed germinated but the roots did not form properly.
- 10 The spatiotemporal expression of *RKD4* was investigated using a two-component reporter construct in which the *RKD4* promoter drives a GAL4:VP16 (GV) transcriptional activator of GFP. Expression of the *RKD4*:GFP fusion in this two-component system completely rescued the *rkd4-1* mutant. Based on sequence similarity, the *RWP-RK* proteins and therefore *RKD4* is suggested to encode a transcription factor and is hypothesised to be a
- 15 regulator of early embryogenesis. In a dexamethasone-inducible, *RKD4*-overexpressing transgenic *Arabidopsis* line, the ectopic *RKD4* expression resulted in upregulation of a number of genes, some of which were identified as being early embryo specific. Longer induction of *RKD4* overexpression was found to trigger somatic embryogenesis. *RKD4* is therefore proposed to directly or indirectly promote expression of genes needed for
- 20 initiating the patterning process in the zygote and early embryo.

- WO2007/073221 (Instytut Hodowli i Aklimatyzacji Roslin (Plant Breeding and Acclimatization Institute)) relates to *in vitro* culture of plant materials, where phenotypic and/or genetic variation arises as a consequence of the culturing process; the
- 25 phenomenon of so-called somaclonal variation. Some of this is thought to be due to genetic changes and some due to epigenetic changes. Disclosed is a method of quantitative and qualitative genetic fingerprinting of induced variability in plants, e.g. double haploid barley regenerated from *in vitro* microspores. The disclosed method estimates the respective percentages of sequence-related and methylation-related
- 30 variability stably passed on to plant progeny. The particular genetic fingerprinting method is that of Amplified Fragment Length Polymorphism (AFLP). Selected primer pairs are used to discriminate presence and absence of methylation at the cleavage sites. By following and comparing the fingerprints of plant material having undergone different paths in the *in vitro* culturing and regeneration process, some of the nature and extent of stable
- 35 somaclonal variability can be described.

**BRIEF SUMMARY OF THE DISCLOSURE**

5 The inventors have discovered that by transforming somatic plant tissues or cells to express zygotic or embryonic transcription factors, and then regenerating plants from those transformed tissues or cells, the resulting plants retain substantially or entirely the epigenetic characteristics of the originally transformed parent plant cells. Further, the inventors have found that the epigenetic characteristics of these plants are substantially or entirely stably and heritably maintained.

10

In essence, the inventors have learned that by reprogramming somatic plant cells from different tissues or exposed to different conditions into a zygotic or early embryonic state, these cells can then be regenerated into fertile plants that retain the epigenetic characteristics of the somatic cells prior to the reprogramming.

15

The inventors therefore provide methods, materials and a system for generating epigenetically uniform plant clones. The epigenetics of these clones are stable and can, in relevant and desired species or varieties, are heritable via sexual reproduction. .

20 Accordingly, the present invention provides a method for generating stable, epigenetic plant clones, comprising:

- a. introducing into a parent plant or plant material an expression construct that comprises a nucleic acid sequence encoding a zygotic and/or an embryonic transcription factor;
- 25 b. controlling the time of expression of the transcription factor in the plant or plant material for a period of time; then
- c. growing transformed plant material in culture without expressing the transcription factor;
- d. identifying a progeny plant initial, plant embryo or plantlet; optionally growing the
- 30 plant initial, plant embryo or plantlet into a progeny plant; and
- e. testing a sample of the progeny material for at least one epigenetic characteristic which is also present in the parent plant.

Where the progeny material, which optionally may be a plant initial, plant embryo, plantlet

35 or plant (including a mature plant) is tested and has the at least one epigenetic

characteristic of the parent plant, then this provides cloned plant material which retains the desired phenotypic character, in that an undesirable somaclonal variant is avoided. Plant material which does not test positively is optionally discarded. Plant material which tests positively for the desired epigenetic character of the parent plant is thereby identifiable as  
5 the stable plant clone of the invention.

Advantageously, the testing of the transformed plant material for epigenetic characteristic may be carried out at the earliest stage of regeneration or onward propagation, meaning that labour, time and cost may be saved in a process of cloning plants for whatever  
10 purpose, whether multiplication of elite material, simple multiplication of material for further use or multiplication of material for selection in a process of plant improvement/breeding.

In instances where parent plant material is not already been characterised in terms of epigenetic character, meaning that the epigenetic character of the parent plant is unknown or uncertain, then a sample of the parent plant may be tested for the at least one  
15 epigenetic characteristic so as to allow for identification of at least one epigenetic characteristic in the progeny plant which is also present in the parent plant.

In preferred aspects, the method of the invention involves testing for a multiplicity of epigenetic characteristics, including specific epigenetic features, all of which are found in  
20 both progeny and parent plants.

The clonal progeny of the methods of the invention are preferably genotypically the same as the parent plant.  
25

In preferred methods of the invention, the plant initial, plant embryo of plantlet is grown into a mature progeny plant before the testing of the sample for at least one epigenetic characteristic which is also present in the parent plant.

Advantageously, the method of the invention may be used for the micropropagation of plants, whether in connection with a program of plant improvement including breeding, or whether simply the multiplication by cloning of agricultural, silvicultural, viticultural or horticultural species.  
30

In essence the invention harnesses the micropropagation of plants from shoot material of parent plants which has been discovered by the inventors to demonstrate the least somaclonal variability on regeneration, together with a screening out and discarding of regenerated plant material which is not epigenetically sufficiently close or identical to the parent plant from which shoot material was taken. The screening can be for one or more particular defined epigenetic features that gives rise to a particular somaclonal variant or trait, e.g. hypomethylation of *Karma* transposon in oil palm leading to the economically undesirable mantle variant.

Alternatively, the epigenetic screening can be comprehensive, whereby any epigenetic difference between regenerated plant and parent material can be identified so that optionally only identical regenerant plants are retained; i.e. epigenetically different plants may be discarded.

The introduction of expression constructs is preferably by way of transformation. Additionally or alternatively, expression constructs may be inducible expression constructs to permit controlling of the time of expression of the transcription factor.

The stability of an epigenetic plant variety produced as a result of the method of the invention is such that the measurable traits of the progeny plant may be transmissible via reproductive processes that can be natural or a consequence of human technical intervention. For example, when the reproductive process is a natural one it may be by selfing or by crossing with another plant, so that they are passed on into at least the next generation of plants without substantial diminution or loss, qualitatively and/or quantitatively. The epigenetic traits that are passed on may be quantitative, observable and/or measurable, and/or they may be based on molecular markers, and/or on gene expression profiling. The stability may be such that they persist from not just the first generation, but preferably to the second, third, fourth or fifth or more generations.

The epigenetic basis of a phenotype, i.e. traits, of the plant may be ascertained from methylation analysis, optionally on a whole-genome profiling basis. Additionally or alternatively, the epigenetic character of a plant may be defined on another basis such as histone modification (chromatin remodelling), e.g. via the likes of histone methylation, acetylation, phosphorylation, ubiquitination, glycosylation, ADP-ribosylation, sumoylation, deamination and proline isomerization. Additionally or alternatively a phenotype may be

characterised based on observation, counting and or measurement of plant traits as will be well known to a person of average skill in the art, e.g. biomass, yield, height, drought tolerance, saline tolerance, flower colour, etc.

5 The expression of the transcription factor by the inducible expression construct occurs during a period of time sufficient to achieve a reprogramming of the cells of the parental plant to a zygotic or embryonic stage, but which does not significantly disrupt or alter the epigenetic characteristics nor the traits or phenotype of the plant governed by the epigenetics of the genome. Whilst not wishing to be bound by any particular theory, the  
10 inventors believe that a genetic reprogramming of the plant cell back to a zygotic or early embryonic state resets the genetic developmental clock, but does not adjust or substantially alter the sequence or the epigenetic character of the plant tissue or cell genome. The period of time of expression of the transcription factor may be empirically determined, but can be a period measured in hours and/or days.

15

If a period of hours, this may be from 1 to 24 hours, e.g. 2 to 24 hours; 3 to 24 hours; 4 to 24 hours; 5 to 24 hours; 6 to 24 hours; 7 to 24 hours; 8 to 24 hours; 9 to 24 hours; 10 to 24 hours; 11 to 24 hours; 12 to 24 hours; 13 to 24 hours; 14 to 24 hours; 15 to 24 hours; 16 to 24 hours; 17 to 24 hours; 18 to 24 hours; 19 to 24 hours; 20 to 24 hours; 21 to 24 hours;  
20 22 to 24 hours; or 23 to 24 hours. If a period of days, then the number of days may be in the range 1 to 12 days; optionally 1 to 11 days; 1 to 10 days, 1 to 9 days, 1 to 8 days; 1 to 7 days; 1 to 6 days; 1 to 5 days; 1 to 4 days; 1 to 3 days; or 1 to 2 days.

The period may be a combination of days and hours, wherein the number of days is as  
25 defined above and is combined with a number of hours as defined above.

A person of average skill will understand that for functionality, the expression construct comprises necessary genetic elements needed to achieve expression of the transcription factor in the transformed cell for the period of time. A suitable promoter is required which  
30 is under a control, directly or indirectly, of an exogenous factor.

In some methods of the invention, parent plant tissue including individual cells are transformed in the whole plant context and then at some stage after transformation, transformed tissues and/or cells are isolated from the parent plant. The step of isolating

the tissue and/or cells may take place before, during or after expression of the transcription factor for the period of time.

5 The introduction of the expression construct into the parental plant or plant material is preferably by way of a transformation of plant cells. Transformation vectors of use in the invention are well known to a person of skill in the art. Particularly well known and preferred are *Agrobacterium* binary Ti vectors, for example. Other methods of transformation may be used to introduce the expression construct into the parental plant or plant material, either alternatively or in addition to the above and to each other, including  
10 ballistics, polyethylene glycol treatment or microinjection.

The transformation of the tissue and/or plant cells with the expression construct may be a transient transformation, for a period of time sufficient to allow inducible expression of the zygotic and/or embryonic transcription factor.

15

In other methods of the invention, organ or tissue and/or cells are isolated from the parent plant prior to transformation with the expression construct.

The expression construct may be an inducible expression system, preferably wherein the induction is selected from alcohol, tetracycline, steroid, metal or a pathogenesis related  
20 protein. For example, the system may be AlcR/AlcA (ethanol inducible); GR fusions, GVG, and pOp/LhGR (dexamethasone inducible); XVE/OlexA ( $\beta$ -estradiol inducible); or heat shock induction. The expression of the transcription factor is thereby inducible for the period of time. In a particularly preferred embodiment, a two-component dexamethasone  
25 inducible system is used.

Transformed plant material, such as organs, tissue or cells, are preferably contacted with the chemical inducer for the period of time, following which the tissue or cells are transferred to a regeneration medium lacking the chemical inducer.

30

In the preferred methods of the invention, prior to step (b) a whole parent plant from step (a) is grown to reproductive maturity, selfed and then grown on to set seed, wherein the plant material of step (b) is the or a seed. In this context a seed is therefore a plant material as referred to herein.

35

In the preferred embodiments, the transcription factor is selected from: RKD4, BBM, LEC2 and FUS3. This also represents a preferred order of effectiveness of transcription factors, with RKD4 being the most preferred. A combination of one or more of these transcription factors or any other zygotic or embryonic transcription factor may be used in accordance  
5 with the invention.

Before and/or after transformation, isolated tissue or cells may be sorted, optionally according to one or more of tissue or cell type, protein marker, methylation profile or gene expression profile. Cells can be sorted according to tissue type and then further by  
10 methylation profile and individual cells used in the cloning. Resulting plants will generally have the same methylation profile as the mother cell, although some variation will naturally occur within the plant.

Tissues and/or cells may be identified by using dyes or specific markers, particularly  
15 fluorescent markers such as GFP, ECFP, EGFP, EYFP, Venus YFP, DsRed, RFP1 or mCherry. A GAL4-UAS system may be used to achieve desired cell and/or tissue specific expression of the fluorescent marker. Various methods of fluorescence microscopy may be used, e.g. confocal laser scanning fluorescence microscopy, spinning disc confocal microscopy, multiphoton microscopy or widefield fluorescence microscopy. Tissues and or  
20 cells can be isolated by microdissection. In other methods, protoplasts may be prepared from the transformed plant cells and labelled with fluorescent labels to allow for fluorescence activated cell sorting (FACS) of the protoplasts.

The inventors have also discovered that when plants are regenerated from shoots or shoot  
25 tissue or cells, then the incidence of epigenetic variation leading to somaclonal change is less than when plants are regenerated from root material. Therefore, in preferred methods of the invention, tissue or cells are obtained from the shoot, any kind of shoot tissue or cells of the parental plant. Other shoot tissues such as leaf, flower, anther, pollen or fruit may be used as the starting material for the targeted expression of zygotic and/or  
30 embryonic transcription factors in accordance with the invention. Also included within the scope of the method of the invention are plant organs or explants, as well as tissues and/or cells. Reference to tissues and/or cells herein thereby includes any plant part where the transcription factor can be delivered and which can then be transiently caused to express the transcription factor, followed by growing the plant part or organ, tissue or

cell in culture so that plant regeneration by a process of somatic embryogenesis can take place.

5 Tissues or cells obtained from roots of parental plants may be used, but are less preferred because they tend to provide a greater range of stable and heritable epigenetic characteristics.

10 The epigenetic character of the plant may be manifested as a change in at least one quantitative trait, such as plant height, crop yield, disease resistance, flower colour and shape, i.e., traits that have continuous, unbroken quasi-normal distributions in a population.

15 The epigenetic character of the plant may be identifiable by its DNA methylation and histone modification profile (i.e. histone marks). This may be carried out in a variety of ways, whether specifically targeted to loci or regions of the genome, or as part of whole genome sequencing. For example, Illumina provides equipment and consumables and software for sequencing-based DNA methylation analysis such as whole-genome bisulfite sequencing (WGBS) or reduced representation bisulfite sequencing (RRBS).

20 The growing of transformed tissues and/or cells in culture employs well known culture media and methods. Often particular media compositions and particular protocols for growing plant tissues and/or cells in culture have already been established as providing optimal growth conditions. Sometimes these may be species-specific, but a person of average skill will be readily able to provide the necessary culture media and protocols from  
25 textbooks and the scientific literature. (See for example: "Plant Cell and Tissue Culture" A Tool in Biotechnology Basics and Application: Eds: Neumann, K. -H. & Imani, J. Springer (2009).)

30 The culture of plant tissues and/or cells may be in solid or in liquid media, as is most appropriate and desirable. Organs and explants may be grown on solid media. When cells are cultured they may form a callus culture.

Plant parts, organs, tissues or cells in accordance with the invention are grown in culture for a period during which expression of the transcription factor takes place, following which  
35 the culturing continues in the absence of such expression. If the expression is induced

chemically then plant material is grown in the absence of the inducer, which in practice may mean that the plant material may need to be transferred to a fresh culture medium.

Following cessation of transcription factor expression (usually corresponding to withdrawal  
5 of the inducer; i.e. a transfer to fresh medium), plant material is continued to be grown in culture for a sufficient time and under conditions conducive to plant regeneration, preferably via embryogenesis. So, in accordance with the invention, plant initials, embryos  
or plantlets that form from the cells and/or tissue being grown in culture are, ideally but not necessarily, visually identified. At some convenient stage, embryonic plant material,  
10 embryos or plantlets are then isolated from the originating tissue or cell culture and grown on to the stage of mature plant, being a plant which is sexually mature in that it has produced ovule(s) and/or pollen.

So, also in accordance with other aspects of the invention, a mature progeny plant from  
15 the above process may be used itself as a parent in a process of producing a second generation of progeny plants that have substantially the same epigenetic character as the first generation. An ovule of the mature progeny plant may be fertilized with pollen of the same plant and the plant grown on, so that it can set seed.

20 Seed of the above plant may be collected, germinated and then a resultant seedling is grown into a mature plant. This then is a third generation progeny plant.

The above process of selfing may be continued any number of times; optionally 2, 3, 4, 5  
or more times to produce fourth, fifth, sixth, etc. generation progeny plants. The stable  
25 and heritable (transgenerational) nature of the epigenetic character of the first generation progeny is determinable by a process involving sexual reproduction, preferably inbreeding, but most by selfing. Other ways of making subsequent generations of progeny plants starting with the first are available, including backcrossing with the first generation progeny. A mixture of selfing and backcrossing may be used. In such ways, individual  
30 plant lines or varieties of stable and heritable epigenetic character are provided.

The invention therefore includes cloned plants of defined genetic and epigenetic character obtained according to any method as hereinbefore described.

Also forming part of the invention is germplasm of such cloned plants. Germplasm may conveniently be in the form of seed, but also included is pollen or ovules.

The invention also includes any plant biomass or plant material of cloned plants of the invention, including leaves, fruit, seed, stems or woody parts. Therefore also included are any plant products involving some form of processing, for example, flour, meal, dried leaves, timber. Diagnostic testing of samples of such plant products where polynucleic acid remains present may permit identification of the particular plant species and epigenetic strain of that species.

Cloned plants of the invention having desired phenotypic traits are therefore useful to incorporate into a plant improvement or breeding program. The invention therefore includes a method of plant breeding comprising combining stable transgenerational epigenetic material of a first cloned parent plant with genetic material of a second parent plant. The second parent plant may also be a cloned plant in accordance with the invention, or it may be another plant differing in genotype and/or epigenetics.

The combining of genetic material may make use of naturally occurring sexual reproduction processes in plants, or it may involve artificial means, e.g. protoplast fusion.

Once a cloned plant in accordance with the invention is provided, this plant and its genetic material and germplasm may be incorporated into any classical or molecular breeding programme with the aim of producing further new varieties which may be themselves hybrids.

Accordingly the invention also provides *in vitro* methods of plant improvement comprising combining stable epigenetic genetic material of a first parental plant with genetic material of a second parental plant. In preferred aspect, the first parental plant, or the first and the second parental plant are produced according to methods as hereinbefore described.

Advantageously the invention helps in providing large numbers of stable epigenetic plants and multiplicities of lines of such plants, each the same or substantially the same as an originating parent plant, thereby assisting in speeding up the development of new plant varieties and hybrids by making an increased pool of such plant clones available more quickly for selection and crossing.

The invention also includes plants or plant cells, tissues, organs or parts, obtainable by any of the methods of the invention as described herein.

- 5 The invention further includes a plant-derived product obtained from any plant obtained or obtainable in accordance with any method of the invention, as described herein. For example, a processed plant product produced by a process of milling or grinding, e.g. flour or meal. Such plant products, whilst not possessing identifiable whole cells, may include sequenceable genetic material such that the particular plant strain or variety can be
- 10 determined. The presence of particular unique nucleotide sequence tags, if incorporated into the genome of the originating plants, will thereby allow for identification of origin and tracking of a processed plant product, as may be desired.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15

Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

- Figure 1: (A) shows a first method of regenerating *Arabidopsis* plants from dissected and
- 20 isolated tissues from transformed root and shoot. (B) shows a method of regenerating *Arabidopsis* plants from de-differentiated cell cultures.

Figure 2 is a heat map showing methylation differences found in leaf and root tissues of plants that have been regenerated from different cell types.

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Figure 3 is a diagram showing the result of a principal component analysis showing DNA methylation differences present in leaf and root tissues of plants regenerated from different cell types.

- 30 Figure 4 is a heat map of unsupervised cluster analysis of whole-genome gene expression of plants regenerated from root (RO) and leaf (LO) tissues.

Figure 5 is a bar chart of data from phenotypic analysis of parents and regenerants subjected to biotic stress.

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Figure 6 is a pictorial representation of the structure of the T-DNA in pTA7002 Ti-plasmid containing *AtRKD4* gene.

Figure 7 shows protocorms of *P. "Sogo Vivien"* orchid. (A) Non transformant plants without DEX induction (positive control). (B) Non transformant plant with DEX induction (negative control). (C) Transformant candidates with somatic embryo formation in the posterior part, followed by the formation of shoot. (D) Transformant candidate plants with more than one bud in the anterior part. (E) Transformant candidate plants with the formation of roots at the new shoots. (F) Transformant candidate plant with the formation of somatic embryos and shoots. Bar 1 mm.

Figure 8 shows the detection of *AtRKD4* gene Insertion into *P. "Sogo Vivien"* orchid genomes. An agarose gel showing the PCR products resulting from using specific primers of *AtRKD4*, which yields 268 bp DNA fragments from each transformant genome. (M) 1000 bp DNA ladder marker. Lanes 1-14: orchid transformant candidates. (K+) Positive control (non-transformed orchid without DEX) (K-) Negative control (non-transformed orchids induced with DEX).

## DETAILED DESCRIPTION

The inventors have used short read sequencing of bisulfite treated genomic DNA and computational analysis to show how a range of epigenetic variation exists between cells in a plant. Also that when an individual plant cell of particular epigenetic character is reprogrammed back to a zygotic or an embryonic developmental stage and regenerated into a plant, that this epigenetic character is stably maintained through more than one generation. Also, qualitatively, the differences in gene expression due to differing methylation, particularly compared to the parent plant, concern genes associated with plant growth regulation or pathogen resistance. Such variation in gene expression has also been shown to manifest itself in advantageous growth of regenerant variant plants in response to particular biotic stress compared to parent controls.

So the inventors have discovered that epigenetic variation in the form of methylation patterns as between somatic cells of plant tissues can be fixed upon cell culture and regeneration of plants, and the individual variants remain fixed into subsequent generations of plants when derived from a single cell.

Using transgenic plant lines carrying zygotic transcription factors, the inventors tested a range of transcription factors, including RKD4, BBM, LEC2 and FUS3. An established two-component inducible expression system was used, activated upon exposure to the chemical inducer dexamethasone. Once somatic plant cells are reprogrammed into undifferentiated initials by the effect of expression of the transcription factor, new plants are recovered simply by growing the transformed and induced cells or tissue in solid culture media, in the absence of the inducer. New plant materials regenerated from this are then grown over three generations (selfing) and assessed in their genetic and epigenetic structure. The analysis shows that whilst no changes in gene sequence take place, the regenerated plants have extensive epigenetic variation. What is more, this epigenetic variation is found to be stable over several generations. A technical advantage of this is that by being able to identify particular epigenetic lines or varieties, the inventors provide the possibility of being able to provide clones which are substantially unaltered in terms of epigenetic character and therefore phenotype, compared to the parent plant from which the clone is derived.

Collectively, the approach now allows the identification and growing of clonal progeny obtained from parent plant material by micropropagation and where the clones are (i) substantially the same; or (ii) identical; or (iii) different, in terms of epigenetic character from the parent material.

The methods and epigenetic plant variants of the invention have wide applicability for plant breeding efforts and biotechnology research. What is now possible is to rapidly generate large numbers of cloned plants with minimal or no somaclonal variation. Stable epigenetic clones can be provided to improve resistance to pathogens and environmental stress, increase yield and enable the production of new metabolic compounds. Another useful application of the invention is in the propagation of recalcitrant plant material (e.g. endangered species or species difficult to reproduce through standard seed propagation).

The methods of the invention are applicable to any plant from any plant taxonomic group, including angiosperms or gymnosperms. Amongst the angiosperms the invention is applicable to monocots and dicots.

The invention is applicable to any angiosperm plant species, whether monocot or dicot.

Preferably, plants which may be subject to the methods and uses of the present invention are plants which when propagated vegetatively, whether directly or as part of a breeding program, may suffer somaclonal variation leading to loss of desired traits in the propagated plants. An important commercial example of a plant which is propagated vegetatively is oil palm (*Elaeis* sp., particularly *E. guineensis* or *E. oleifera*).

Generally, the plants to which the present invention relates include all agricultural, horticultural, silvicultural and viticultural species. Agricultural species include, but are not limited to, corn (*Zea mays*), Brassica sp. (e.g. *B. napus*, *B. rapa*, *B. juncea*), particularly those Brassica species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g. pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea utilis*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, duckweed (*Lemna*), barley, vegetables, ornamentals, and conifers.

Vegetables within the scope of the invention include tomatoes (*Lycopersicon esculentum*), lettuce (e.g. *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*), Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga ultilane*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and  
 5 balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).

Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek,  
 10 soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc. Legumes include, but are not limited to, *Arachis*, e.g, peanuts, *Vicia*, e.g, crown vetch, hairy vetch, adzuki bean, mung bean, and chickpea, *Lupinus*, e.g, lupine, trifolium, *Phaseolus*, e.g, common bean and lima bean, *Pisum*, e.g, field bean, *Melilotus*, e.g, clover, *Medicago*, e.g, alfalfa, *Lotus*, e.g, trefoil, *Lens*, e.g, lentil, and false indigo.

15 Preferred forage and turf grass for use in the methods of the invention include alfalfa, orchard grass, tall fescue, perennial ryegrass, creeping bent grass. Also; redtop, switchgrass, reed canary grass, prairie cordgrass, tropical grasses, *Brachypodium distachyon*, and *Miscanthus*.

20 Other plants within the scope of the invention include acacia, aneth, artichoke, arugula, blackberry, canola, cilantro, clementines, escarole, eucalyptus, fennel, grapefruit, honey dew, jicama, kiwifruit, lemon, lime, mushroom, nut, okra, orange, parsley, persimmon, plantain, pomegranate, poplar, willow, silver maple, black locust, sycamore, sweetgum,  
 25 eucalyptus, radiata pine, radicchio, Southern pine, sweetgum, tangerine, triticale, vine, yams, apple, pear, quince, cherry, apricot, melon, hemp, buckwheat, grape, raspberry, chenopodium, blueberry, nectarine, peach, plum, strawberry, watermelon, eggplant, pepper, cauliflower, Brassica, e.g. broccoli, cabbage, utilian sprouts, onion, carrot, leek, beet, broad bean, celery, radish, pumpkin, endive, gourd, garlic, snapbean, spinach,  
 30 squash, turnip, utilane, and zucchini.

Ornamental plants within the scope of the invention include species of *Impatiens*, *Begonia*, *Pelargonium*, *Viola*, *Cyclamen*, *Verbena*, *Vinca*, *Tagetes*, *Primula*, *Saint Paulia*, *Ageratum*, *Amaranthus*, *Antihirrhinum*, *Aquilegia*, *Cineraria*, clover, *Cosmos*, cowpea, *Dahlia*, *Datura*,

*Delphinium*, *Gerbera*, *Gladiolus*, *Gloxinia*, *Hippeastrum*, *Mesembryanthemum*, *Salpiglossos*, and *Zinnia*.

Another important area where the invention can be applied is in ornamental horticultural plants, for example, orchids of any species, or *Nepenthes* sp.

## EXAMPLES

### Example 1: Preparation of *Arabidopsis* plants transformed to express transcription factor RKD4 on dexamethasone induction

*Arabidopsis thaliana* (Col-0 genetic background) plants were transformed to have a two-component dexamethasone inducible expression vector to provide for transient expression of RKD4 transcription factor upon induction with dexamethasone.

15

#### ***Molecular cloning***

An RKD4 inducible expression construct was made by chemically synthesizing a DNA fragment encoding for the RKD4 protein (At5g53040). Restriction enzyme sequences were included at both ends of the synthetic fragment to facilitate cloning into a pOp6-OCS cassette (see Craft *et al* (2005) Plant J. 41(6): 899 – 918). This fragment was later subcloned into a binary vector containing a CaMV 35s-LhGR-N construct (see Samalova *et al.*, (2005) Plant J. 41(6): 919 – 935). The final vector, pBIN-LhGR>>RKD4 was transformed into *Agrobacterium tumefaciens* (strain GV3101) and this new strain was used for plant transformation by floral dipping (see Clough and Bent, (1998) Plant J. 16(6): 735 - 743).

25

Seeds from flowers exposed to *Agrobacterium* were sown on MS media containing 50ug/ml Kanamycin to identify transformed plants. To identify lines that were suitable for our experiments, we sowed progenies of transformed plants in MS media. Five days after germination plantlets were transferred to media containing 20μM dexamethasone, which acts a transcriptional chemical inducer in this system. Lines suitable for controlled RKD4 expression were selected based on the formation of embryogenic structures in the root tips. Selected lines were allowed to self-pollinate over two generations and homozygous lines were identified by selection on Kanamycin MS media.

35

Example 2: Induction of transformed *Arabidopsis* plant material and regeneration of epigenetic variant progeny plants

***Plant materials and growth condition***

- 5    *A. thaliana* seeds were sterilized with 5% bleach for 5 minutes, then washed five times with sterile double distilled water. A few drops of 0.1% agarose was added after the final washing. The seeds were then sowed into MS media containing 2% of sucrose and 8 g/L of plant agar. The seeds were stratified for four days at 4°C to break the dormancy. After four days, the plates were moved into control environment with following conditions; 10 kLux light for 16 h, dark for 8 h; 22°C/18°C day/night temperature; 50% / 60% relative humidity at day/night. The plants were allowed to grow for six days and then transferred to soil until they reached maturity.

***Somatic embryo regeneration***

- 15    *Arabidopsis* plants were grown for six days in MS media, and plantlets were moved into MS media containing 20µM dexamethasone. After six days, plants were moved to MS media (without dexamethasone) and developing embryos were dissected from somatic embryos forming in roots (named RO) or leaves (named LO) by micromanipulation and transferred to MS media until roots and leaves developed fully. Finally, plantlets were 20 moved to soil and grown until maturity to collect dry seeds.

Example 3: Comparison of genetic methylation features between parent and regenerant plants

- 25    Leaves and root samples were collected from regenerated plants and their progeny in order to assess changes in DNA methylation and gene expression profiles. The molecular analysis was carried out using Next Generation sequencing and data were analysed using computational methods. This strategy allowed precise identification of stable changes in DNA methylation and gene expression. In addition, plants were grown in order to conduct 30 phenotypic analyses. Changes in flowering time were assessed by growing plants in short and long day conditions. Further, enhanced resistance/susceptibility to three different pathogens was assessed: *Botrytis cinerea*, *Hyaloperonospora parasitica* and *Pseudomonas syringae* pv. tomato DC30000 (Pst DC3000).

- 35    ***Nucleic acid extraction***

Leaf samples were collected from five individual five-weeks-old plants. The leaf samples were collected in 1.5 mL Eppendorf tubes and flash-frozen in liquid nitrogen and stored under -80°C until further use. The samples were grounded in a mortar with the addition of liquid nitrogen to prevent sample from thawing. After the samples were completely pulverized, the genomic DNA was extracted using Qiagen Plant DNeasy kit (Qiagen). The quality and quantity of genomic DNA was checked using agarose gel electrophoresis and NanoDrop (Thermo Scientific).

The total RNA sample is extracted from leaf samples using Qiagen Plant RNeasy kit (Qiagen) following the manufacturing manuals. The quality and quantity of total RNA will be analyzed using agarose gel electrophoresis and NanoDrop (Thermo Scientific).

#### ***Methylation analysis - library preparation***

Preparation of DNA libraries for bisulphite sequencing was adapted from Lister *et al.* (2008) Cell 133: 1 – 14. Libraries were constructed starting from 100ng of purified genomic DNA using the Illumina TruSeq DNA Sample Prep kit (San Diego, CA, USA) according to the manufacturer's instructions with the following modifications. After adapter ligation, non-methylated cytosine residues were converted to uracil using the EpiTect Plus DNA Bisulfite kit (Qiagen) according to the manufacturer's guidelines. For higher conversion efficiency the bisulphite incubation was doubled. Library enrichment was performed with the Kapa Hifi+ Uracil Hotstart Polymerase (PeqLab) and 14 PCR cycles.

#### ***Methylation analysis - bisulphite sequencing:***

Bisulphite sequencing was performed on an Illumina HiSeq2000 instrument. Bisulphite-converted libraries were sequenced with 2 × 101-bp paired-end reads and a 7-bp index read. For bisulphite sequencing, conventional *A. thaliana* DNA genomic libraries were analysed in control lanes. For image analysis and base calling, we used the Illumina OLB software version 1.8.

#### ***Methylation analysis - processing and alignment of bisulphite-treated reads:***

The method adapted from Becker *et al.*, (2011) was used. The SHORE pipeline was used to trim and filter the reads. Reads with more than 2 bases in the first 12 positions with quality score less than 3 were deleted. The reads with quality values equal to or greater than 5 were trimmed to the right-most occurrence of two adjacent bases. All trimmed reads shorter than 40 bp were deleted. All the high quality reads were aligned to TAIR9

(<http://www.arabidopsis.org>) by using a modified version of the mapping tool GenomeMapper.

5 All alignments with the least amount of mismatches for each read were reported by GenomeMapper. However, only reads mapping uniquely to a single position were used for this study. Furthermore, all but one read were removed from further analysis if their 5' ends aligned to the same genomic position, to account for amplification biases. A paired-end correction method was used to discard repetitive reads by comparing the distance between reads and their partner to the average distance between all read pairs. Reads  
10 with abnormal distances were removed if there was at least one other alignment of this read in a concordant distance to its partner. Finally, read counts on all cytosine sites were obtained with SHORE. The 'scoring matrix approach' of SHORE assigns a score to each site by testing against different sequence and alignment related features. For comparisons across lines, cytosines were accepted if at most one intermediate penalty on its score was  
15 applicable to at least one strain (score  $\geq 32$ ). In this case, the threshold for the other strains was lowered, accepting at most one high penalty (score  $\geq 15$ ). In this way, information from other strains is used to assess sites from the focal strain under the assumption of mostly conserved methylation patterns, allowing the analysis of additional sites. The methylation statistics on each single strain assumed a quality score of 25 or higher, which  
20 means no more than two intermediate penalties.

***Methylation analysis - determination of methylated sites:***

To minimize false-positive methylation detection, an independent binomial model to the relative proportions of converted and unconverted reads that cover cytosines in the  
25 chloroplasts were fitted. The binomial rate of false-positive methylation from the maximum likelihood was estimated separately for each library and for different bins of total read coverage:

To account for the variability in error rates in the downstream analyses, specific error  
30 models for each strain and for read-coverage bins of multiples of fivefold, yielding error rates between 0.2% and 5.0% were used. For coverage bins with too few sites for robust statistical estimation ( $< 50$ ), the false methylation rate from the closest sufficiently populated coverage bin was imputed. Given the estimated rates for false methylation, a genome-wide test for significant methylation of cytosines was carried out. For each site,  
35 the P value under the background model was calculated. Storey's method, an extension

of the Benjamini–Hochberg stepdown procedure, to assess genome-wide significance using q values was used, and a joint false discovery rate (FDR) of 5% was used.

***Methylation analysis - identification of differentially methylated positions:***

- 5 From total cytosines obtained which fulfill high-quality reads criteria in each strain, one that has significant methylation in at least one strain was chosen. Sites with statistically significant methylation differences were identified with Fisher's exact test. P values from individual tests per site were combined into single P values via conservative Bonferroni correction. Genome-wide FDRs were then estimated using Storey's method. To limit  
10 false-positive DMPs, FDR of 10% was used. The pairwise tests were performed for each RO generated plants against LO generated plants.

- The same strategy was applied to identify DMPs that differed either between the RO-plants and LO-plants, or between RO/LO-plants. Count data from replicates were  
15 combined for each site, followed by pairwise Fisher's exact tests between all combinations of strains. P values for at least one differential pair were estimated using a Bonferroni correction, followed by Storey's method to assess genome-wide significance.

***Methylation analysis - identification of differentially methylated regions:***

- 20 Differentially methylated regions (DMRs) were identified as described in Hagmann *et al.*, (2015) PLOS Genetics, Volume 11, Issue 1, e1004920. First, contiguous stretches of methylation, herein referred to as methylated regions (MRs) were detected using a Hidden-Markov-Model based approach in every individual sample (Hagmann *et al.*, (2015)). For differential analysis of MRs, all LO samples were grouped as replicates. MR  
25 segments were then tested for differential methylation between these groups.

***Expression analysis:***

- All RNA samples were prepared with the TruSeq RNA sample prep kit (Illumina, SanDiego, CA), according to the manufacturer's instructions. Samples were sequenced  
30 on a HiSeq2000 at a depth of 20-30 million reads per sample. Transcript abundance was calculated by mapping reads to the combined transcript models of the Arabidopsis reference genome using bwa. Reads were filtered to allow for only uniquely mapped reads. Differential expression was calculated using the DESeq package in R (v3.0.1).

Short read sequencing of bisulphite treated genomic DNA and computational analysis identified regions of the genome that differed between parents and regenerants of Example 2. Figure 2 is an unsupervised cluster analysis of differentially methylated regions (DMRs). The analysis shows that for the two organs tested, leaves of root-cell-regenerants (RO) contained DNA methylation features typically found in roots, whereas leaf-cell-regenerants (LO) displayed methylation features typically only found in leaves. The progenies of RO and LO plants were analysed and it was found that these DNA methylation features were stably inherited over two generations. These results show that the method of reprogramming somatic cells with zygotic or early stage transcription factors can be used to engineer a range of plants, each with specific epigenetic character, which is stably inherited by the offspring over subsequent generations.

#### Example 4: Determination of the extent of epigenetic variation between regenerant plants

The level of novel epigenetic variation generated was assessed in the regenerant plants of Example 2. The DNA methylation profiles of each individual regenerant were analysed by a principal components analysis (Figure 3), which revealed that whilst RO regenerants displayed highly diverse DNA methylation patterns, LO regenerants were largely uniform. These results indicate that roots contain a highly complex source of epigenetic cellular variation and that our method is able to fix and stabilize such epigenetic variation in whole plants.

#### Example 5: Correlating epigenetic variation to changes in gene expression

Given that individual regenerant plants have unique epigenetic character, it was verified whether these plants would have patterns of gene expression not found in the parents. RNA was extracted from leaves of parents and regenerants, and a genome-wide gene expression analysis was carried out using Next Generation Sequencing. Figure 4 shows the unsupervised cluster analysis of whole-genome expression of plants regenerated from root (RO) and leaf (LO) tissues. Leaves of RO regenerants displayed a novel pattern of gene expression not present in the parents. A computational analysis of the gene expression data found that >400 genes were differentially expressed in leaves of RO regenerants. Further analysis revealed that 73% of these genes are known to be activated in response to pathogens and also regulate plant growth.

Example 6: Analysis of difference in gene expression between parents and regenerants in response to stress

5 Changes in gene expression between parents and regenerants are associated with growth phenotypes in response to biotic stress. Parents and regenerants were grown under controlled growth conditions and both groups were subjected to infection with three different pathogens.

10 ***Plant pathogen infection assays:***

*Arabidopsis* plants were grown from 2 weeks under short day conditions (8h light/16h dark). Rosette leaves were infected with an inoculum of three different pathogens: *Botrytis cinerea*, *Hyaloperonospora parasitica* and *Pseudomonas syringae* pv. tomato DC30000 (Pst DC3000). Resistance to infection was determined after 5 days by microscopic  
15 investigation. We determined in infected leaves the size of necrosis formed after *Botrytis* infection, bacterial growth on leaf surfaces by *Pseudomonas* growth and number of spores on leaf surfaces after *Hyaloperonospora* infection.

Plants were collected two weeks after infection and dry weights measured. Figure 5  
20 shows the results in which regenerants displayed phenotypes not previously observed in the parents. In particular, RO regenerants grew more vigorously than LO regenerants and parental lines. These new phenotypes were stable in subsequent generations. These results show how the methods of the invention generate novel beneficial phenotypic traits that can be further selected for in plant improvement programs.

25 Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of them mean “including but not limited to”, and they are not intended to (and do not) exclude other moieties, additives, components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the  
30 plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in  
35 conjunction with a particular aspect, embodiment or example of the invention are to be

understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

The reader's attention is directed to all papers and documents which are filed concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

Example 7: Cloning oil palm to produce plants which are not "mantle" variants

Young stems of selected *Elaeis guineensis* plants are excised and dissected to provide explants. The stem explants are washed and then transformed with *Agrobacterium tumefaciens* (strain gv3101) containing the vector pBIN-LhGR>>RKD4 as described in Example 1. The explants are transformed by dipping (see Clough and Bent, (1998) Plant J. 16(6): 735 – 743).

A sample of each explant is obtained and subjected to methylation analysis of the genetic material it contains, according to methods described in Ong-Abdullah M. et al. (2015) Nature vol 525: 533 – 537, including whole genome bisulphite sequencing and measurement of the methylation state of a LINE retrotransposon related to rice *Karma*, in the intron of the homeotic gene DEFICIENS. Each explant is determined as to whether or not the LINE retrotransposon is hypomethylated or not.

The transformed explants are then cultured on MS agar without hormones until embryo structures are identifiable. The embryos are moved onto MS media containing 20µM dexamethasone. After six days dexamethasone exposure, embryos are moved to MS media (without dexamethasone) and developing embryos dissected from somatic embryos

forming in leaves by micromanipulation and transferred to MS media until roots and leaves develop fully. Lastly, plantlets are moved to soil and grown on.

Each cloned plant which has been grown on is sampled and subjected to whole genome  
5 bisulphite sequencing and measurement of the methylation state of the LINE retrotransposon. Plants which are hypomethylated at the LINE retrotransposon are rejected.

Plants which are densely methylated near the Karma splice site are retained as not being  
10 somaclonal “mantle” variants. These are grown on in readiness for planting for palm oil production.

Example 8: Preparation of *Phalaenopsis* plants transformed to express transcription factor RKD4 under dexamethasone induction.

15

*Phalaenopsis* “Sogo Vivien” is a type of *Phalaenopsis* orchid hybrids that is a preferred ornamental potted plant because of its mini-size, numerous flowers and inflorescences. Somatic embryogenesis is a micropropagation technique which can be used to produce uniform seedlings in large numbers.

20

***Plant materials, bacterial strain and plasmid vector***

Three month old *P.* “Sogo Vivien” orchid pods were obtained from self-pollinated *P.* “Sogo Vivien” flowers. Seeds were sown on new phalaenopsis basal medium (NP) (Islam *et al.* 1998) enriched with 150 ml of coconut water, incubated at 25°C under continuous white  
25 light. For gene transfer, 16 days after sowing (das) protocorms were used as transformation targets.

*Agrobacterium tumefaciens* strain EHA105 carrying pTA7002 Ti-plasmid with T-DNA 35S::GAL::AtRKD4::GR was used for genetic transformation (Figure 6).

30

The genetic transformation was followed by a glucocorticoid-inducible system with Dexamethasone (DEX) at gradual concentrations (10, 20 or 40) µM for a duration of 1 or 2 weeks.

35 ***Transformation and regeneration of protocorms***

Before transformation, the 16 das-protocorms were cultured on solid Callus Induction Medium (CIM) which consisted of NP medium + 2 mg.L<sup>-1</sup> 2,4-D + 100 g.L<sup>-1</sup> tomato extract. This treatment was carried out 3 days before the protocorms were inoculated with *A. tumefaciens*. A suspension culture of *Agrobacterium* in LB liquid medium + 100 mg.mL<sup>-1</sup> kanamycin + 30 mg.L<sup>-1</sup> rifampicin was cultured for 24 hours, which was then supplemented with 40 mL of NP liquid medium + 50 mg.L<sup>-1</sup> Acetosyringone (AS) + 40 µL tween. The mixture was then vortexed and the resulting mixture was used for inoculation. Protocorms were soaked with *Agrobacterium* suspension culture for an hour, dried, cultured on CIM medium and then co-cultivated for 3 days.

10

After 3 days, protocorms were washed with 5 ml mixture of NP medium without sugar + 25 mg.L<sup>-1</sup> meropenem and shaken overnight at a speed of 100 rpm. *Agrobacterium* elimination process was carried out for 3 days. On day 3, protocorms were cultured on solid NP medium + 25 mg.L<sup>-1</sup> meropenem for 3 weeks for the recovery process. Protocorms were further sub-cultured in selection medium (NP + 25 mg.L<sup>-1</sup> meropenem + 10 mg.L<sup>-1</sup> hygromycin) for 2 weeks.

15

In this study, *P. "Sogo Vivien"* orchid protocorms that were 16 das germinated protocorms in NP medium with the addition of coconut water, then sub-cultured on solid CIM medium (NP + 2 mg.L<sup>-1</sup> 2,4-D + 100 g.L<sup>-1</sup> tomato extract + 25 mg.L<sup>-1</sup> acetosyringone). This treatment was carried out 3 days before protocorms were inoculated with *A. tumefaciens*. Co-cultivation began with protocorm immersion in the culture of *Agrobacterium* and NP liquid medium at a ratio of 1:4 according to the method conducted by Semiarti *et al.* (2007) for genetic transformation of the *GFP* gene into *P. amabilis* orchid. Soaking protocorm was carried out for 1 hour, then dried for 1 hour on sterile filter paper and then cultured on CIM for 3 days to optimize the integration of the T-DNA from *Agrobacterium* into the protocorms. To eliminate the growth of *Agrobacterium*, protocorms were washed using a liquid NP medium without sugar + 25 mg.L<sup>-1</sup> meropenem antibiotic over 3 days. Protocorms were then cultured on NP medium + 25 mg.L<sup>-1</sup> meropenem. After 3 weeks, selection was performed with the addition of 10 mg.L<sup>-1</sup> hygromycin for 2 weeks. The percentage of survived protocorms after the selection process was calculated, based on the number of green protocorms, as being 0.08% (Table 1).

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25

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**Table 1. Percentage of survived *P. "Sogo Vivien"* protocorms in selection medium**

|                  | Number of co-cultivated protocorms | Number of green protocorms | Percentage of survival protocorms |
|------------------|------------------------------------|----------------------------|-----------------------------------|
| Non-transformant | 404                                | 0                          | 0 %                               |
| Transformant     | 350                                | 30                         | 0.08 %                            |

### ***Somatic embryo induction***

Green protocorms that were maintained in the selection medium were sub-cultured in induction medium (NP + 25 mg.L<sup>-1</sup> meropenem + 10 mg.L<sup>-1</sup> hygromycin + (10, 20 or 40) 5 µM dexamethasone (DEX). Half of the protocorms were induced for 1 week, and the rest were induced for 2 weeks. Once induced, protocorms were transferred into NP medium without antibiotics and inducer. The growth of the protocorms was observed every week and the number of somatic embryos were counted.

Confirmation of the successful insertion and integration of *AtRKD* genes into the candidate 10 transformants' plant genome was conducted by direct PCR with *AtRKD4* specific primers. The results showed that the induction of somatic embryogenesis through genetic transformation using the *AtRKD4* gene in *35S::GAL4::AtRKD4::GR* construction generated 47 shoots from 14 transformant protocorm candidates. The highest number of transformed 15 protocorms were obtained from the 10 µM DEX induction for 1 week treatment (80%). The highest number of somatic embryos and shoots were obtained from the 40 µM DEX induction for 2 weeks treatment (13 shoots). Confirmation of the successful insertion of *AtRKD* gene into the candidate transformants by PCR showed that all of the transformant 20 protocorms contained 268 bp *AtRKD4* DNA fragment. The efficiency of *AtRKD4* transformation on *P. "Sogo Vivien"* orchid was 0.04%. These results suggest that the *AtRKD4* gene can be used as a new tool for micropropagation of orchids through somatic embryogenesis.

### ***Shoot formation***

After selection, surviving protocorms were transferred onto induction medium (NP + 25 25 mg.L<sup>-1</sup> meropenem + 10 mg.L<sup>-1</sup> hygromycin + DEX) for 1 or 2 weeks. Concentrations of DEX varied from 10, 20 to 40 µM. After induction by DEX, the protocorms were sub-cultured into basal NP medium without any plant growth regulator. As a positive control, non-induced protocorms were used, while negative controls were non-transformed protocorms that were induced by DEX for 2 weeks.

30

The results showed that somatic embryos formed at all concentrations of DEX treatment. Formation of somatic embryos continued with the formation of shoots. The time of first shoot formation varied between the different treatments. Some of the candidate protocorm transformants failed to survive after the induction process, some underwent necrosis and death (Table 2).

**Table 2. Number of protocorms were induced with DEX and shoot formation on protocorm of transformant candidates**

| Duration of DEX incubation | DEX concentration ( $\mu\text{M}$ ) | Number of induced protocorms | Number of survived protocorms 12 weeks after induced | First shoot formation | Total shoots |
|----------------------------|-------------------------------------|------------------------------|------------------------------------------------------|-----------------------|--------------|
| 1 week                     | 10                                  | 5                            | 4                                                    | Week-2 and 4          | 9            |
|                            | 20                                  | 5                            | 2                                                    | Week-4                | 4            |
|                            | 40                                  | 5                            | 2                                                    | Week-2                | 6            |
| 2 week                     | 10                                  | 5                            | 2                                                    | Week-6                | 11           |
|                            | 20                                  | 5                            | 2                                                    | Week-8                | 4            |
|                            | 40                                  | 5                            | 2                                                    | Week-8                | 13           |
| Total                      |                                     | 30                           | 14                                                   |                       | 47           |

There were 4 surviving protocorms at week 12 on the 10  $\mu\text{M}$  DEX induction treatment for 1 week. The 4 protocorm forming somatic embryos continued to grow shoots, 2 of the protocorm forming shoots developed at week 2 post-induction, and the other 2 protocorm forming shoots developed at week 4 post-induction. A total of 9 shoots were formed from the 4 protocorms at week 12 post-induction. Whereas on the other treatments, the number of surviving protocorms was up to 2.

The first buds on the 20 and 40  $\mu\text{M}$  DEX induction treatment for 1 week formed during week 4 and week 2 respectively. Formation of buds on the DEX induction treatment for 2 weeks occurred more slowly, during week 6 (DEX treatment 10  $\mu\text{M}$ ) and week 8 (DEX treatment of 20 and 40  $\mu\text{M}$ ). However, a high number of shoots formed in protocorms incubated with 40  $\mu\text{M}$  DEX induction for 2 weeks (total 13 shoots).

The positive control plants (non-transformant) protocorm grew normally, the apical buds appear at the anterior end of protocorm and developed into shoots (Figure 7A). However, the growth of transformed candidates showed the formation of more than one bud on one protocorm in the anterior (upper) (Figure 7D). In addition to the shoot, globular somatic embryos also formed (Figure 7F). In some protocorms, shoot growth began with the formation of somatic embryos in the posterior (bottom), followed by the formation of new shoots (Figure 7C). Root formation did not occur in transformed candidates, until week 12 post-DEX induction. The same phenomenon was observed in non-transformed protocorms with DEX induction (Figure 7B). However, root growth occurred in the form of new shoots (Figure 7E). When compared with the positive control protocorm, root growth was very slow, the new roots began to appear at week 8 post-DEX induction.

#### ***Detection of transformants by PCR***

To confirm the successful insertion of *AtRKD4* gene into the genome of candidate transformants, *direct* PCR was performed by amplifying the gene using *AtRKD4* specific primers (*AtRKD4\_RT\_Fw* 5'-ACGACGGTCTCATTTCC AAC-3' and *AtRKD4\_RT\_Rv* 5'-CTCTTCCATTCCAACATTCTTGAG-3') with KAPA3G Plant PCR Kit (Kapa Biosystem). PCR was performed with pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, elongation at 72°C for 1 minute. The denaturation, annealing and elongation steps were repeated for a total of 40 cycles. This was followed by extension at 72°C for 5 minutes and then the reaction was held at 10°C for 30 minutes. PCR products were separated on 1.2% agarose gel, stained with EtBr and visualized with a UV-Transilluminator.

#### ***Molecular analyses***

Amplification of genomic DNA from 14 plant transformation candidates with *AtRKD4* specific primers showed that all plant transformants contained the gene that was inserted by the transgene. They were characterized by the existence of DNA bands with the sizes of 268 bp (Figure 8).

#### ***Transformation efficiency calculation***

Transformation efficiency was calculated based on the number of protocorms grown on antibiotic containing medium for selection, and comparing to the number of positive transformants detected by PCR.

**Efficiency of transformation**

Transformation efficiency was 0.04%, which was calculated based on the number of positive transformant plants carrying transgenes *AtRKD4* (14 protocorms) and comparing to the total number of protocorm infected with *A. tumefaciens* T-DNA carrying 35S::GAL4::*AtRKD4*::*GR* (350 protocorms).

**Conclusion**

*AtRKD4* gene transfer in *P. "Sogo Vivien"* orchid via agrobacterium mediated transformation was successfully performed. The efficiency of genetic transformation was 0.04%. The presence of the *AtRKD4* gene in *P. "Sogo Vivien"* orchids, led to a developmental phenotype, which was characterized by a change in form during somatic embryo formation and the presence of new shoots. Molecular analysis confirmed that the transformants contained *AtRKD4* DNA fragment with a length of 268 bp. Overall this resulted in the formation of 47 shoots from 14 plant transformants

## CLAIMS

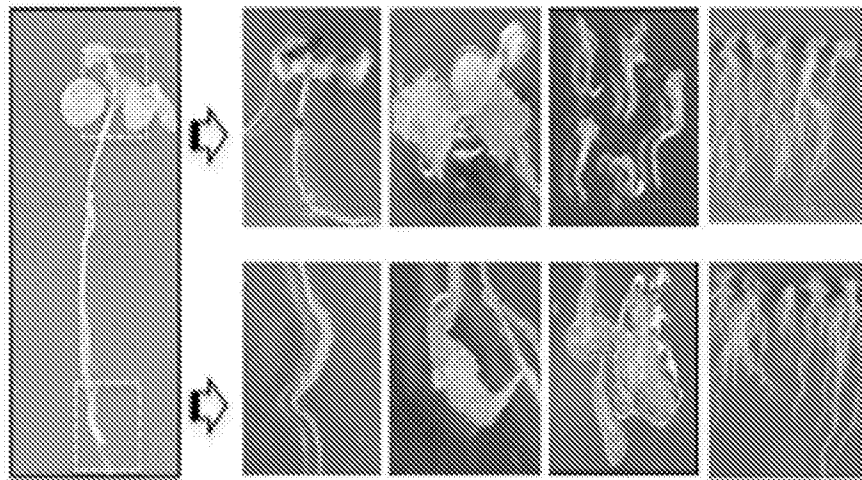
5

1. A method of making stable epigenetic plant clones from a parent plant, comprising:
  - a. introducing into a parent plant or plant material, an expression construct that comprises a nucleic acid sequence encoding a zygotic and/or an embryonic transcription factor;
  - 10 b. regulating the expression of the transcription factor in the plant or plant material for a period of time; then
  - c. growing transformed plant material in culture without expressing the transcription factor;
  - d. identifying a regenerant progeny plant initial, plant embryo or plantlet;
  - 15 e. optionally growing the progeny into a plant; and
  - e. obtaining a sample of the progeny material and testing this sample for at least one epigenetic characteristic which is also present in the parent plant.
2. A method as claimed in claim 1, wherein a sample of the parent plant is tested for  
20 at least one epigenetic characteristic.
3. A method as claimed in claim 1 or claim 2, wherein the plant initial, plant embryo or plantlet is grown into a progeny plant, optionally a mature plant, and then tested.
- 25 4. A method as claimed in claim 1 or claim 2, wherein the expression construct is introduced into the parental plant or plant material by a process of transformation; preferably *Agrobacterium*.
- 30 5. A method as claimed in any preceding claim, wherein the plant material is an explant, an organ, a tissue, a seed or cell; optionally wherein the plant material is isolated from the parent plant prior to transformation of that material with the expression construct.

6. A method as claimed in any of claims 1 to 3, wherein prior to step (b) a whole parent plant from step (a) is grown to reproductive maturity, selfed and then grown on to set seed, wherein the plant material of step (b) is the or a seed.
- 5 7. A method as claimed in any preceding claim, wherein the expression construct is an inducible expression construct; optionally one part of a two-part inducible expression system.
- 10 8. A method as claimed in any preceding claim, wherein expression of the transcription factor is inducible for the period of time.
- 15 9. A method as claimed in claim 7 or claim 8, wherein the expression is with a chemical; optionally wherein the chemical inducer is selected from alcohol, tetracycline, steroid, metal or pathogenesis related protein; preferably wherein the chemical is dexamethasone.
- 20 10. A method as claimed in claim 8 or claim 9, wherein following contacting transformed material with the chemical inducer for a period of time the tissue or cells are transferred to a regeneration medium lacking the chemical inducer.
- 25 11. A method as claimed in any preceding claim, wherein the transcription factor is selected from: RKD4, BBM, LEC2 and FUS3.
12. A method as claimed in any preceding claim, wherein before and/or after transformation isolated tissue or cells are sorted; optionally according to one or more of tissue or cell type, protein marker, methylation profile or gene expression profile.
- 30 13. A method as claimed in any preceding claim, wherein the tissue or cells are from the shoot of the parent plant.
14. A method as claimed in any preceding claim, wherein the epigenetic character is manifest as at least one quantitative trait.

15. A method as claimed in any preceding claim, wherein the epigenetic character is identifiable by DNA methylation profile; optionally by methylation state of at least one genetic element, e.g. transposon.
- 5 16. A method as claimed in any preceding claim, wherein an ovule of the progeny plant is fertilized with pollen of the same plant and the plant then grown on to set seed.
- 10 17. A method as claimed in claim 16, wherein a seed of the plant is collected, germinated and then the resultant seedling is grown into a mature plant.
- 15 18. A method as claimed in claim 16 and then claim 17; preferably wherein the step of claim 16 followed by the step of claim 17 is repeated a number of times; optionally 2, 3, 4, 5 or more times.
- 20 19. An *in vitro* method of plant improvement comprising combining stable epigenetic genetic material of a first parent plant with genetic material of a second parent plant.
- 25 20. A method of plant improvement as claimed in claim 18, wherein the first parent plant, or the first and the second parent plant are produced according to a method of any of claims 1 to 17.
21. Plants, plant cells, tissues, organs or parts, obtainable by a method of any preceding claim.
22. A plant-derived product obtained from a plant of claim 21.

A



B

Embryonic cell culture after controlled RKD4 expression

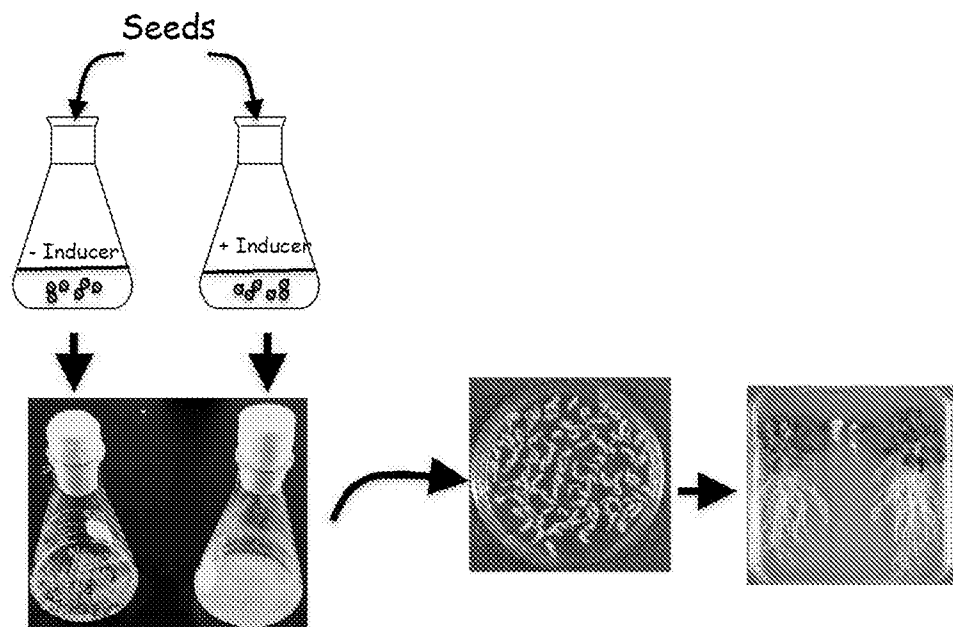
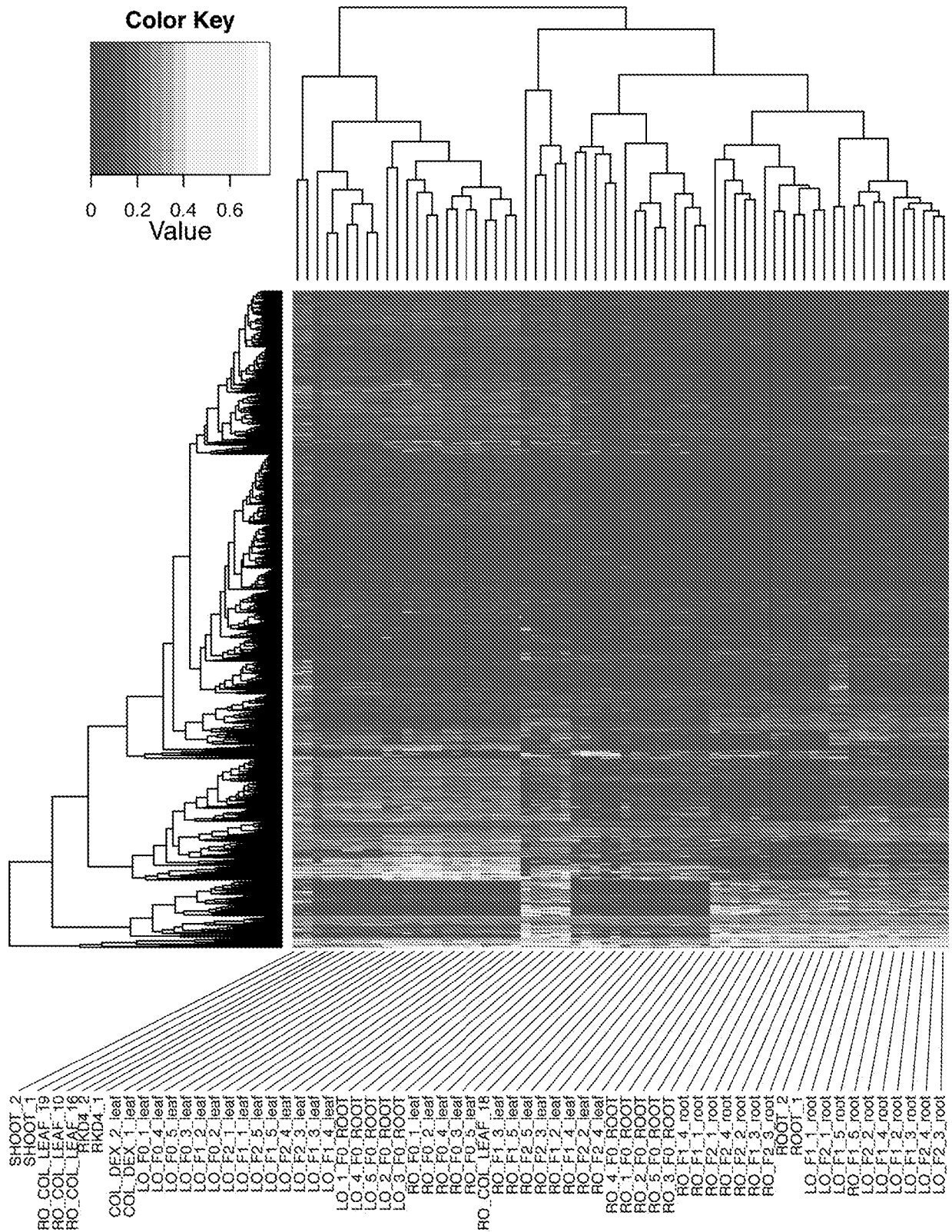


Figure 1

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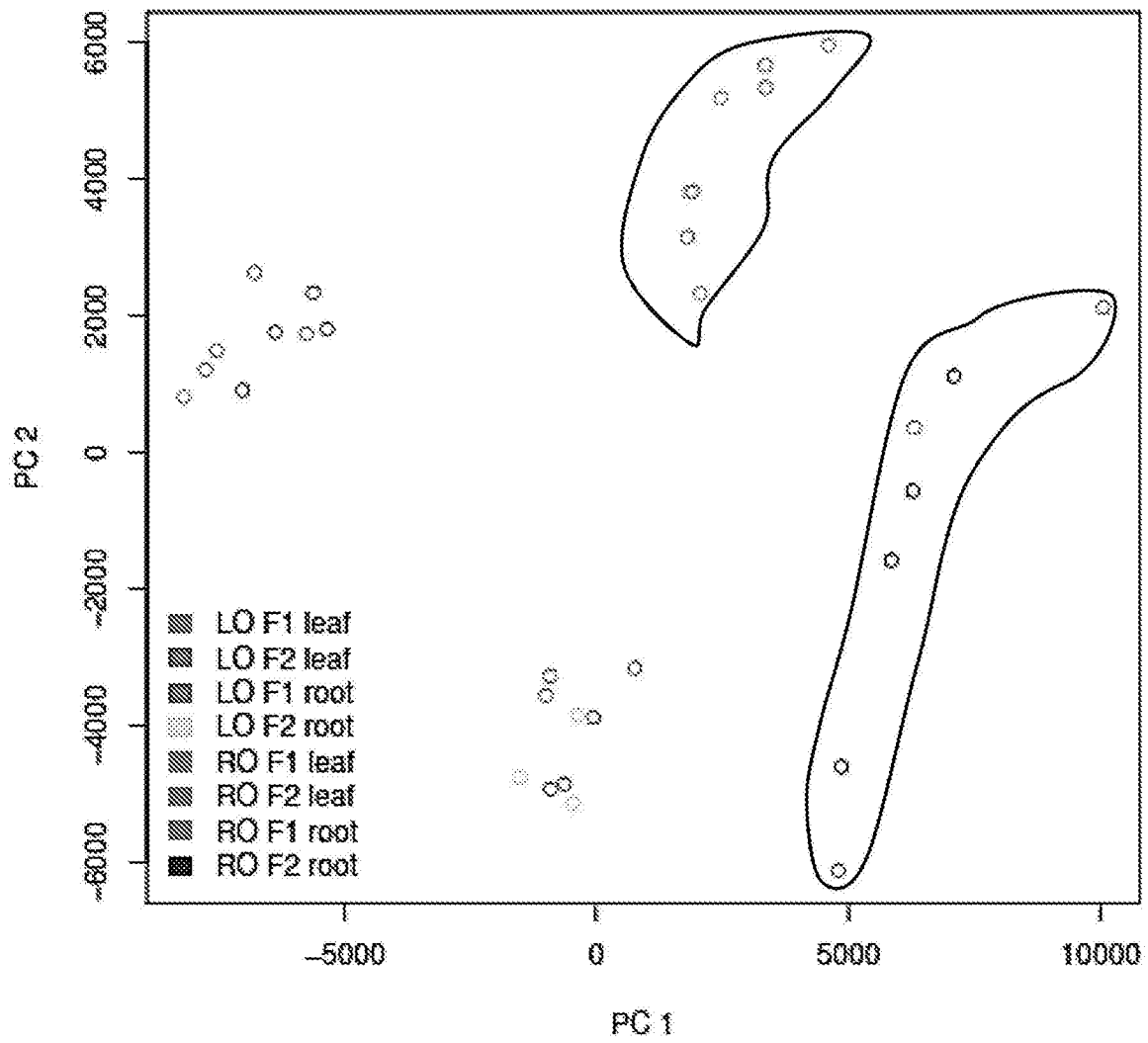


Fig. 3

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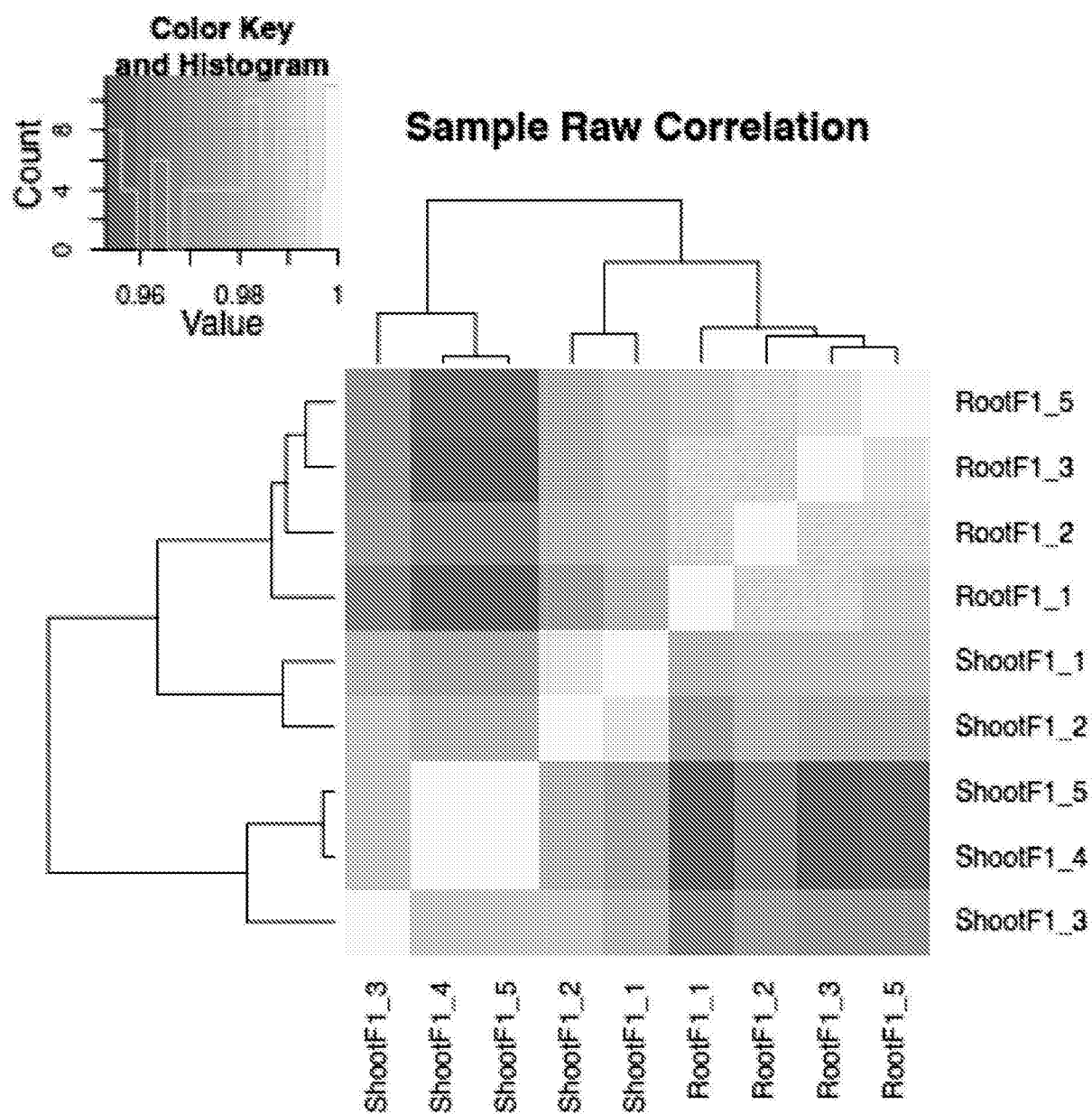


Fig. 4

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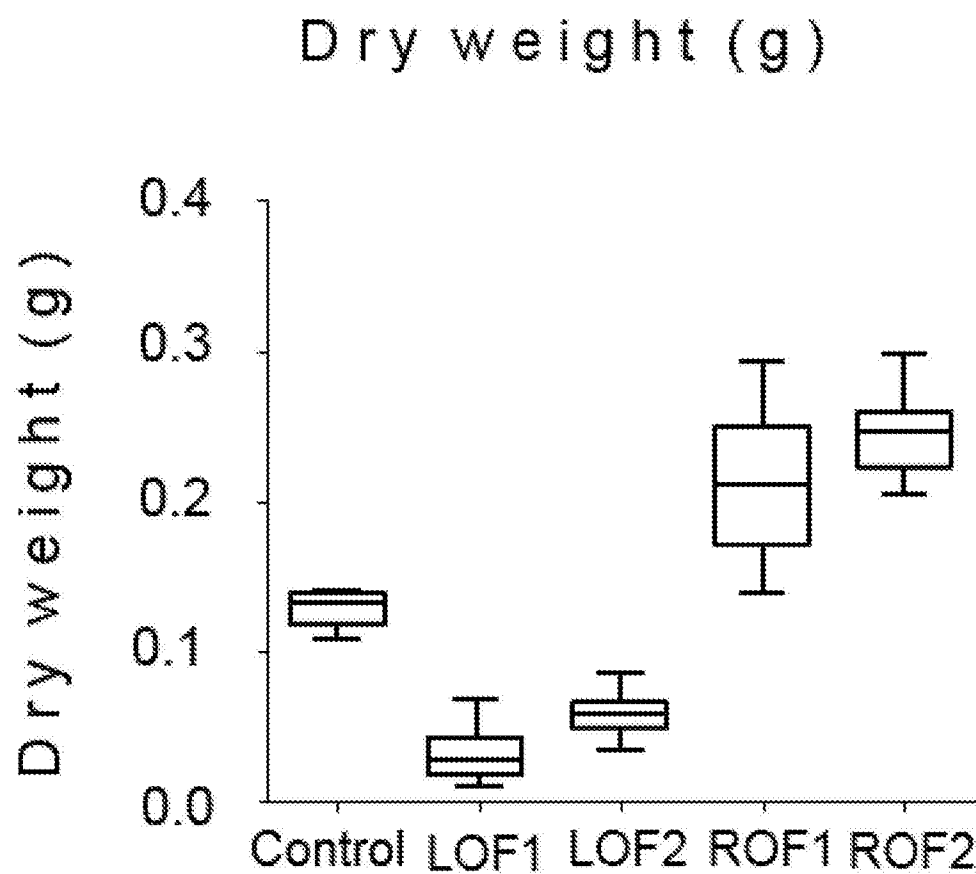


Fig. 5



Figure 6

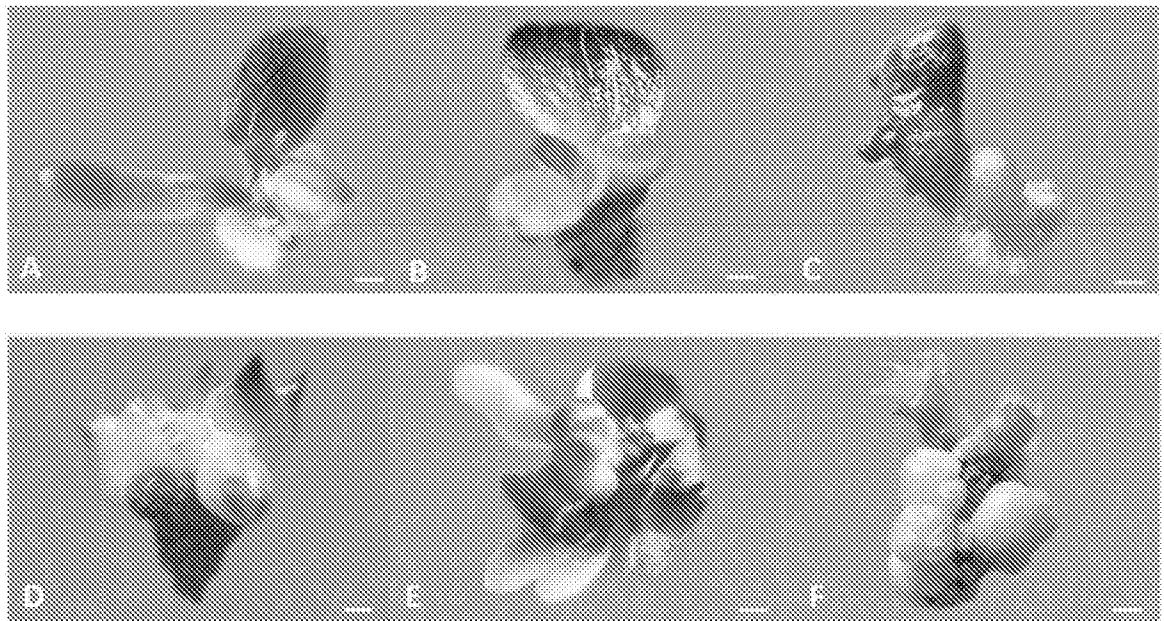


**Figure 7**

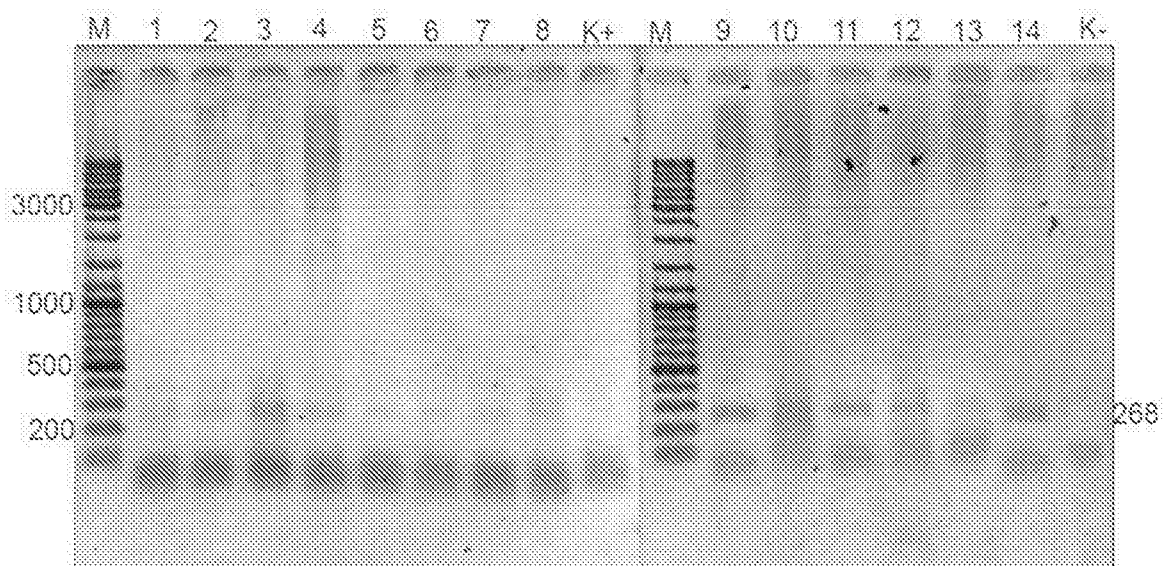


Figure 8

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB2017/050640

A. CLASSIFICATION OF SUBJECT MATTER  
INV. 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, EMBASE, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                       | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | M. IKEUCHI ET AL: "Plant Callus: Mechanisms of Induction and Repression", THE PLANT CELL, vol. 25, no. 9, 1 September 2013 (2013-09-01), pages 3159-3173, XP55271537, US | 1-10,<br>12-22        |
| Y         | ISSN: 1040-4651, DOI: 10.1105/tpc.113.116053<br>abstract<br>table 1<br>figure 4<br>page 10, column 2<br>-----<br>-/--                                                    | 11                    |



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

9 May 2017

Date of mailing of the international search report

18/05/2017

Name and mailing address of the ISA/

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Keller, Yves

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/GB2017/050640

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                       |                       |
|------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                    | Relevant to claim No. |
| X                                                    | TAKAMITSU WAKI ET AL: "TheRWP-RK Protein RKD4 Triggers Gene Expression and Pattern Formation in Early Embryogenesis", CURRENT BIOLOGY, CURRENT SCIENCE, GB, vol. 21, no. 15, 30 June 2011 (2011-06-30), pages 1277-1281, XP028254639, ISSN: 0960-9822, DOI: 10.1016/J.CUB.2011.07.001 [retrieved on 2011-07-06]                                       | 1-22                  |
| Y                                                    | abstract<br>page 1279, column 2, paragraph 4                                                                                                                                                                                                                                                                                                          | 1-22                  |
| X                                                    | A. SMERTENKO ET AL: "Somatic embryogenesis: life and death processes during apical-basal patterning", JOURNAL OF EXPERIMENTAL BOTANY, vol. 65, no. 5, 1 March 2014 (2014-03-01), pages 1343-1360, XP55271155, GB<br>ISSN: 0022-0957, DOI: 10.1093/jxb/eru005                                                                                          | 1-22                  |
| Y                                                    | page 8                                                                                                                                                                                                                                                                                                                                                | 1-22                  |
| A                                                    | Anonymous: "Plant Breeding and Biotechnology",<br>30 December 2013 (2013-12-30), XP55271172,<br>Retrieved from the Internet:<br>URL: <a href="http://www.plantbreedbio.org/journal/view.html?uid=79&amp;vmd=Full&amp;">http://www.plantbreedbio.org/journal/view.html?uid=79&amp;vmd=Full&amp;</a><br>[retrieved on 2016-05-09]<br>the whole document | 1-22                  |
| X,P                                                  | WO 2016/146552 A1 (UNIV OF WARWICK [GB]; MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WSS E) 22 September 2016 (2016-09-22)<br>the whole document                                                                                                                                                                                                        | 1-22                  |

## INTERNATIONAL SEARCH REPORT

### Information on patent family members

International application No

PCT/GB2017/050640

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| WO 2016146552                             | A1                  | 22-09-2016                 | NONE                |
| -----                                     |                     |                            |                     |