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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 January 2009 (08.01.2009)

PCT

(10) International Publication Number
WO 2009/003429 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/CZ2008/000075

(22) International Filing Date: 2 July 2008 (02.07.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PV 2007-454 4 July 2007 (04.07.2007) CZ
10-2008-0038610 25 April 2008 (25.04.2008) KR

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

WO 2009/003429 A2

(54) **Title:** METHOD OF REGULATION OF BIOMASS PRODUCTION IN PLANTS, DNA SEQUENCES AND METHOD OF PREPARATION THEREOF

(57) **Abstract:** The invention relates to a method of regulation of biomass production in plants, wherein the gene CKI1 or a homologue or an orthologue thereof is identified in the plant and the expression of the gene CKI1 or a homologue or an orthologue thereof or the activity of the gene product is modified. The identification of the homologue or the orthologue of the gene CKI1 in the plant species comprises the following steps: i) identification of the nucleotide sequence homology or amino acid sequence homology, ii) proving the histidine kinase activity of the gene product of the identified gene, iii) identification of the expression in vascular tissues of the respective plant species. Modification of the expression of the gene includes increasing or decreasing of the expression.

Method of regulation of biomass production in plants, DNA sequences and method of preparation thereof

Technical Field

The invention relates to a method of regulation of biomass production in plants by means of modification of activity of a gene or a gene product, to DNA sequences and a method of preparation thereof.

Background Art

A substantial dependence on petroleum products exists in the current EU economy as well as in the economy of other countries. This dependence is negative in its time limitation and in the necessity to search for other, alternative energy resources and material resources for chemical industry. Another disadvantage consists in the negative CO_2 balance, which contributes substantially to deterioration of the environment due to the so-called greenhouse effect. An important issue not only for the Czech economy and other EU-member countries economies is therefore the use of biotechnologies with an equal CO_2 balance as biomass sources in the concept of sustainable development economy.

The European Union has set the targets in increasing the use of renewable resources (RR) in two fundamental documents: „White paper" has set the fundamental target in increasing the share of RR in primary energy resources consumption in the whole European Union from 6 % in 1995 to 12 % in 2010 and the Directive 2001/77/EC determines the aim of the EU to increase the RR share in electric energy production from 13.9 % in 1997 to 22.1 % in 2010. Biomass should substantially contribute to fulfilling these aims, both waste biomass and biomass cultivated for this purpose. By assessing the overall potential of energy biomass in the Czech Republic it was determined that almost one half of the amount necessary for accomplishing the set objectives (8 % of electricity and 6 % of heating energy from the total consumption in the Czech Republic until 2010) must be obtained by cultivation of energy crops – the so-called residual biomass (wood and forest waste, haulm, etc.) is not sufficient for fulfilling this urgent requirement.

The most common method of increasing biomass production is the cultivation of the crops recommended for energetic use (e.g. white sweetclover, weedy mallows (*Malva vertillicata*), various species of high grass), whereas the most elaborated energy crop is fodder crop sorrel (*Rumex sp.*). The cultivation, however, requires due care, so that e.g. weeds or pests do not appear, and this method does not increase the plant biomass as such (Petříková V.: Energetické plodiny - nová šance pro zemědělce. *Biom.cz* [online]. 2004-04-21).

For increasing the biomass production in plants as such, various fertilizers are used, which should compensate for the consumed soil nutrients. Mineral fertilization, however, has a negative total effect on the economic efficiency of the agricultural production, at the same time it can endanger the environment and the high concentration of mineral nutrients in the soil solution does not even comply with the environmental requirements of the crops. The yields of the fertilized crops do not correspond to the invested resources and also the quality of the harvested crops gradually decreases. Although it was found that the reason for the decrease in efficiency of mineral fertilizers are not the elements themselves, but the disturbance of the ratio of mineral nutrients and organic substances, the efforts directed to the optimizing of the increase in efficiency of mineral fertilizers do not take into account the risks relating to the fertilizer use (Vrba V., Huleš L.: Humus - půda - rostlina (15) Minerální hnojiva. *Biom.cz* [online]. 2007-04-06).

The fastest method of preparation of novel productive and energy plants is undoubtedly the genetic modification of plants (Váňa J.: Nové cíle v energetickém využití biomasy a příprava high-technologií k jejich zabezpečování. *Biom.cz* [online]. 2001-11-29). There exists a whole range of methods for targeted intervention into the genetic information of plants. Among the most widely used methods belong the methods based on the natural ability of the bacteria *Agrobacterium tumefaciens* to insert part of its genetic information (so-called T-DNA) into the plant genome, while it is possible to modify them so that their ability of the transfer of genes into the plant genome is preserved but they transfer also other inserted genes. From other methods, direct „shooting" of genes into plant cells by means of an apparatus commonly designated as „gene gun", i.e. shooting of a microscopic particle of gold or wolfram

coated with the gene which has to be inserted into the plant genome, can be mentioned. At present, genes for the herbicide resistance of the plants, the genes for resistance to insect pests and the viral diseases resistance genes predominate among the genes used for the genetic modification of plants (Miloš Ondřej, Jaroslav Drobník: Transgenoze rostlin, Academia, Praha, 2002) – the first genetically modified plant entered the Czech Republic officially on the 14th May 2001, when genetically modified soybean resistant against the herbicide Roundup (soybean line GTS 40-3-2 and all progenies derived from this line by traditional cultivation methods) was approved for processing (not for cultivating). The genetic methods leading solely to the increase of the volume of biomass are rare, some of them were performed only for the underground parts of the plants.

The conductive tissues are the place of production of xylem cells, i.e. wood, wherein the lignification of the cell walls and the cellulose deposition occurs. These processes are critical for the production of exploitable biomass and can therefore have an economic impact.

The gene *CYTOKININ INDEPENDENT1* (*CKI1*) was identified in the year 1996 by activation mutagenesis as a dominant regulator of cytokinin response in *Arabidopsis thaliana* (Kakimoto T., Science *274*, 982-985 (1996)). An increased expression of the gene *CKI1* (*CKI1* under the control of a strong constitutive promoter, tetramer of enhancers of the promoter of tobacco mosaic virus for 35S RNA [CaMV 35S RNA promoter]) leads during cultivation on the growth medium without cytokinins to phenotypic features that are dependent on the presence of cytokinins in the cultivation medium (greening and shooting). Based on the similarity of the amino acid sequence of CKI1 with the sensor histidine kinases of bacteria, the function of CKI1 was predicted to be a cytokinin receptor. Nevertheless, further experiments did not show any cytokinin binding to CKI1. Later, *CKI1* homologues were identified, genes *AHK2*, *AHK3* and *AHK4/CRE1/WOL*, and their function as genuine receptors in cytokinin perception in plants was identified (Inoue et al., Nature *409*, 1060-1063 (2001); Yamada et al., Plant Cell Physiol *42*, 1017-1023 (2001)). In case of CKI1, its constitutive activity, independent of the presence or absence of cytokinins, was found, and therefore, the hypothesis was pronounced that it is not a genuine cytokinin receptor (Yamada et al., Plant Cell Physiol *42*, 1017-1023 (2001)). The role of the

gene *CKI1* in the development of female gametophyte was identified (Hejátko et al., Mol Genet Genomics *269*, 443-453 (2003); Pischke et al., Proc Natl Acad Sci U S A *99*, 15800-15805 (2002)) and it was also found that *CKI1* is expressed not only during the entire process of female gametophyte development, but also later in the sporophyte.

The expression of the *CKI1* homologues, genes *AHK2, AHK3* and *AHK4*, was identified in vascular tissues of *A. thaliana* and their function in the response to exogenous cytokinins, root growth, hypocotyl elongation, number of cells in leaves and size of the leaf in *A. thaliana* was proven (Higuchi et al., Proc Natl Acad Sci U S A *101*, 8821-8826 (2004); Nishimura et al., Plant Cell *16*, 1365-1377 (2004)). The influence of AHK2, AHK3 and AHK4 on the vascular tissues production in shoot is not known, while only the results of phenotype analysis in the vascular tissues of the root were published (Mahonen et al., Genes Dev *14*, 2938-2943 (2000); Scheres et al., Development *121*, 53-62 (1995)).

Considering that the vascular tissues and particularly the stem cells of vascular tissue (procambium and cambium, respectively) are place of biomass production in plants, changes in the capacity of plant vascular tissues to produce biomass seems to be one of the most efficient ways to modulate biomass production in plants. Since cytokinin signalling is considered to be essential for formation and differentiation of vascular tissue in the root, it might imply a possibility to develop a technique to increase the production of plant biomass through growth of plant via regulating cytokinin-associated genes in the aerial part of the plants (i.e. shoots), too.

Disclosure of the Invention

Object of the present invention is a method of regulation of biomass production in a plant, wherein the gene *CKI1* (Sequence ID No. 1) or a homologue or an orthologue thereof is identified in the plant and the expression of the gene *CKI1* or the homologue or the orthologue thereof or the activity of the gene product (the encoded protein) is modified. A homologue is a gene sequentially similar to the gene *CKI1* – similarity on the level of nucleotide sequences -, or a gene, the product of which is similar to the CKI1 protein, – similarity on the level of amino acid sequence -, which had developed

in the past from the same sequence. An orthologue is a gene which has the same or a similar function in a different species, i.e. in this case a gene the function of which in another plant species corresponds to the function of the gene *CKI1* in *A. thaliana*.

The identification of homologues or orthologues is carried out on the basis of the following criteria:

i) sequence homology on the level of nucleotides or amino acids, containing particularly the conserved regions of sensor histidine kinases. When the genome of the concerned species is already known, the homologous genes can be identified by comparing these sequences with the genome database of this species and by the identification of the homologous sequence by means of a suitable computer software, e.g. BLAST. When the genome of the concerned species is not known so far, it is possible e.g. to perform the identification of the conserved regions by comparing the sequences of the already identified CKI1 homologues (preferably by means of comparing the identified or assumed amino acid sequences), to propose degenerated primers, covering these conserved or partly conserved regions and to perform the identification of the sequence of the searched-for gene by amplification of the conserved region using the genomic DNA or cDNA from the gene library of the respective species as a template and subsequent isolation of its cDNA or genomic DNA by means of polymerase chain reaction (PCR) or similar methods. The conserved regions of sensor histidine kinases can be found also in publicly available databases (e.g., database PRINTS, http://www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/). Alternatively, it is possible to identify the cDNA of homologous genes e.g. by means of hybridization with cDNA library or genome library of the respective species using the probe obtained from the genomic DNA or cDNA of *CKI1* or any of the already identified homologues thereof, e.g. the genes *AHK2, AHK3* or *AHK4/CRE1/WOL* or by similar methods.

ii) proving and analysis of the histidine kinase activity of the gene product of the respective homologue, e.g. by means of gene expression in heterologous or homologous expression system, preferably in the form of recombinant protein, and subsequent analysis of its activity (Mahonen et al., Curr Biol *16*, 1116-1122 (2006)) or by means of complementation of bacterial, yeast or plant mutants in the genes encoding sensor histidine kinases (e.g. *ahk2, ahk3, ahk4* or *cki1*) using the encoding sequence of the homologue put under the control of the promoter of the respective

gene and in the respective mutant background by methods generally known in the art or by means of analysis of the activity of the gene product in the cytokinin signal transduction using the analysis in plant protoplasts, as described earlier (Hwang and Sheen, Nature *413*, 383-389 (2001)) and

iii) expression of the homologue or the orthologue in vascular tissues or tissues adjacent to vascular tissues of the respective plant species.

It is an aspect of the invention that the modification of the expression of the gene CKI1 or the homologue or the orthologue thereof comprises the modification of the expression of histidine kinase coding region of said gene.

It is an aspect of the invention that the expression of the gene *CKI1* or the homologue or the orthologue thereof is modified so that it is decreased or eliminated, e.g. by means of RNA interference, insertion mutagenesis or similar methods. Alternatively, the activity of the gene product of the homologue or the orthologue can be regulated by means of methods of site-directed mutagenesis in selected amino acids, preferably in amino acids participating in the transfer of phosphate by the respective sensor histidine kinase, in potential interactions with other, e.g. regulatory, proteins or in amino acids with a regulatory activity. The thus modified DNA under the control of a constitutively or conditionally active promoter is then used for the preparation of stable transformants of the concerned plant species using the methods and approaches generally known in the art.

When RNA interference is used for decreasing the activity of the homologue or the orthologue, it is necessary to prepare the recombinant DNA by methods of molecular biology generally known in the art, said recombinant DNA containing parts of cDNA sequence of the homologue or the orthologue in reverse repetition, separated by another DNA sequence, e.g. part of the encoding sequence *uidA* (Sequence ID No. 5; its part *GUSp*, Sequence ID No. 6) or a natural intron of the homologue or the orthologue. For decreasing the activity of the homologue or orthologue, it is possible to choose various regions of its cDNA, probably with differing results in the decrease of the expression of the homologue or the orthologue; this shall be tested by methods known by those skilled in the art (e.g. analysis of the amount of the protein encoded by the homologue or the orthologue by Western blot or *in situ* immunolocalization on

sections of inflorescence stem or other tissues). Suitable part of the cDNA sequence is the sequence of nucleotides, the order of which corresponds at least in 20 % to the nucleotide sequence of *CKIp2*, Sequence ID No. 4. This construct is then inserted under the control of a constitutively active promoter (e.g. CaMV 35S RNA promoter) or a conditionally active promoter (e.g. dexamethasone inducible promoter) and terminated by a transcription terminator. The transcription terminator may be any element capable of terminating transcription in plant cells, such as terminator of the *NOPALINE SYNTHASE* (*NOS*) gene. The resulting construct is then cloned into a binary vector, which is used for the transformation of a suitable strain of *Agrobacterium tumefaciens* (e.g. GV3013::pMP90; strain with rifampicine resistance, bearing a helper plasmid pMP90 resistant to gentamicine as a selection marker [Koncz and Schell, Mol Gen Genet *204*, 383-396 (1986)] or similar). This is followed by the preparation of transgenic plants of the concerned species using the methods known in the art, either by infiltration of inflorescence using the transgenic *Agrobacterium*, carrying the binary vector, part of which is the said gene construct, or by another method suitable for the concerned plant species, e.g. by means of infiltration of tissue explants by the transgenic Agrobacterium, carrying the binary vector, part of which is the said gene construct, and *in vitro* regenerations of the transgenic plants or similar methods. After selecting several independent lines of primary transformants on the basis of the presence of the selection marker, which is part of the inserted construct, it is necessary to identify homozygous lines in their progeny and to analyze their phenotype, this is carried out by methods known in the art of molecular biology of plants. For the analysis of the phenotype of vascular tissues development, it is possible to use a specific staining, e.g. with a mixture of orange GG and aniline blue, of hand-made sections or morphological and histological analyses of thin sections prepared using the methods known in the art.

Object of the invention is further a nucleotide sequence, having the sequence of nucleotides at least in 20 % identical with the nucleotide sequence of CKIp2 (Sequence ID No. 4).

Object of the invention is a sequence of recombinant DNA for the regulation of gene expression by means of RNA interference, having the nucleotide sequence at least in

20 % identical with the nucleotide sequence of *35S::CKIi2::pA* (Sequence ID No. 7). This sequence is also shown in Fig. 7.

Object of the invention is further a method of preparation of the recombinant DNA for the regulation of gene expression by RNA interference, wherein a specific part of the cDNA or the encoding region of said gene is amplified using primers containing a sequence of 18-21 nucleotides specific for the cDNA or the encoding sequence of said gene and regions inserted to the 5′ end of these primers, designated herein RNAi_up and RNAi_down,

RNAi_up:

BamHI HindIII
5′ - TAT AGG ATC CAA GCT T - 3′

RNAi_down:

XbaI SalI
5′ - CAC TTC TAG AGC [illegible] C - 3′

a connecting sequence is subsequently amplified, preferably a part of the encoding sequence of the gene *uidA* (Sequence ID No. 5; its part *GUSp*, Sequence ID No. 6), which is amplified using the primers Bgus and Hgus,

Bgus:

BamHI
5′ - CAG CGG ATC CCT CTA CAC CAC GCC GAA CAC C - 3′
LOOP_up

Hgus:

HindIII
5′ - TTC CAA GCT TTT CTC TGC CGT TTC CAA ATC G - 3′
LOOP_down

and by means of digestion by restriction endonucleases BamHI, HindIII, XbaI and SalI and connection of the resulting fragments by ligase, these three parts are bound to form one resulting construct, which is then inserted under the control of a suitable promoter. The thus prepared sequence is then used for the preparation of the transgenic organism of the concerned species.

If necessary, other sequences than *uidA* can be used as the connecting sequence, and in such a case, they are amplified using primers consisting of 18-21 nucleotides,

specific for the respective sequence, and further sequences, inserted to the 5′ end of these primers, designated herein LOOP_up and LOOP_down.

LOOP_up:

BamHI
5′ – CAG CGG ATC C – 3′

LOOP_down:

HindIII
5′ – TTC CAA GCT T – 3′

The sequence of 4 nucleotides at the 5′ end of the sequences RNAi_up, RNAi_down, LOOP_up and LOOP_down was optimized for the amplification of the sequences *CKIp2*, resp. *GUSp*. These sequences of 4 nucleotides can optionally be modified so that the amplification of said sequence is enhanced (improvement of the thermodynamic properties of the primers after the insertion of the 18-21 nucleotides specific for the sequence of the homologue or the orthologue and the connecting sequence, respectively, prevention of the formation of secondary structure and mutual similarities etc.), optionally, these 4 nucleotides can be omitted.

The regulation of the activity of the gene product (encoded protein) of the homologue or the orthologue can be carried out by means of the preparation of recombinant DNA, consisting of modified cDNA or genomic DNA of the homologue or the orthologue under the control of a suitable promoter (constitutively or conditionally active promoter). The modification of the cDNA or the encoding region of the genome DNA consists in the replacement of a part of the nucleotide sequence by methods known in the art (site-directed mutagenesis) in such a way that during the translation of mRNA into protein, the substitution of selected amino acids of the homologue or the orthologue by other selected amino acids occurs. Modified can be the amino acids participating in the transfer of phosphate by the respective histidine kinase, encoded by the homologue or the orthologue and/or it is possible to modify other amino acids, respectively, that can participate in the phosphate transfer as such or in potential interactions with other, e.g. regulatory proteins, or amino acids with a regulatory activity. These amino acids must be identified by methods known in the art, particularly by comparing the amino acid sequence of the homologue or the orthologue with the amino acid sequences of similar proteins and experimentally. The

resulting construct is inserted into a binary vector and stable transformant lines of the respective plant species are prepared by the above described methods and by methods known in the art, said lines expressing the modified allele of the homologue or the orthologue. This step is followed by the selection of several independent lines of primary transformants on the basis of the presence of a selection marker which is a part of the inserted construct, by identification of a homozygous line in their progeny and by analysis of their phenotype, all these steps being carried out by methods known in the art of molecular biology of plants. For the analysis of the phenotype of vascular tissue development, it is possible to use a specific staining, e.g. with a mixture of orange GG and aniline blue, on hand-made sections, or morphological and histological analyses on thin section prepared using the methods known in the art.

In another embodiment of the present invention, the expression of the gene *CKI1* or the homologue or the orthologue thereof or the activity of the gene product is modified so that it is increased, e.g. by means of overexpressing said gene. This can be achieved by means of an overexpression vector comprising a promoter for the expression in plant cell; one or more genes selected from the group consisting of *CKI1* and the homologues and the orthologues thereof, linked operable to the promoter; and a transcriptional terminator for plant cell. Preferably, the promoter is a CaMV 35S promoter. Alternatively, the plant can be treated with the gene product.

Another aspect of this embodiment of the invention is an agent for regulating the growth in volume of a plant comprising one or more active ingredients selected from the group consisting of one or more proteins selected from the group consisting of gene products of *CKI1* and the homologues and the orthologues thereof; and the overexpression vector.

In one embodiment of the invention, the gene *CKI1* or a homologue or an orthologue thereof can preferably be selected from the group consisting of CKI1 (CYTOKININ-INDEPENDENT 1, SEQ ID NO: 2) coding gene, AHK2 (ARABIDOPSIS HISTIDINE KINASE 2, SEQ ID NO: 8) coding gene, AHK3 (ARABIDOPSIS HISTIDINE KINASE 3, SEQ ID NO: 9) coding gene, and AHK4 (WOL, CYTOKININ RESPONSE 1, SEQ ID NO: 10) coding gene.

It is another aspect of the invention that it further comprises the histological staining of sections of living plants with a mixture of orange GG and aniline blue, suitable for the analysis of the phenotype of vascular tissue development in plants with modified expression of the *CKI1* gene or the homologue or orthologue thereof or with modified activity of the gene product thereof. The staining by this method allows to distinguish between lignified cell walls, which are stained in yellow to orange together with cytoplasma, and living, i.e. not yet fully lignified cell walls, which are stained in blue. Using fluorescence, it is possible to identify lignified cells and cells with cellulose deposition. This method provides for colour distinguishing of the cell components of vascular tissues, using microscopic observation in transmitted light, particularly differential interference contrast (DIC microscopy): Phloem is stained in blue, metaxylem in orange and protoxylem in deep blue to magenta.

Generally, cytokinin signalling pathways are conserved in plants and it can be assumed that the formation of vascular bundles (VB) in other plant species is directed by similar (homologous) genes as in the model plant *A. thaliana*. As model plants for the research of economic utility in wood production are used particularly poplar tree (*Populus trichocarpa*) and birch tree (*Betula sp.*). In case of the poplar tree, the genome of which is already known, homologues of the *CKI1* gene, participating in osmoregulation, were identified (Chefdor et al., FEBS Lett *580*, 77-81 (2006)). Our results of searching for homologous sequences show that genes homologous to the *CKI1* gene exist in the poplar tree genome (Hejátko, unpublished results). The homologues of the *CKI1* gene and of other sensor histidine kinases were identified in many others, economically important species (e.g., rice, corn, barley, wheat).

The CKI1 protein is a positive regulator of the cell division in cambium, therefore increasing the expression of CKI1 encoding gene or the homologue or the orthologue thereof or increasing the activity of the gene product increases the number of cells in the cambial region of vascular tissues of a plant. It is at the same time a negative regulator of the cell differentiation and might be involved in the regulation of the width of the VB, leading to formation of wide VB with enlarged area of the metaxylem region. In view of the fact that only differentiated cells are able to deposit cellulose and lignify, decreasing of the expression of CKI1 encoding gene or the homologue or the orthologue thereof or decreasing of the activity of the gene product

increases the lignification in vascular tissues, thereby affecting the economical potential of the plant biomass produced.

The invention is further illustrated by the following examples, which should not be construed as further limiting.

Figures

Fig. 1 shows the analysis of the biomass production in transgenic lines bearing the construct for decreasing the expression of *CKI1* according to example 3. DMSO1=0.0001%; DMSO2=0.001% and DMSO3=0.01% (v/v) corresponds to the increasing DMSO (dimethylsulphoxide) concentration in the cultivation medium.

Fig. 2 represents the comparison of several independent transgenic lines with a decreased *CKI1* expression (lines CJH4T3) with mutants in the genes homologous to *CKI1* and plants with overexpression of the gene for *CYTOKININ OXIDASE/DEHYDROGENASE3*, leading to a decreased level of endogenous cytokinins (35S::AtCKX3), with wild-type plants (wt Col-0). Shown are rossette diameter and number of leaves in a rossette during the plant growth (A, 8 weeks; B, 10 weeks; C, 12 weeks and D, 14 weeks old plants cultivated under the short day conditions).

Fig. 3 shows the phenotypic analysis according to example 3: A faster development of vascular bundles and an increased deposition of cellulose during the secondary thickening in *A. thaliana* as a result of the repression of the *CKI1* gene activity. The vascular bundles in the inflorescence lateral branches of wild type (a-d), *35S::CKIi* plants (transgenic plants bearing the construct *35S::CKIi2::pA*, causing the suppression of the *CKI1* gene, line CJH4T3, 5-3, [e-h]), in double mutants in the genes homologous to the *CKI1* gene, *ahk2 ahk3*, (i-l) and *ahk2 ahk4* (q-t) and in the transgenic line with an overexpression of the gene for *CYTOKININ OXIDASE/DEHYDROGENASE3* (35S::AtCKX3) leading to a decreased level of endogenous cytokinins (m-p). Hand-made sections were stained with a mixture of aniline blue and orange GG and observed using DIC optics (a, c, e, g, i, k, m, o, q, s) or using fluorescence (b, d, f, h, j, l, n, p, r, t), with the use of triple filter (U-M61000,

DAPI/FITC/TRITC triple filter block, Olympus). if, interfascicular arch fibers; mx, metaxylem; p, phloem; px, protoxylem. Scale bars 100 μm (a, b, e, f, i, j, m, n, q, r) and 50 μm (c, d, g, h, k, l, o, p, s, t). The arrows depict the regions of interfascicular arch fibres, the arrowheads depict the metaxylem region.

Fig. 4 represents the quantification and the comparison of the thickness of the layer of lignified cells in interfascicular arcs of two independent transgenic lines with a decreased *CKI1* expression, CKI1, *35S::CKI1i*, 1-5 and 5-3 (shortened designations of the lines CJH4T3, 1-5 and CJH4T3, 5-3), in double mutants *ahk2 ahk4, ahk3 ahk4* and *ahk2 ahk3* and in the transgenic line with overexpression of the gene *AtCKX3* (35S::AtCKX3) for CYTOKININ OXIDASE/DEHYDROGENASE3 with a decreased endogenous cytokinin levels in comparison with the wild-type line wt Col-0. Shown are the dimensions in μm.

Fig. 5 shows the analysis of the effect of CKI1 to the cytokinin signal transduction in protoplasts of *A. thaliana* according to example 5.

Fig. 6 shows the phenotypic analysis of transgenic lines with an increased expression of wild-type *CKI1* gene and of the dominant negative mutant $CKI1^{H405Q}$. **A, B.** Ectopic expression of *CKI1* leads to sterility and an increased production of trichomes (A) and thick fasciated inflorescence stems (B). **C.** Changes in the architecture of vascular bundles in the plants with an increased expression of *CKI1* (transgenic line *35S::CKI1*). Transverse sections (a, b, d and e) and longitudinal sections (c and f) of the inflorescence stems of wild-type plants (a-c) and transgenic lines *35S::CKI1* (d-f). The arrows indicate the formation of ectopic tissues that resemble vascular bundles. **D, E.** Ectopic expression and overexpression of *CKI1* leads to the formation of additional vegetative tissues initiated from lateral meristems. The node structures of wild-type and transgenic lines *35S::CKI1* (D), transverse and longitudinal sections from wild-type plants (E.a) and transgenic lines *35S::CKI1* (E.b). The arrow indicates an ectopic axillary bud formed in transgenic lines *35S::CKI1*. Scale bars 100 μm. **F.** Ectopic overexpression of *CKI1* leads to disruptions in proper development of vascular bundles in *A. thaliana*. Transverse section of the inflorescence stems of transgenic lines with overexpression of $CKI1^{H405Q}$ (35S::CKI1 ^{H405Q}, a, b a c), wild-type (d) and double mutant *ahk2 ahk3* (e). Scale bars 100 μm. (f)

Expression analysis of transgenic lines *35S::CKI1* ^{H405}. Total proteins from 6-day-old transgenic plants was analysed using an immunoblot assay (Western blot) with an anti-HA-epitope antibody and anti-actin antibody.

Fig. 7 represents the sequence of recombinant DNA *35S::CKIi2::pA* (Sequence ID No. 7).

Fig. 8 demonstrates expression of CKI1 in vascular tissue. Specificity of *CKI1* promoter as determined in *pCKI1::uidA* transgenic plants. **A.** Transgenic seedlings at various stages of development. **a.** GUS staining in the primary root at 1 DAG (arrowhead). Two-day-old (**b**) and five-day-old seedlings (**c-h**). **c.** Tip of cotyledon (arrowhead). **d.** Staining in the lateral parts of SAM (arrowheads). **e.** Expression in the region close to the root-shoot junction. **f.** LRP at stage III-IV. **g.** Staining in the region adjacent to the root tip (arrowhead). **h.** Lateral root cap. Scale bars, 500 μm (b), 200 μm (e), 100 μm (a, c, g), and 50 μm (d, f, h). **B.** GUS activity in flowering transgenic plants harboring *pCKI1::R12-uidA* (a, c-e) or *pCKI1::uidA* (b). **a.** Top of the *Arabidopsis* inflorescence. Note the maximum of the signal in the inflorescence stem, vascular tissue of floral organs and the flower pedicels (arrowheads). **b-c.** Floral organs and flower pedicels before (b) and at/just after anthesis (c). Note the predominant GUS staining in the pistil in the flowers before anthesis (b, arowheads); *vice versa*, the signal in the vascular tissue of stamens is stronger in flowers at/just after anthesis (c, arrows). **d.** Male sporophytic tissue (arrowhead). Scale bars 500 μm (a, c, e) and 100 μm (b, d). **C.** *CKI1* expression in the VB of inflorescence stem. **a.** GUS staining in cross-section of the inflorescence stem of *pCKI1::R12-uidA*. GUS activity is located in cells of the vascular bundle sheath located at the lateral borders of the phloem and cambium (arrowhead) and xylem (arrows, see also figures b and c). **b.** *In situ* localization of *CKI1* mRNA in cross-sections of the inflorescence stem. **c-h.** *In situ* immunolocalization of CKI1 using $\alpha CKI1_{ED}$ in cambium of VB (deep purple signal, arrowheads) on the cross sections of inflorescence stem. **c, d** wt, **e, f** *35S::CKIi* line, **g, h** *35S::CKI1*. Note the localization of CKI1 in cambium even in case of *35S::CKI1* plants, which suggests presence of cambium localization signal in CKI1. px, protoxylem; arrows point to the first differentiated metaxylem cells. Scale bars 100 μm (b, d, g, j), 50 μm (a, c, e, h, k) and 25 um (f, i, l).

Fig. 9 shows that CKI1, AHK2 and AHK3 are involved in the VB development of inflorescence stems. **A.** Suppression of CKI1 activity in RNAi lines (**c, d** and **j, k**) results into reduced and disorganized files of cambial cells. *Vice versa*, the overexpression of *CKI1* or *CRE1-CKI1* (**e, f** and **g, h,** respectively) leads to increase of the number of cambium layers. **l.** Overexpression of dominant negative allele $CKI1^{H405Q}$ leads to a dramatic reduction of cambium formation. **B.** Reduction of *CKI1* expression by T-DNA insertion resembles *CKI1* RNAi plants. Transverse sections of the inflorescence stems of wild-type (WS-2, a) and the heterozygous *CKI1* T-DNA insertion lines, *cki1-5/CKI1* (b) and *cki1-6/CKI1* (c). Staining of hand-made sections (a-h) with toluidine blue (TB) and thin sections made from fixed and embeded material (i-l). c, cambium; ic, interfascicular cambium; mx, metaxylem; p, phloem. Scale bars 100 μm (a, e, c, g) and 50 μm (b, f, d, h).

Fig. 10 demonstrates that cytokinin signalling via AHK2 and AHK3 affects vascular bundle development in *Arabidopsis*. **A.** The loss-of-function *ahk2* or *ahk3* mutations disrupt proper development of the vascular bundles. Transverse sections of the inflorescence stems of **a, f, k,** wild-type (Col-0), **b, g, l** *ahk2,* **c, h, m** *ahk3,* **d, i, n** *ahk2, 3* and **e, j, o** *35S::AtCKX* lines. Note reduction of cambial layers in *ahk2, ahk3* and particularly in *ahk2, 3* line. The overall reduction in the VB size is apparent in *ahk2, 3* and in *35S::AtCKX* lines, suggesting positive regulation of CK signalling via *AHK2/AHK3* pathway in VB development of *Arabidopsis*. Staining of hand-made sections (a-j) with toluidine blue (TB) and thin sections made from fixed and embeded material (k-o). Scale bars, 100 μm (a-e) and 50 μm (f-j).

Fig. 11 demonstrates lowered expression of CKI1 in RNAi lines and T-DNA tagged mutant lines. Transcript levels of *CKI1* in RNAi lines (upper pannel) and T-DNA insertion heterozygous lines (lower panels). Quantitative real time PCR was performed with total RNA extracted from inflorescence stems of wild type (Col-0 and WS-2), *CKI1* RNAi, *cki1-5/CKI1* and *cki1-6/CKI1* transgenic plants and gene-specific primers for *CKI1*. Error bars indicate standard deviation (n=3).

Fig. 12 demonstrates that CKI1 is functionally conserved with AHK2 and AHK3 in the vasculature development. A. The dwarf architecture resulting from deletion of ahk2/ahk3 was rescued in the presence of CKI1. Three-week-old wild type (Col-0)

and transgenic plants expressing 35S::CKI1-HA or 35S::CKI1H405Q-HA in the ahk2/ahk3 genetic background were used for phenotypic analysis. B. Expression analysis of HA-tagged CKI1 and CKI1H405Q proteins under the control of 35S promoter in the transgenic lines. Total proteins from three-week-old of each designated plants were subjected to 7.5% SDS-PAGE. Actin proteins served as the input controls. C, D. Ectopic expression of CKI1 rescues the abnormal vasculature of the ahk2/ahk3 mutant. The microscope images of ten (C, a), twenty (C, b-d) and forty (D) magnification of transverse sections of the inflorescence stems of wild type (a), ahk2/ahk3 (b), 35S::CKI1-HA/ ahk2/ahk3 (c) and 35S::CKI1H405Q-HA/ahk2/ahk3 (d). The arrow indicates the immature vasculature. Scale bars, 100 μm.

Sequence listing

Sequence 1 – genomic sequence of *CKI1*
Sequence 2 – amino acid sequence of CKI1
Sequence 3 – cDNA sequence of *CKI1*
Sequence 4 – sequence *CKIp2* (part of the cDNA sequence of *CKI1*)
Sequence 5 – sequence *uidA*
Sequence 6 – sequence *GUSp* (part of *uidA*)
Sequence 7 – sequence of the recombinant DNA of *35S::CKIi2::pA*
Sequence 8 – amino acid sequence of AHK2 (ARABIDOPSIS HISTIDINE KINASE 2)
Sequence 9 – amino acid sequence of AHK3 (ARABIDOPSIS HISTIDINE KINASE 3)
Sequence 10 – amino acid sequence of AHK4 (WOL, CYTOKININ RESPONSE 1)

Examples

Several independent transgenic lines of *A. thaliana*, Col-0, bearing the recombinant DNA for the repression of the *CKI1* gene activity using the RNA interference were prepared.

Example 1

Preparation of the construct for RNA interference

The region of cDNA of the *CKI1* gene designated *CKIp2* and delimited by the sequences of CKIp2_up and CKIp2_down primers was amplified by PCR. Similarly, part of the encoding sequence of the *uidA* gene (Sequence ID No. 5) was amplified with Bgus and Hgus primers, and was designated *GUSp* (Sequence ID No. 6). The primers used contain, in addition to the nucleotides derived from the *CKI1* and *uidA* sequences, respectively, inserted sites that are recognized by specific restriction endonucleases, see the sequences of the primers, wherein the inserted regions are underlined (non-specific to the sequence of the template used for amplification) and the sites recognized by restriction endonucleases are marked in colour. In the CKIp2_up primer, these are BamHI and HindIII, in the CKIp2_down primer, these are XbaI and SalI specific sites, the Hgus primer contains site recognised by HindIII, the Bgus primer contains the recognition sequence for BamHI.

Sequences of the primers used:

CKIp2_up:

BamHI HindIII
5′ - TAT AGG ATC CAA GCT TGC AGT TGT CCT TTT GGT GAT T - 3′
RNAi_up

CKIp2_down:

XbaI SalI
5′ - CAC TTC TAG A[illegible] CTG ATG CCA TTA TCC TCT TAC C - 3′
RNAi_down

Bgus:

BamHI
5′ - CAG CGG ATC CCT CTA CAC CAC GCC GAA CAC C - 3′
LOOP_up

Hgus:

HindIII
5′ - TTC CAA GCT TTT CTC TGC CGT TTC CAA ATC G - 3′
LOOP_down

The amplification with the thus modified primers and with cDNA of the *CKI1* gene and DNA of the *uidA* gene, respectively, as a template led to the production of DNA comprising said restriction sites. With the inserted sites for restriction endonucleases, all these fragments could be cloned at once by the following process: The DNA fragment of *CKIp2* (Sequence ID No. 4) was divided into halves, one half was

digested by BamHI and XbaI (i.e., enzymes recognizing the outer restriction sites), the other half was digested by HindIII and SalI (i.e., enzymes recognizing the inner restriction sites). The DNA chains obtained by the amplification with the primers Hgus and Bgus were digested by HindIII and BamHI. After purification using QIAquick PCR Purification Kit (Qiagen), all three different fragments were mixed in equimolar amounts and added was the vector pBluescript (Stratagene), cleaved by the enzymes XbaI and SalI and dephosphorylated by thermo labile alkaline phosphatase (Alkaline Phosphatase, shrimp, Roche, as described by the manufacturer) so that the resulting molar ratio between the inserted fragments and the plasmid DNA was 1:1 to 3:1. This step was followed by ligation using T4 DNA ligase (T4 DNA Ligase, Roche), for 12 h at 16 °C, transformation into *Escherichia coli*, strain DH10β, and control digestion of the DNA (by SalI), isolated from the positively selected bacterial colonies on a selection medium (ampicillin). All the above-captioned procedures were carried out either as described by manufacturer of the chemicals and enzymes or according to molecular biology methods known in the art (Ausubel et al., Current protocols in molecular biology. John Wiley and Sons, New York (2003)). The resulting construct was then designated *pBsc::CKIp2i*. In this case, we have achieved more than 50% efficiency (13 positive samples out of 20 tested DNA isolates from the positively selected bacterial clones), that is an above-average value, illustrating a relatively high efficiency of this method of preparation of the recombinant DNA. Other parts of the *CKI1* gene were cloned with similar results for the purposes of silencing thereof by RNA interference, which illustrates the versatility of this procedure also for other sequences. This was followed by the control of the selected clones by sequencing, which has confirmed a successful cloning of all three fragments (*CKIp2* in antisense orientation, *uidA* fragment and *CKIp2* in sense orientation), that were altogether designated *CKIp2i*. The above-described procedure can be used universally for the preparation of constructs for silencing genes by RNA interference as far as the parts of the cDNA of the silenced gene are chosen which do not contain recognition sites for the restriction enzymes BamHI, HindIII, XbaI and SalI. Furthermore, it is necessary to prepare primers containing approximately 18-21 nucleotides specific for the selected part of the cDNA and in addition RNAi_up sequence at the 5′ end of the „left (forward)“ primer, and RNAi_down at the 5′ end of the „right (reverse)“ primer, respectively (see the underlined parts of the sequences of the primers CKIp2_up and CKIp2_down). When the *uidA* gene sequence cannot be

used as the connecting sequence, it is possible to modify primers, containing a specific sequence of the selected DNA, which, however, must not contain the recognition sites for the restriction enzymes BamHI and HindIII, by similar methods (insertion of the sequences LOOP_up and LOOP_down, containing the sites recognized by the restriction enzymes BamHI and HindIII, respectively).

Example 2

Preparation of transgenic lines

The binary vector pVKH*35SGUSpA* (Reintantz et al., Plant Cell *13*, 351-367 (2001)) was modified by digestion with the restriction enzymes BamHI and HindIII, creating blunt ends using Klenow enzyme (Roche) and by subsequent religation by T4 DNA polymerase (all enzymes from Roche). The resulting vector, which was designated *pVKH::(35S::pA)*, contained the 35S expression cassette (CaMV 35S RNA promoter and transcription terminator). Subsequently, the construct *CKIp2i* was cleaved out by SalI from the vector *pBsc::CKIp2i* and it was cloned into the binary vector *pVKH::(35S::pA)*, digested by SalI. The resulting construct was designated *pVKH::(35S::CKIi2::pA)* (Sequence ID No. 7), abbreviated to pJH_006. The construct was checked by sequencing (see Sequence ID No. 7) and transformed by electroporation into *Agrobacterium tumefaciens*, strain GV3013::pMP90 (Koncz and Schell, Mol Gen Genet *204*, 383-396 (1986)). By infiltration of the inflorescence of wild-type *A. thaliana*, Col-0 (Clough and Bent, Plant J *16*, 735-743 (1998)) and subsequent selection on the medium with hygromycine (*in vitro* on MS medium, without sucrose, 15 mg/l hygromycine, 400 mg/l ticarcillin/potassium clavulanate, 15:1), primary transformants (T1 generation, lines designated CJH4T1, 1 to CJH4T1, 58) were obtained, from the progenies thereof, the presumed single-copy lines were selected on the basis of segregation ratios (frequency of resistance and/or sensitiveness, respectively, to the selection marker hygromycine). In the progenies of the presumed single-copy lines, lines homozygous for a given transgene (T2 generation, lines designated CJH4T2) were identified. The seeds from these lines were then collected and used for further analyses (T3 generation, lines designated CJH4T3, 1 to CJH4T3, 16).

Example 3

Phenotypic analysis of the lines with a decreased *CKI1* expression

We have identified an interesting phenotypic feature in several independent homozygous lines. Selected transgenic lines (see Fig. 1 and Fig. 2) were cultivated *in vitro* in square-shaped Petri dishes (12x12 cm) for 21 days on MS medium supplemented with 1% sucrose and solidified with 1.5 % phytagel, containing various concentrations of dimethylsulphoxide (DMSO), in vertical position in cultivation chambers with adjustable growth conditions (Percival Scientific, Ltd., relative air humidity 70 %, short day conditions [8 hours light, 16 hours dark], temperature during light period 21 °C, temperature during dark period 19 °C). DMSO1=0.0001%; DMSO2=0.001% and DMSO3=0.01% (v/v) corresponds to an increasing concentration of DMSO in the cultivation medium. After harvesting, roots and shoots were separated. The weight of the produced biomass of roots and shoots was measured by weighing. It was found that in at least 4 out of the 6 tested lines, an increased production of biomass in comparison to wild-type lines occurs, both in roots and in shoots (Fig. 1). At cultivation in soil under short day conditions, the transgenic lines grew faster and produced larger vegetative part of the plant (so-called leaf rosette, Fig. 2) in comparison to wild-type plants.

Further, a more detailed phenotypic analysis in the development of vascular tissues was carried out. Hand-made sections from living plants (without fixation) were prepared and stained with aniline blue and orange GG. The sections were prepared by razor-blade from basal nodes of inflorescence lateral branches, growing from basal node of the main inflorescence stem of *A. thaliana* and were immediately stained for 15 minutes in a staining solution containing 0.25% aniline blue and 1% orange GG, destained for 10-15 min. in water and mounted into 50% glycerol. The staining solution was prepared by the following procedure: 0.25 g of water-soluble aniline blue and 1 g of orange GG were dissolved in 100 ml of water. This mixture was brought to boil and after cooling-down, 5 ml of acetic acid was added, and the mixture was filtered (Braune, Leman, Taubert, Pflanzenanatomisches Praktikum II, p. 401, VEB Gustav Fischer Verlag, Jena (1982)). Staining by this method allows distinguishing between lignified cell walls, which are stained in yellow to orange together with cytoplasm, and living, i.e. not yet fully lignified cell walls, which are stained in blue. When using fluorescence, it is possible to identify lignified cells. This

method also allows the colour distinction between particular cell and functional components of vascular tissues in plants: phloem is stained in blue, metaxylem in orange and protoxylem deep blue to magenta (see Fig. 3). The sections were observed on the same day and the documentation was performed by motorized microscope (BX 61, Olympus) equipped with digital camera (DP50, Olympus) and controlled by a special software (AnalySIS, Soft Imaging System, GmbH). It was found on the sections that abnormalities occur in the development of vascular (conductive, vasal) tissues of the inflorescence stem in transgenic lines. It was found that in transgenic lines with presumed decreased expression of the *CKI1* gene, formation of thicker fibres of interfascicular arcs between vascular bundles occurs (Fig. 3). The thickness of the arcs was measured and analysed by a special software (AnalySIS, Soft Imaging System, GmbH) on microphotographs obtained by observing fluorescence in the above-captioned sections of living plants, stained with a mixture of orange GG and aniline blue, see Fig. 3. Fig. 3 shows the phenotype of vascular bundles in lateral inflorescence branches of wild-type (a-d), *35S::CKIi* plants (transgenic plants bearing the construct for silencing the gene *CKI1 35S::CKIi2::pA*, line CJH4T3, 5-3 [e-h]) and in mutants in the genes homologous to the *CKI1* gene, genes *AHK2, AHK3* and *AHK4: ahk2 ahk3*, (i-l) and *ahk2 ahk4* (q-t). Figures (m-p) show the phenotype of the transgenic line with overexpression of the gene for CYTOKININ OXIDASE/DEHYDROGENASE3 (*35S::AtCKX3*) leading to decreased levels of endogenous cytokinins. Hand-made sections were stained with a mixture of aniline blue and orange GG and observed using DIC optics (a, c, e, g, i, k, m, o, q, s). Phloem is stained in blue, metaxylem in orange and protoxylem in deep blue to magenta. Cells of interfascicular arcs fibres (if) were observed on the same sections using fluorescence and using triple filter (U-M61000, DAPI/FITC/TRITC triple filter block, Olympus; [b, d, f, h, j, l, n, p, r, t]). The specimens were obtained from plants approx. 16 weeks old, 7 weeks after transfer into long day conditions and approx. 5 weeks after induction of flowering. When comparing figures a-d with figures e-h, an increased deposition of cellulose, particularly in the metaxylem region, can be observed in lines with a decreased *CKI1* expression (more intense staining with orange GG and thicker cell walls, arrowhead). In the lines with a decreased *CKI1* activity and in double mutant *ahk2 ahk4*, a radial expansion of the layer of interfascicular arcs cells (if) can be observed. This difference is statistically significant ($P<0.05$, see Fig. 4). The partly lignified cells on the outer edge of the central nucleus

of medulla, arrowheads on figures d, h, l and t) were not included into the measurement. In the lines *35S::CKIi* and *ahk2 ahk4* mutants, an increased cellulose deposition was observed during the secondary thickening as an intense orange GG staining (compare figures c, g and s, the arrowhead points to the cell walls in xylem, the arrow indicates the cell walls in interfascicular arcs; the arrows indicating these differences in figures a, e, i, m and q are in the same positions as in the corresponding pictures from the fluorescence microscope b, f, j, n and r). The increased intensity of staining with orange GG in the if region in *35S::CKI1i* lines (e and g) reflects the increased cellulose deposition and lignification in comparison to wild-type lines, which are in these regions stained in blue (a and c). if, interfascicular arch fibres; mx, metaxylem; p, phloem; px, protoxylem. Scale bar 100 μm (a, b, e, f, i, j, m, n, q, r) and 50 μm (c, d, g, h, k, l, o, p, s, t).

Analysis of the phenotype in the vascular tissue of mutant lines with decreased expression of CKI1 has been also performed on hand-made sections that were prepared from the main inflorescence stem. The sections were stained with toluidine blue (TB, 1% w/v solution in water) for 1 min., destained in water, mounted and observed with microscope as described above using DIC. Using TB staining, the phloem is stained in blue, undifferentiated cambial zone in pink, metaxylem in blue-green and protoxylem in purple (see Fig. 9). We have found that in lines with decresed expression of CKI1, there is a lateral expansion of VB, particularly of MX region (see Fig. 9). In contrast to that, the VB in 35S::CKI1 lines are rather narrow, with decreased amount of MX. This analysis has also shown increased dedifferentiated region in case of 35S::CKI1 lines while in the RNAi lines thickness of the cambial (undifferentiated) region was reduced. These results are consistent with the positive role of CKI1 in the regulation of division and maintenance of cells in undifferentiated (cambial) zone while negative role in the cell differentiation. CKI1 seems to be also involved in the regulation of the width of VB, which is in good accordance to the localization of *CKI1* transcriptional activity and localization of CKI1 in cells located at the outer borders of VB (see Fig. 8).

The results of the histochemical staining show that in these lines, an increased deposition of cellulose and lignification during the secondary thickening of the stem in both xylem and interfascicular arcs occurs as a result of the decreased expression of the *CKI1* gene. That means that CKI1 is a negative regulator of said processes (i.e. particularly of development and differentiation of vascular bundles, formation

(lignification) of interfascicular arc fibres and secondary thickening). Further, CKI1 activity seems to regulate the amount and quality of water and nutrient conducting tissue that might also affect plant growth and biomass production. Thus, these facts can be used in biotechnological applications connected with the biomass production in plants.

Example 4

Phenotypic analysis of the mutants in the genes homologous with CKI1

The analysis of the phenotype of vascular tissues in the lines with insertion mutations in the genes homologous to the gene *CKI1, AHK2, AHK3* and *AHK4*, was carried out in a similar way as for the lines with a decreased *CKI1* expression. The results of the phenotypic analyses in double mutants in the genes homologous to the gene *CKI1*, genes *AHK2, AHK3* and *AHK4,* confirmed the participation of the homologues of the gene *CKI1* in the development of vascular tissues in *A. thaliana.* In case of double mutants *ahk2, ahk3* (double homozygous for the respective mutation, Nishimura et al., Plant Cell *16*, 1365-1377 (2004)), the identified phenotype of vascular tissues was opposite to the phenotype in the lines with a decreased expression of *CKI1*, as found by the analysis of the phenotype of vascular tissues of the inflorescence stem (see above), it means that in comparison with the wild-type there is a delayed and incomplete differentiation of protoxylem and metaxylem, formation of thinner interfascicular arc fibres among vascular bundles and a decreased deposition of cellulose in both xylem and the region of interfascicular arc fibres ; compare the intensity of the orange GG staining in the region of interfascicular arcs in Fig. 3: In double mutants *ahk2 ahk3* (i and k), the region of interfascicular arcs (arrows) is more stained in blue than the wild-type line (a and c) and particularly the *35S::CKIi* line (e and g). However, in the cambial zone, the phenotype of *ahk2 ahk3* double mutant resembles the phenotype of RNAi lines. Downregulation of CKI1 leads to formation of narrow cambial zone with abnormal cell morphology (Fig. 10). A similar phenotype was observed also for the transgenic lines with an overexpression of the gene for CYTOKININ OXIDASE/DEHYDROGENASE3 *AtCKX3* (Fig. 3, m - o). We have found a decrease in the endogenous levels of cytokinins of the zeatine type down to approx. 1 % of the level in the wild-type lines in the inflorescence of these lines (Hejátko, unpublished results). On the contrary, in case of double mutants in the genes

ahk3, ahk4 (Nishimura et al., Plant Cell *16*, 1365-1377 (2004)) and particularly *ahk2, ahk4* (Higuchi et al., Proc Natl Acad Sci U S A *101*, 8821-8826 (2004)), the phenotype similar to the phenotype of the transgenic lines with a decreased *CKI1* expression was identified, wherein in comparison with the wild-type lines the formation of thicker interfascicular arc fibres between vascular bundles (Fig. 3 and 4) and an increased deposition of cellulose in both the region of interfascicular arc fibres and xylem (compare Fig. 3 a, c vs. q, s) occurs.

We have shown that CKI1 shares downstream signalling components with the cytokinin receptors for its action. Proteins involved in the two-component signalling were known to regulate vascular tissue formation in the *Arabidopsis* root (Hutchitson et al., Plant Cell, 18, 3073-3087 (2006); Mahonen et al., Science 311, 94-98 (2006); Mahonen et al., Genes Dev 14, 2938-2943 (2000)). We have tested the histological vasculature morphology of AHK null mutants and their combinational series to examine potential roles of cytokinin-mediated two-component signals in vascular bundle development of the inflorescence stem. In ahk2 mutant line, the number of cell layers in the cambial region was dramatically decreased, and the cell arrangements in the xylem and phloem were markedly changed (Fig. 10b, g, l). Weaker phenotype was identifiable in the *ahk3* line (Fig. 10c, h, m). In the *ahk2, ahk3* double mutants, the reduction of the cambium and size of VB was more pronounced in comparison to both single mutants (Fig. 10d, i, n). Reduction of cambium layer and VB size was identified also as a result of endogenous CK depletion in *35S::AtCKX3* and *35S::AtCKX1* lines (Fig. 10e, j and 10o, respectively). Taken together, two-component signalling cascade initiated by CKI1, AHK2, and AHK3 is likely to play critical roles in normal vascular bundle development, especially in maintenance of cambial cell identity.

To confirm that CKI1 is functionally conserved with AHK2 and AHK3 in the vasculature development, we ectopically expressed *CKI1* or $CKI1^{H405Q}$ in the *ahk2 ahk3* mutants (Figure 12A). *CKI1* expression in the transgenic lines was determined by immunoblotting (Figure 12B). Ectopic expression of *CKI1* partially rescued the growth defects of the *ahk2 ahk3* mutants (Figure 12A, C, and D). The rosette leaves and petioles of the transgenic plants were similar to those of the wild type (Figure 12A). In comparison to the *ahk2 ahk3* mutants, overexpression of *CKI1* in the *ahk2 ahk3* background resulted in a 2–3 fold increase in the diameter of inflorescence stems (Figure 12C), and irregular sized cells were restored in the xylem, phloem, and

cambial layers (Figure 12D). However, ectopic expression of a dominant negative *CKI1*^{H405Q} further accentuated the mutant phenotypes of the *ahk2 ahk3* mutants. The aerial parts and diameter of the inflorescence stem were much smaller (Figure 12A and 12C). The cambial cell layers were unidentifiable and vascular tissue differentiation were nearly abolished (Figure 12D).

These results collectively suggest that a constitutively active CKI1 shares two-component signalling pathway with the cytokinin receptors, but still have its own specificity in the vasculature development. These results have further shown that the observed effects are gene-specific for a respective cytokinin signal pathway and that for the purpose of modulation of the vascular tissue development in shoots of plants it is possible to use also the regulation of gene expression and/or modification of the activity of gene products, respectively of the genes homologous or orthologous to the gene *CKI1*.

Example 5

Preparation of recombinant DNA encoding CKI1 with a modified activity using the methods of site-directed mutagenesis

Recombinant DNA encoding CKI1 with a modified activity was prepared. The modification of the activity of CKI1 was tested using expression in plant protoplasts and using genetic and biochemical analyses. Subsequently, transgenic lines expressing *CKI1* with the modified activity were prepared. The phenotype analysis of these transgenic lines has shown changes in the formation of vascular tissues. These results have shown that by modulating the activity of CKI1 and its orthologues, it is possible to affect the production of vascular tissues in plants.

The genomic DNA for CKI1 was amplified using primers CKI1gen_fwd and CKI1gen_rev.

CKI1gen_fwd:

5′-GGA TCC ATG ATG GTG AAA GTT ACA AAG C-3′ (BamHI)

CKI1gen_rev:

5′-AGG CCT GTG ACG TTT GCT TTC GAT TTC-3′ (StuI)

The resulting DNA (*CKI1gen*) was digested using BamHI and StuI and cloned into the vector pCB302ES, which was obtained by modifying the vector pCB302-2 (Xiang et al., Plant Mol Biol *40*, 711-717 (1999)) in such a way that the coding region of *CKI1* became under the control of the promoter *35SC4PPDK* (Hwang and Sheen, Nature *413*, 383-389 (2001)). The resulting vector was designated *pCB302ES::(35S::CKI1gen)*. Using the kit for site-directed mutagenesis (QuickChange site-directed mutagenesis, Stratagene) and the primer His405_CAA, the sequence *CKI1gen* was modified so that the resulting DNA encoded the mutant $CKI1^{H405Q}$ with the replacement of histidine in the position 405 (nucleotide sequence CAC) by glutamine (CAA). The resulting construct was designated *pCB302ES::(35S::CKI1genH405Q)*.

His405_CAA:
5′-GCA AAT GCT AGC **CAA** GAT ATT AGA GGT GCC-3′

Similarly, using the primer Asp1050_AAC, genomic DNA encoding the mutant $CKI1^{D1050N}$ with the replacement of aspartic acid in the position 1050 (GAC) by asparagine (AAC) was prepared and the resulting construct was designated *pCB302ES::(35S::CKI1genD1050Q)*.

Asp1050_AAC:
5′-GAC TAC ATA TTC ATG **AAC** TGC CAA ATG CCA G-3′

This DNA was then used for transformation of the protoplasts of *A. thaliana* and the activity of the encoded proteins was analysed as to their effect to the transduction of a signal in the cytokinin signalling pathway by known methods (Hwang and Sheen, Nature *413*, 383-389 (2001)). The transduction of the signal in the cytokinin signalling pathway is in the protoplasts of *A. thaliana* quantified using the luciferase activity measurements, the expression of which is controlled by the cytokinin inducible promoter of the gene *ARR6*. As a control of the constitutive (independent of cytokinins) activity of CKI1, in addition to protoplasts isolated from the wild-type *A. thaliana*, Col-0 (wt), protoplasts isolated from the transgenic line *A. thaliana* with an overexpression of the gene *AtCKX2* encoding CYTOKININ OXIDASE/DEHYDROGENASE2 (35S::CKX2-9) were used for the transformation. In these transgenic lines, a decrease of the endogenous level of the active forms of cytokinins (isopentyladenine and zeatine) to approx. 45 % of the wild-type level

occurred (Werner et al., Plant Cell *15*, 2532-2550 (2003)) as a result of the overexpression of *AtCKX2*. By this analysis it was found that in case of an overexpression of the wild-type allele *CKI1*, constitutive, i.e. independent of cytokinin presence activation of the cytokinin signalling pathway in *A. thaliana* occurs. On the contrary, overexpression of the genes for the mutant forms of CKI1, $CKI1^{H405Q}$ and $CKI1^{D1050N}$, leads to a dominant negative effect on the signal transduction in the cytokinin signalling pathway in *A. thaliana* (see Fig. 5).

The DNA of *pCB302ES::CKI1gen* and *pCB302ES::CKI1genH405Q* was transformed into *Agrobacterium tumefaciens*, strain GV3101, and by infiltration of the inflorescence (Clough and Bent, Plant J *16*, 735-743 (1998)) transgenic plants were obtained, producing increased quantities of CKI1, resp. $CKI1^{H405Q}$ in the form of recombinant proteins (a fusion protein, containing in addition to the amino acid sequence of CKI1 and $CKI1^{H405Q}$, respectively, also the sequence of two copies of the so-called epitope HA (hemaglutinine), allowing the detection of the fusion protein using the anti-HA epitope antibodies, see Fig. 6Ff). Three independent transgenic lines bearing the construct *35S::CKI1gen,* designated 2-2, 2-13, 1-13 and four independent transgenic lines bearing the construct *35S::CKI1genH405Q* , designated 2, 6, 7 and 9 were analysed.

By the analysis of the above described transgenic lines it was found that changes in the development of vascular tissues in the transgenic lines occurred, both in the lines with an overexpression (increased expression) of the wild-type allele *CKI1* (lines 2-2, 2-13, 1-13 and 4) and in the lines overexpressing the mutant allele $CKI1^{H405Q}$ (lines 2, 6, 7 and 9).

In the lines with an increased expression of the wild-type allele *CKI1*, the formation of ectopic vascular bundles and disruption in their differentiation occurred (in addition to other phenotype features, such as different levels of sterility of the transgenic plants or shortening of siliques, shown in Fig. 6). In the lines with an increased expression of *CKI1*, absence or a substantial decrease in the lignification was identified in comparison to the wild-type lines. On the transverse sections of the stems of the transgenic lines, cellulose deposits in the corners of cells were identifiable, which is the stage preceding the lignification of these tissues in *A. thaliana* (Altamura et al., New Phytologist *151*, 381-389 (2001)). The inflorescence stems of the transgenic lines with an increased expression of *CKI1* also contained an increased number of cells in comparison to the wild-type (Fig. 6).

Fig. 6 shows the results of the phenotypic analysis of the transgenic lines with an increased expression of the wild-type *CKI1* gene and of the dominant negative mutant $CKI1^{H405Q}$. **A, B.** Ectopic expression of *CKI1* leads to sterility and an increased production of trichomes (A) and thick fasciated inflorescence stems (B). **C.** Changes in the architecture of vascular bundles in the plants with an increased expression of *CKI1* (transgenic line *35S::CKI1*). Transverse sections (a, b, d and e) and longitudinal sections (c and f) of the inflorescence stems of wild-type plants (a-c) and transgenic lines *35S::CKI1* (d-f). The arrows indicate the formation of ectopic tissues that resemble vascular bundles. **D, E.** Ectopic overexpression of *CKI1* leads to the formation of additional vegetative tissues initiated from lateral meristems. The node structures of wild-type and transgenic lines *35S::CKI1* (D), transverse and longitudinal sections from wild-type plants (E.a) and transgenic lines *35S::CKI1* (E.b). The arrow indicates an ectopic axillary bud formed in transgenic lines *35S::CKI1*. Scale bars 100 μm. **F.** Ectopic overexpression of *CKI1* leads to disruptions of proper development of vascular bundles in *A. thaliana*. Transverse sections of the inflorescence stems of transgenic lines with overexpression of $CKI1^{H405Q}$ (35S::CKI1 ^{H405Q}, a, b a c), wild-type (d) and double mutant *ahk2 ahk3* (e). Scale bars 100 μm. (f) Expression analysis of transgenic lines $35S::CKI1^{H405}$. Total protein from 6-day-old transgenic plants was analysed using an immunoblot assay (Western blot) with an anti-HA-epitope antibody and anti-actin antibody. Actin served as an internal control. In the lines with an overexpression of the mutant allele of *CKI1* ($CKI1^{H405Q}$), the phenotype identified in vascular tissues was to a large extent opposite to the phenotype in lines with an overexpression of the wild-type allele of *CKI1*. In the inflorescence stems, a smaller number of cell layers, particularly in case of cambium cells, and an uneven size of cells in both xylem and phloem were identified (see Fig. 6). A similar phenotype was identified also in double mutants *ahk2 ahk3*.

These results show that both qualitative change in the expression of the wild-type allele of *CKI1* (i.e. both decrease and increase) and expression of a mutant allele, encoding CKI1 with a modified activity, lead to the change in the phenotype of vascular tissues. This fact can be used for the regulation of the production properties of plants with an economic effect. At the same time, the results of the analysis of the lines bearing mutations in the genes homologous to the gene *CKI1* have shown that it is possible to use the gene engineering methods for the regulation of the development

of vascular tissues in plants by means of genetic manipulations in the genes belonging to the family of sensor histidine kinases, i.e. the homologues and the orthologues of *CKI1*.

Example 6

CKI1 participating in the development of vascular tissues in plant

It was observed that approximately 7 to 8 layers of cambial tissue were formed in transgenic plant *35S::CRE1-CKI1* modified so as to overexpress only the kinase region of CKI1 and CKI1 (*35S::CKI1*), while only 3 layers were formed in wild type line (Col-0) (see Fig. 9A). To the contrary, it was observed that the cambial tissues lost their normal shapes in *35S::CKI1*^{H405Q} where the kinase activity of CKI1 was deleted, CKI1 RNAi where the amount of the expressed product was decreased by RNA interference (RNAi), and other plants where the expression amounts were decreased by T-DNA insertion (*CKI1/cki1-5*: approximately 20% of abnormal vascular tissues and approximately 80% of normal vascular tissues, *CKI1/cki1-6*: approximately 50% of abnormal vascular tissues) (Figs. 9A and 9B) (Pischke et al. 2002. PNAS. 99: 15800-15805).

The results shown in Fig. 9 reveal that CKI1 plays an important role in development of phloem and xylem consisting in vascular tissue. The wild type line used as a control was ecotype Ws-2 of *Arabidopsis thaliana*.

The amount of protein *CKI1*^{H405Q} expressed in plant was determined by immunoblotting, and the obtained results are shown in Fig. 6. Further, the decrease of gene expression in *CKI1* RNAi was determined by Quantitative Real Time Polymerase Chain Reaction, and the obtained results are shown in Fig 11. Fig. 6 shows the amount of protein *CKI1*^{H405Q} determined by immunoblotting, indicating the increase of the foreign protein expression in *CKI1*^{H405Q} overexpressing plant lines Nos. 6 and 9. In addition, Fig. 11 shows that the expressed amounts in the plants with decreased expression of gene CKI1 by RNA interference or T-DNA insertion is decreased by at least 50% compared with those in wild type.

The above results shows that in the secondary growth for increasing biomass of plant, gene CKI1 and/or homologues or orthologues thereof are essential to vascular tissue formation for the growth in volume of plant, and that the activity of cambial cells functioning as mother cells for vascular tissue is regulated by CKI1 and/or homologues or orthologues thereof.

Industrial Applicability

The present invention comprising genetic manipulations of the activity of *CKI1* and the homologues and the orthologues thereof, provides for targeted obtaining of plants with an increased production of biomass. Another possible uses are in e.g. biotechnological applications allowing the decontamination of contaminated soils by phytoremediation. In the development of these technologies, the use of transgenic plants producing enzymes allowing the decontamination by decomposition of pollutants, e.g. petrol products, is considered and an increased production of biomass can increase the efficiency of their use. An increased deposition of cellulose, observed in transgenic lines, is suitable for use in paper and wood-processing industry, wherein it can particularly facilitate and reduce the prices of the time-consuming wood production. The increase of biomass in some plant species will be advantageous also for the food-processing and pharmaceutical industry. An important area of utility is the production of the so-called energy plants, e.g. Uteuša sorrel, wherein such genetic modification can enhance the energetic balance of the plants. Potentially, the invention can be used in breeding of plants, wherein the regulation of the formation of lignified tissues and of lignification of vascular tissues and interfascicular arc fibres can be used in breeding of plant varieties with improved stem erectness (e.g. to avoid procumbent-like phenotype of cereals or rapeseed).

CLAIMS

1. A method of regulation of biomass production in a plant, characterized in that the gene *CKI1* or a homologue or an orthologue thereof is identified in the plant and the expression of the gene *CKI1* or the homologue or the orthologue thereof or the activity of the gene product is modified.

2. The method according to claim 1, characterized in that the identification of the homologue or the orthologue of the gene *CKI1* in the plant species comprises the following steps:

i) identification of the nucleotide sequence homology or amino acid sequence homology,

ii) proving the histidine kinase activity of the gene product of the identified gene,

iii) identification of the expression of the homologue or the orthologue in vascular tissues or the tissues adjacent to vascular tissues of said plant species.

3. The method according to claim 2, characterized in that the step i) is carried out by searching genome databases for sequences similar to conserved regions of sensor histidine kinases, obtained by comparing the already identified homologues of *CKI1* or experimentally by means of hybridization of the genome library or cDNA library of the respective species with a probe obtained from genomic DNA or cDNA of *CKI1* or any of the already identified homologues thereof, preferably the genes *AHK2, AHK3* or *AHK4/CRE1/WOL.*

4. The method according to claim 2, characterized in that the step ii) is carried out by means of the expression of said gene in heterologous or homologous expression system and subsequent analysis of its activity.

5. The method according to claim 2, characterized in that the step ii) is carried out by means of complementation of bacterial, yeast or plant mutants in the genes for sensor histidine kinases using the encoding sequence of the homologue or the orthologue, inserted under the control of promoter of said gene and in the respective mutant background.

6. The method according to claim 2, characterized in that the step ii) is carried out by means of analysis of the activity of the gene product in the cytokinin signal transduction in plant protoplasts.

7. The method according to claim 1, characterized in that the modification of the expression of the gene CKI1 or the homologue or the orthologue thereof comprises the modification of the expression of histidine kinase coding region of said gene.

8. The method according to claim 1, characterized in that the expression of the gene *CKI1* or the homologue or the orthologue thereof or the activity of the gene product is modified in such a way that it is decreased or eliminated.

9. The method according to claim 8, characterized in that the expression of the gene *CKI1* or the homologue or the orthologue thereof is decreased by RNA interference.

10. The method according to claim 9, characterized in that the construct for the RNA interference is prepared by insertion of recombinant DNA containing parts of the sequence of cDNA of the homologue or the orthologue in inverse repetition, separated by a sequence of another DNA, preferably by a part of the encoding sequence *uidA* or a natural introne of the homologue or the orthologue of *CKI1*, under the control of a constitutively active promoter or a conditionally active promoter and termination by transcription terminator.

11. The method according to claim 8, characterized in that the expression of the gene *CKI1* or the homologue or the orthologue thereof is decreased by insertion mutagenesis.

12. The method according to claim 8, characterized in that the activity of the gene product is modified by site-directed mutagenesis.

13. The method according to claim 12, characterized in that the mutation is carried out in a triplet encoding a selected amino acid, preferably an amino acid participating in

the phosphate transfer by the respective sensor histidine kinase, in potential interactions with regulatory proteins, or an amino acid with a regulatory activity.

14. The method according to claim 13, characterized in that a replacement in a triplet encoding the amino acid histidine in the position corresponding to the position 405 in CKI1 (H405) or the aspartic acid in the position corresponding to the position 1050 (D1050) in CKI1 is performed by the mutation.

15. The method according to claim 1, characterized in that the expression of the gene *CKI1* or the homologue or the orthologue thereof or the activity of the gene product is modified in such a way that it is increased.

16. The method according to claim 15, characterized in that the expression of the gene *CKI1* or the homologue or the orthologue thereof or the activity of the gene product is increased by means of an overexpression vector comprising

a promoter for the expression in plant cell;

one or more genes selected from the group consisting of *CKI1* and the homologues and the orthologues thereof, operable linked to the promoter; and

a transcriptional terminator for plant cell.

17. The method according to claim 1, characterized in that it further comprises the step of analysis of the phenotype of the development of vascular tissues, comprising histological staining of sections of living plants with a mixture of orange GG and aniline blue and the analysis of the stained sections by DIC or fluorescence microscopy.

18. The method according to claim 1, wherein the plant is selected from the group comprising rice, corn, barley, wheat, poplar and birch.

19. A nucleotide sequence, characterized in that the sequence of nucleotides is at least in 20 % identical to the sequence of nucleotides of the sequence *CKIp2* (Sequence ID No. 4).

20. A recombinant DNA sequence for the regulation of gene expression by RNA interference,

comprising

- a constitutively active or conditionally active promoter,
- recombinant DNA containing parts of cDNA sequence of said gene in reverse repetition, separated by another DNA sequence, preferably by part of the sequence uidA (Sequence ID No. 7) or a natural intron of the silenced gene,
- a transcription terminator.

21. The recombinant DNA sequence according to claim 20, having the sequence of nucleotides at least in 20 % identical to the sequence of nucleotides of the sequence *35S::CKIi2::pA* (Sequence ID No. 7).

22. A method of preparation of the recombinant DNA for the regulation of gene expression by RNA interference, characterized in that a specific part of the cDNA or the encoding region of the silenced gene is amplified using primers, which contain in addition to the sequence of 18-21 nucleotides specific for cDNA or the encoding sequence of said gene also regions inserted to the 5′ end of these primers, designated RNAi_up and RNAi_down,

RNAi_up:

BamHI HindIII
5′ – TAT AGG ATC CAA GCT T – 3′

RNAi_down:

XbaI
5′ – CAC TTC TAG A[illegible] C – 3′

a connecting sequence is then amplified using primers consisting of 18-21 nucleotides specific for said connecting sequence and sequences LOOP_up and LOOP_down, inserted to the 5′ end of these primers,

LOOP_up:

BamHI
5′ – CAG CGG ATC C – 3′

LOOP_down:

HindIII
5′ – TTC CAA GCT T – 3′

and by means of digestion by restriction endonucleases BamHI, HindIII, XbaI and SalI and joining of the resulting fragments by ligase, these three parts are connected into one resulting construct which is then inserted under the control of a suitable promoter.

23. The method according to claim 22, wherein the connecting sequence is the part *GUSp* (Sequence ID No. 6) of the encoding sequence of the gene *uidA* (Sequence ID No. 5), which is amplified using the primers Bgus and Hgus:

Bgus:

BamHI
5′ – CAG CGG ATC CCT CTA CAC CAC GCC GAA CAC C – 3′
LOOP_up

Hgus:

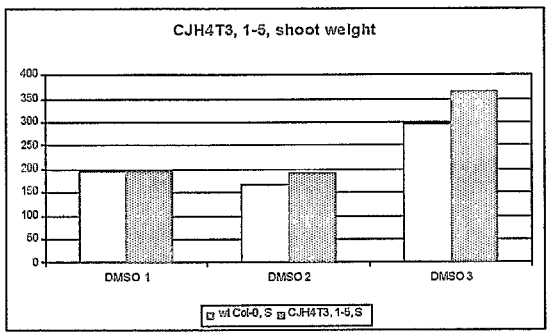
HindIII
5′ – TTC CAA GCT TTT CTC TGC CGT TTC CAA ATC G – 3′
LOOP_down

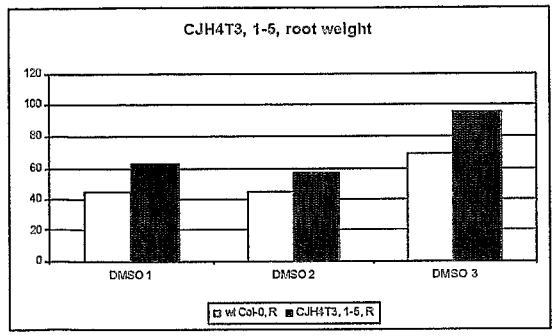
24. A recombinant DNA for the regulation of the activity of a gene product, containing a modified cDNA or genomic DNA of said gene under the control of a constitutively or conditionally active promoter.

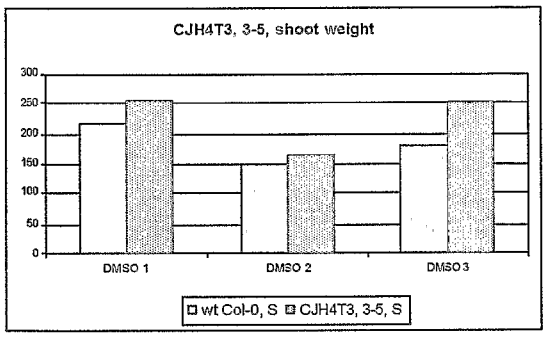
25. An expression vector for overexpression of one or more genes selected from the group consisting of CKI1 or the homologue or the orthologue thereof, comprising
- a promoter for the expression in plant cell, preferably CaMV 35S promoter;
- one or more genes selected from the group consisting of *CKI1* and the homologues and the orthologues thereof, operable linked to the promoter; and
- a transcriptional terminator for plant cell.

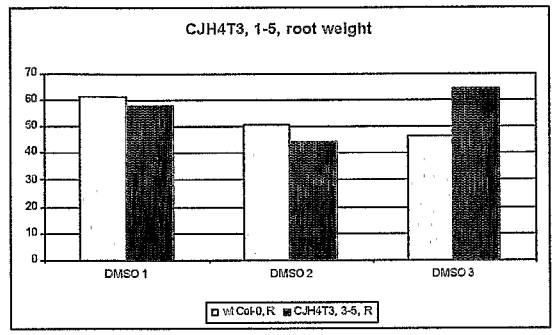
26. An agent for regulating the growth in volume of a plant comprising one or more active ingredients selected from the group consisting of one or more proteins selected from the group consisting of gene products of *CKI1* and the homologues and the orthologues thereof; and the expression vector according to claim 25.

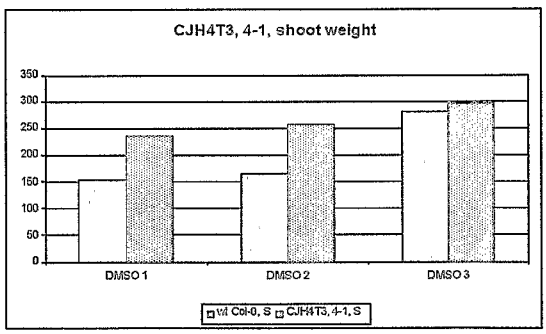
Fig. 1


CJH4T3, 1-5, shoot weight
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350
300
250
200
150
100
50
0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, S
CJH4T3, 1-5, S


CJH4T3, 1-5, root weight
120
100
80
60
40
20
0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, R
CJH4T3, 1-5, R


CJH4T3, 3-5, shoot weight
300
250
200
150
100
50
0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, S
CJH4T3, 3-5, S


CJH4T3, 1-5, root weight
70
60
50
40
30
20
10
0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, R
CJH4T3, 3-5, R


CJH4T3, 4-1, shoot weight
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300
250
200
150
100
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0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, S
CJH4T3, 4-1, S

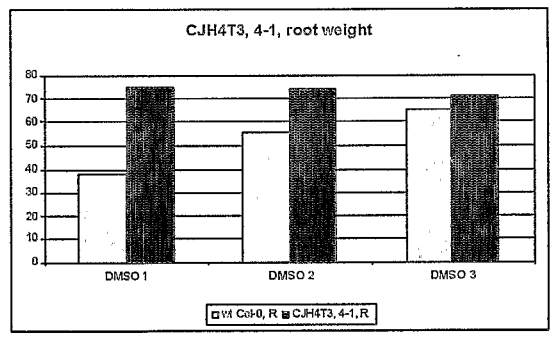
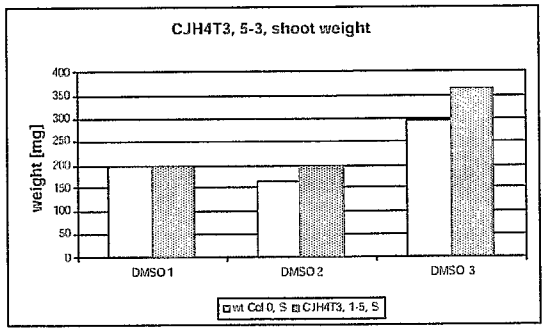
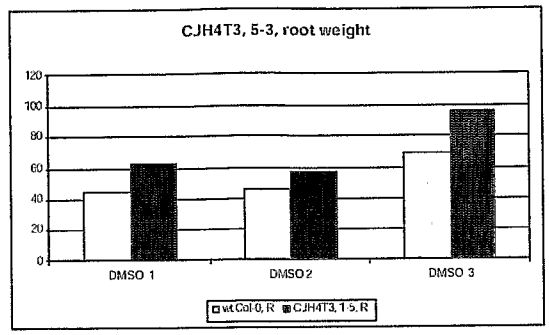
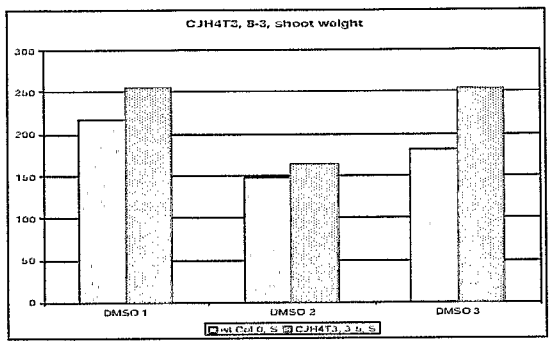
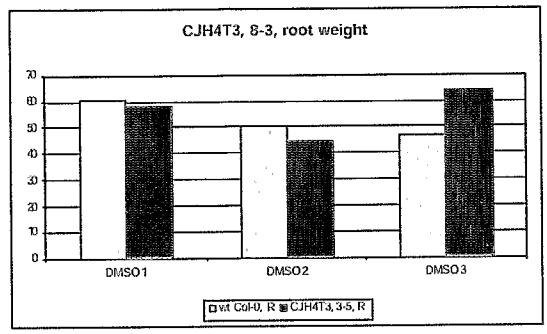

CJH4T3, 4-1, root weight
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60
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30
20
10
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DMSO 1
DMSO 2
DMSO 3
wt Col-0, R
CJH4T3, 4-1, R

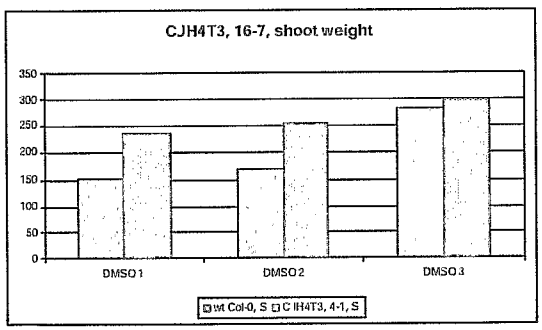
Fig. 1, cont.


CJH4T3, 5-3, shoot weight
weight [mg]
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350
300
250
200
150
100
50
0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, S
CJH4T3, 1-5, S


CJH4T3, 5-3, root weight
120
100
80
60
40
20
0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, R
CJH4T3, 1-5, R


CJH4T3, 8-3, shoot weight
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250
200
150
100
50
0
DMSO 1
DMSO 2
DMSO 3


CJH4T3, 8-3, root weight
70
60
50
40
30
20
10
0
DMSO1
DMSO2
DMSO3
wt Col-0, R
CJH4T3, 3-5, R


CJH4T3, 16-7, shoot weight
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300
250
200
150
100
50
0
DMSO1
DMSO2
DMSO3
wt Col-0, S
CJH4T3, 4-1, S

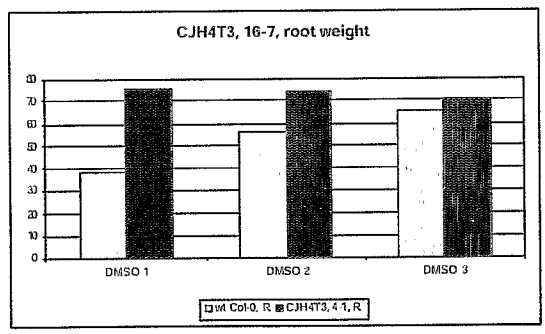

CJH4T3, 16-7, root weight
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70
60
50
40
30
20
10
0
DMSO 1
DMSO 2
DMSO 3
wt Col-0, R
CJH4T3, 4-1, R

Fig. 2

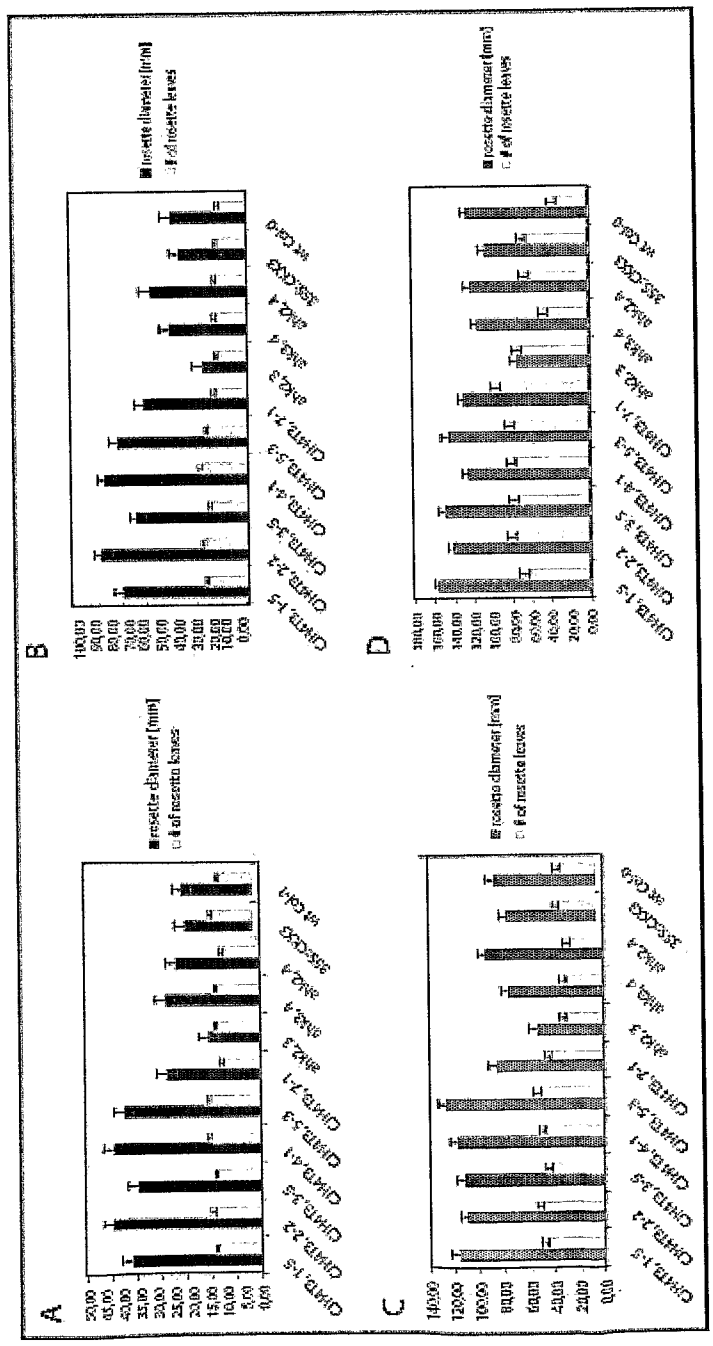


Fig. 3

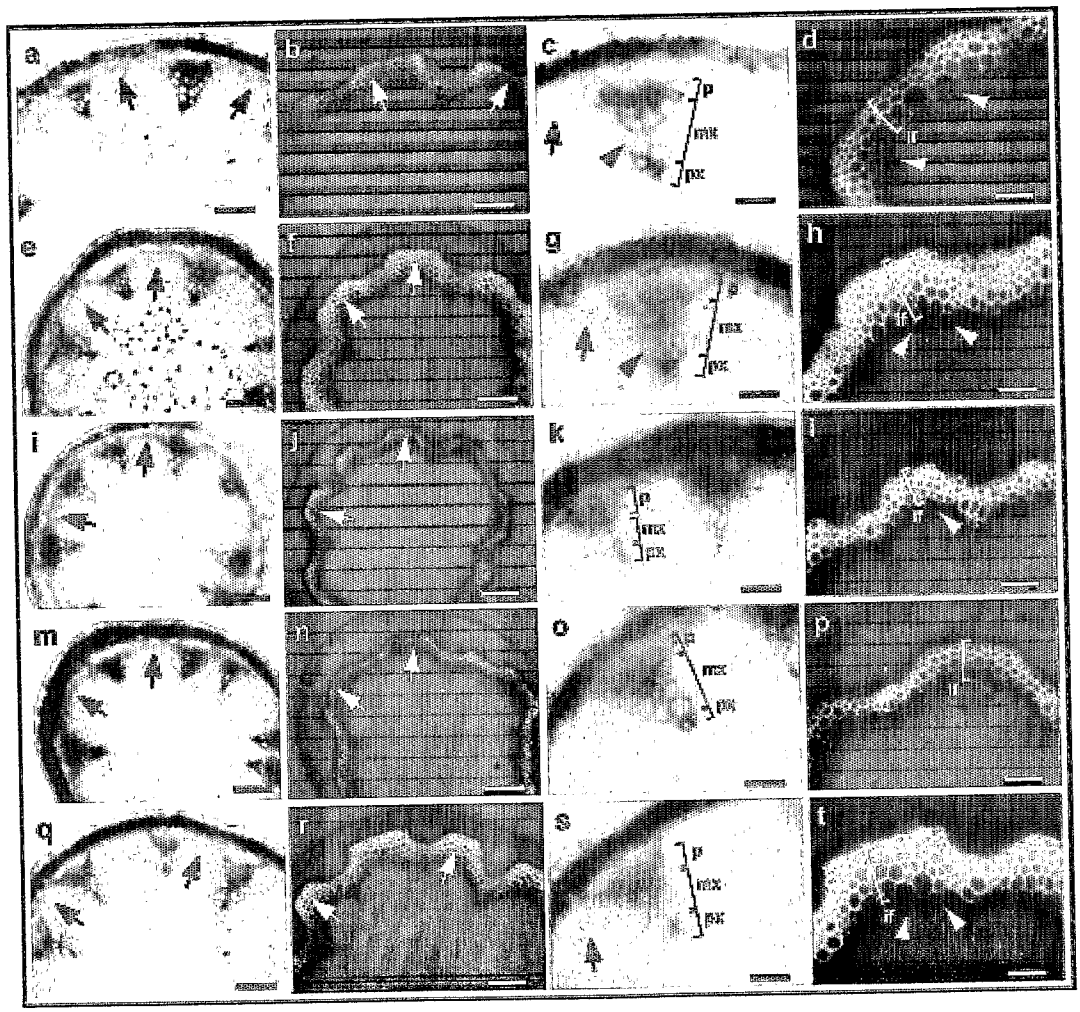


Fig. 4

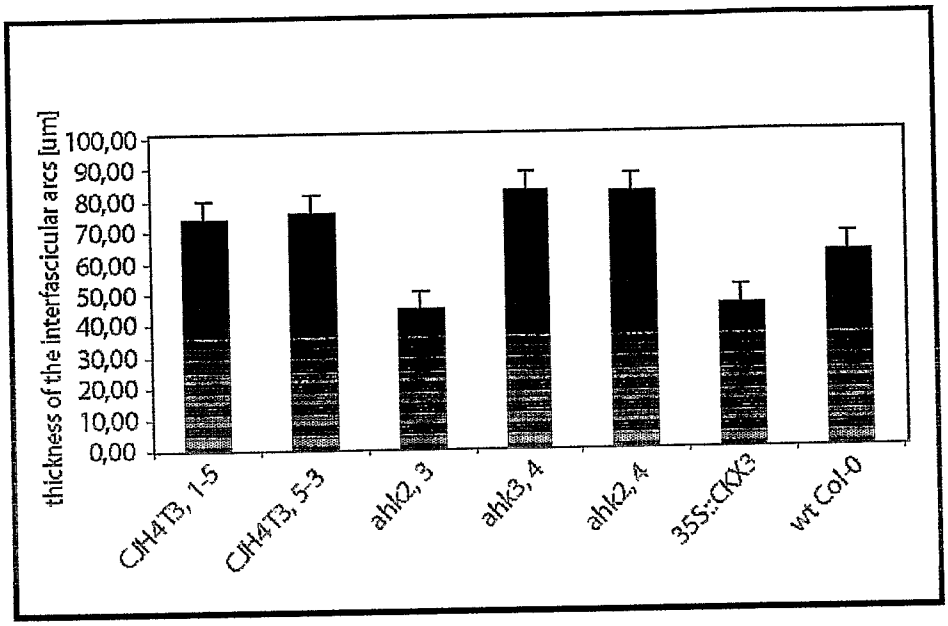

thickness of the interfascicular arcs [um]
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
CJH4T3, 1-5
CJH4T3, 5-3
ahk2, 3
ahk3, 4
ahk2, 4
35S::CKX3
wt Col-0

Fig. 5

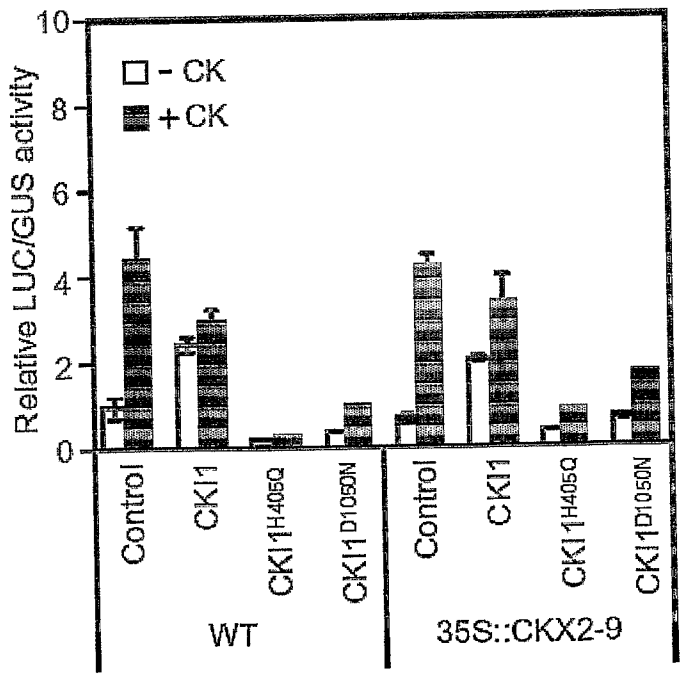

Relative LUC/GUS activity
10
8
6
4
2
0
- CK
+ CK
Control
CKI1
CKI1H405Q
CKI1D1050N
WT
Control
CKI1
CKI1H405Q
CKI1D1050N
35S::CKX2-9

Fig. 6

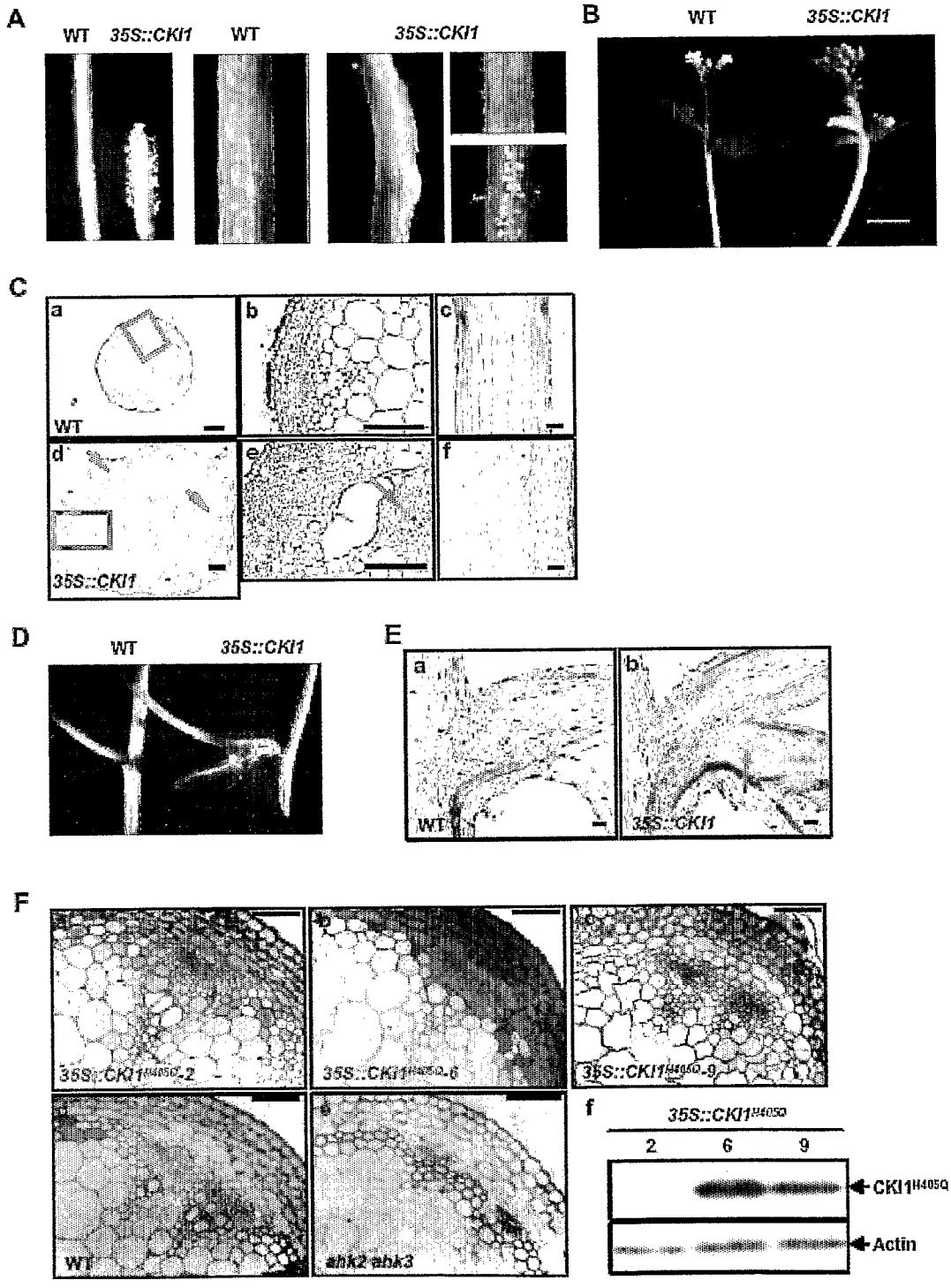

A
WT
35S::CKI1
WT
35S::CKI1
B
WT
35S::CKI1
C
a
b
c
WT
d
e
f
35S::CKI1
D
WT
35S::CKI1
E
a
b
WT
35S::CKI1
F
35S::CKI1H405Q-2
35S::CKI1H405Q-6
35S::CKI1H405Q-9
WT
ahk2 ahk3
f
35S::CKI1H405Q
2
6
9
CKI1H405Q
Actin

Fig. 7

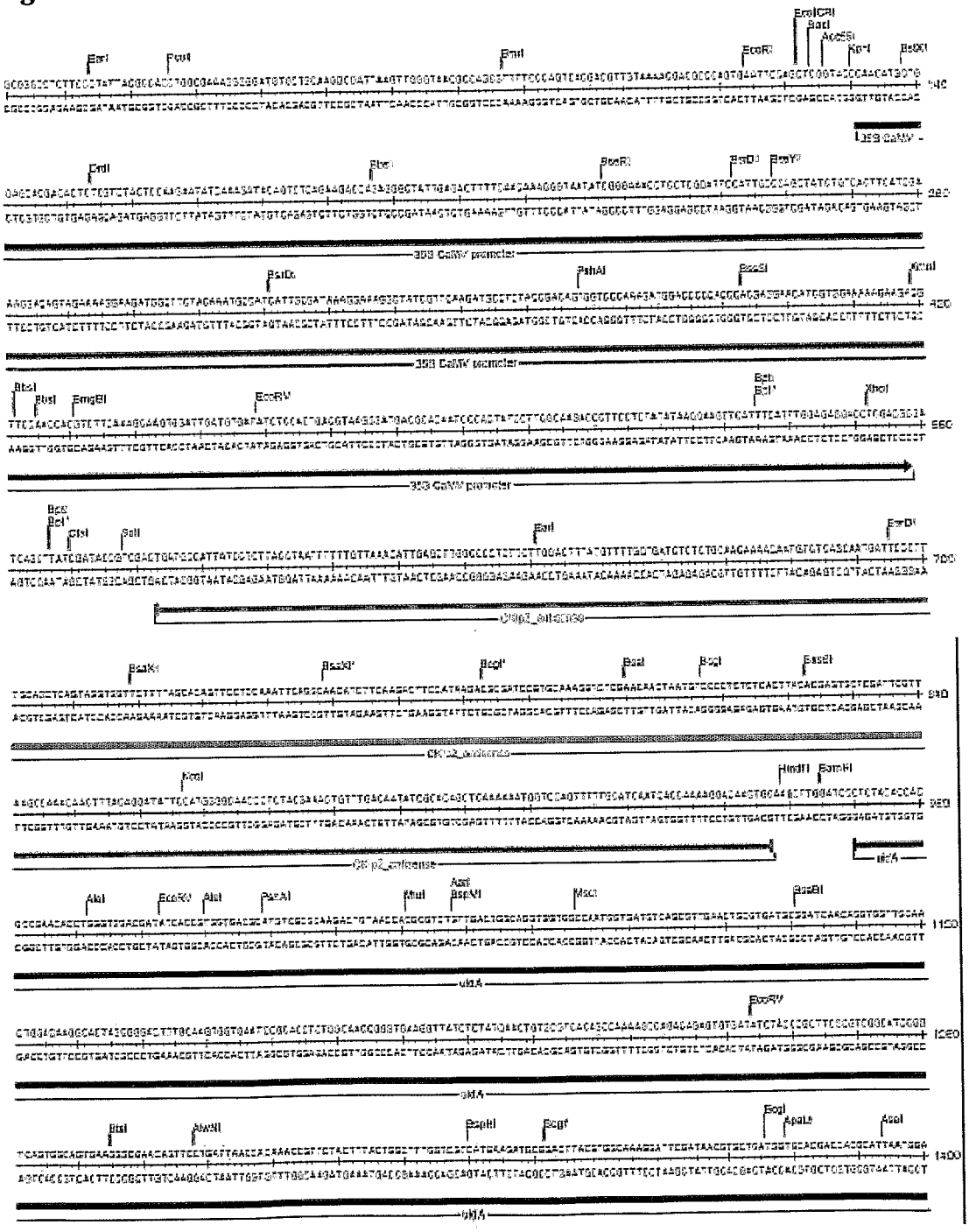

EcoICRI
BstI
Acc65I
KpnI
BstXI
EcoRI
EcoRV
BsaBI
BsaAI
BsiEI
BsrBI
BsrGI
BsiWI
AflII
BspHI
ScaI
ApaLI
AseI
35S CaMV promoter
35S CaMV terminator
CP12-2_antisense
CP12-2_promoter
GBSS1_antisense
GBSS1_promoter

Fig. 7 – cont.

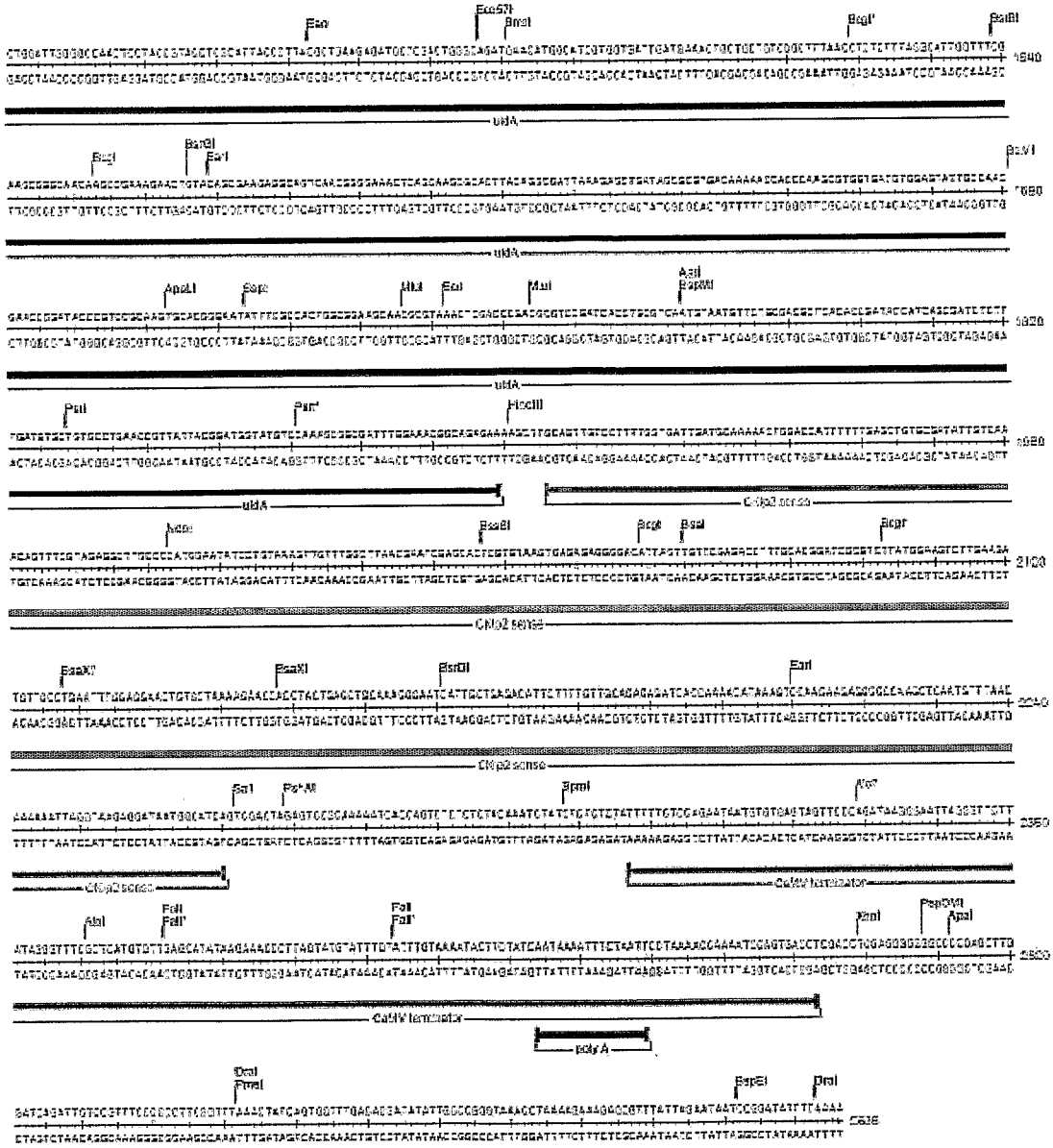

EcoRV
BmgI
BsiWI
uidA
BanGI
BsrI
EarI
BclVI
ApaLI
SspI
AhdI
EcoI
MunI
AsiI
PspXI
PstI
PsrI
HindIII
uidA
C-Vip2 sense
NdeI
BsaBI
BcgI
BsaI
BcgI
C-Vip2 sense
BsaXI
BsaXI
BsrDI
EarI
C-Vip2 sense
SacI
PstAI
BmgI
XcmI
C-Vip2 sense
CaMV terminator
AbaI
FatI
FatI'
FatI
FatI'
XcmI
PspOMI
ApaI
CaMV terminator
polyA
DraI
PmeI
BspEI
DraI

Fig. 8

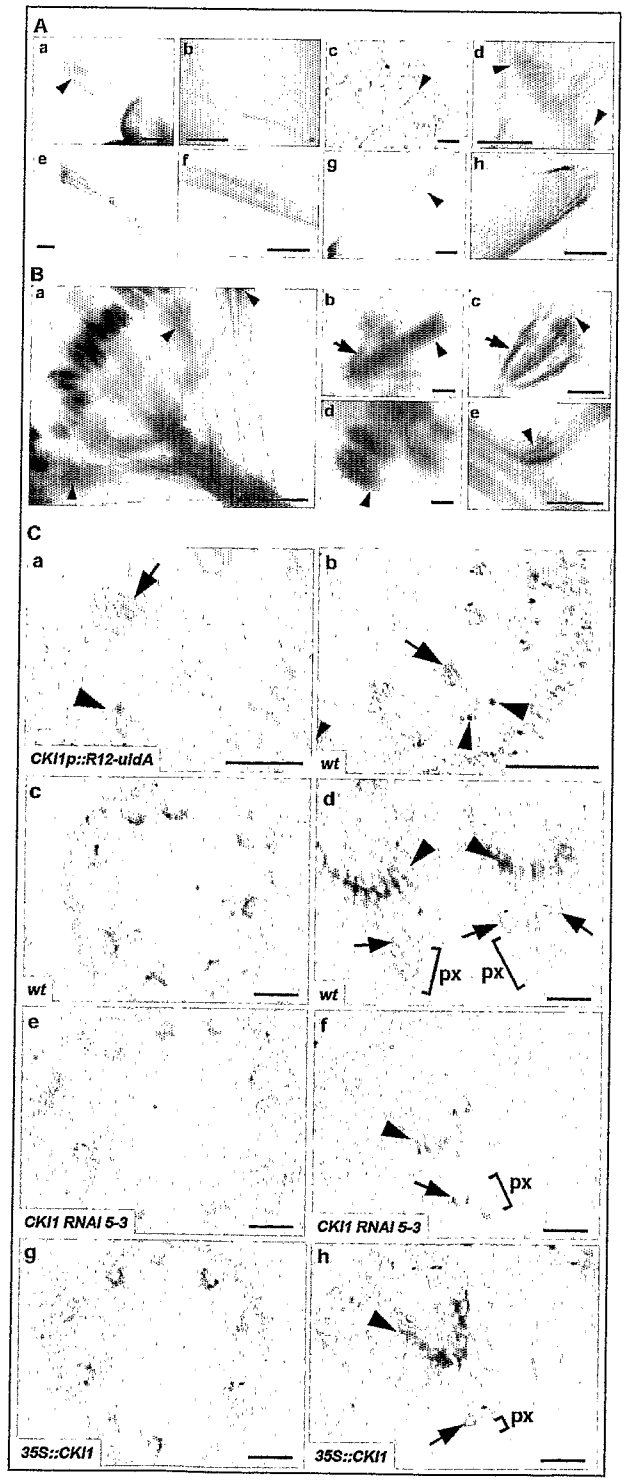

A
a
b
c
d
e
f
g
h
B
a
b
c
d
e
C
a
b
CKI1p::R12-uidA
wt
c
d
wt
px
px
wt
e
f
CKI1 RNAi 5-3
px
CKI1 RNAi 5-3
g
h
35S::CKI1
px
35S::CKI1

Fig. 9

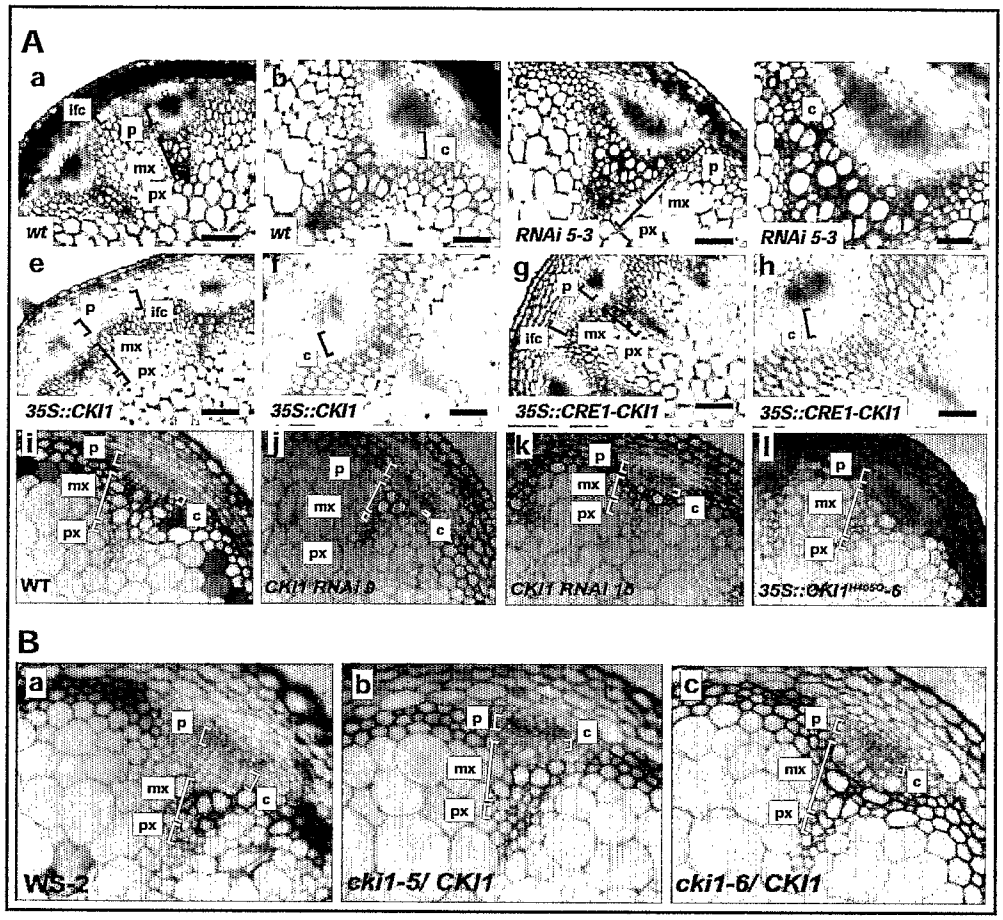
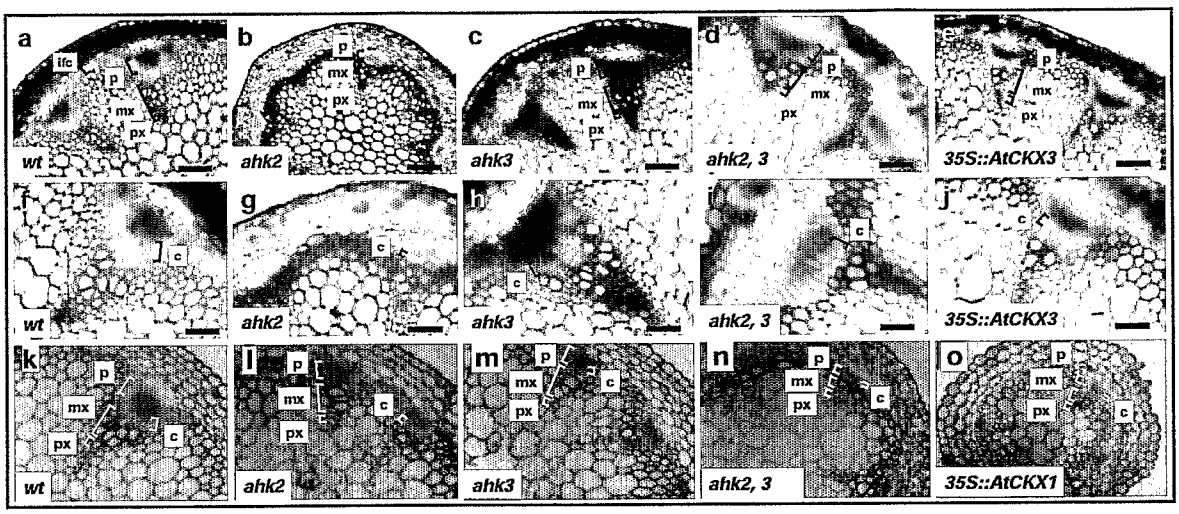
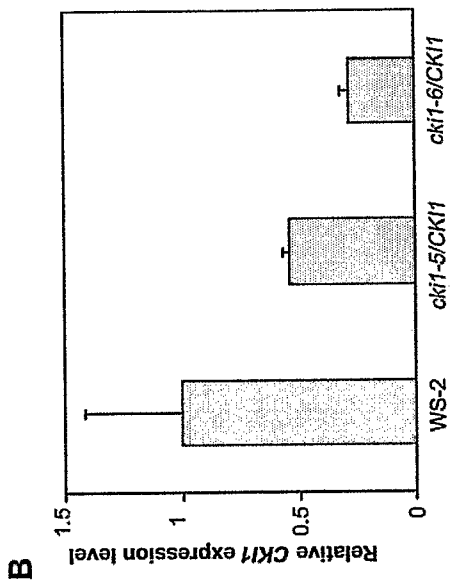

A
a
ifc
p
mx
px
wt
b
c
wt
RNAi 5-3
RNAi 5-3
e
35S::CKI1
35S::CKI1
35S::CRE1-CKI1
35S::CRE1-CKI1
WT
CKI1 RNAi 1b
B
WS-2
cki1-5/ CKI1
cki1-6/ CKI1

Fig. 10


a
ifc
p
mx
px
wt
b
p
mx
px
ahk2
c
p
mx
px
ahk3
d
p
mx
px
ahk2, 3
p
mx
px
35S::AtCKX3
c
wt
g
c
ahk2
h
c
ahk3
c
ahk2, 3
c
35S::AtCKX3
k
p
mx
px
c
wt
l
p
mx
px
c
ahk2
m
p
mx
px
c
ahk3
n
p
mx
px
c
ahk2, 3
o
p
mx
px
c
35S::AtCKX1


B
Relative CKI1 expression level
1.5
1
0.5
0
WS-2
cki1-5/CKI1
cki1-6/CKI1

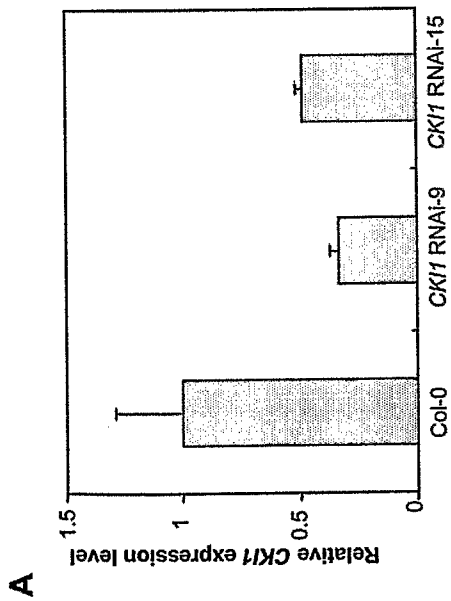
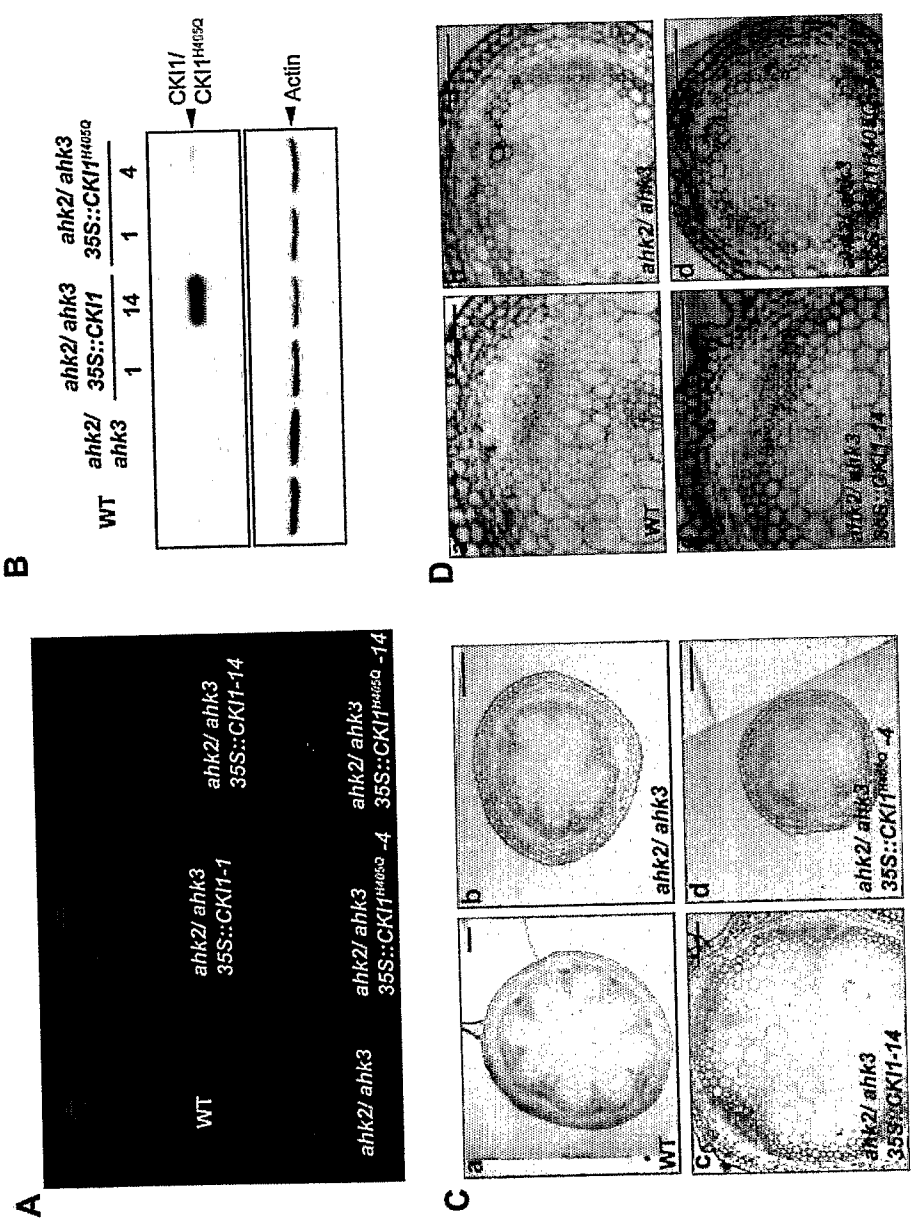

A
Relative CKI1 expression level
1.5
1
0.5
0
Col-0
CKI1 RNAi-9
CKI1 RNAi-15

Fig. 11

Fig. 12


A
WT
ahk2/ ahk3
ahk2/ ahk3 35S::CKI1-1
ahk2/ ahk3 35S::CKI1-14
ahk2/ ahk3 35S::CKI1H405Q -4
ahk2/ ahk3 35S::CKI1H405Q -14
B
WT
ahk2/ ahk3
ahk2/ ahk3 35S::CKI1
1
14
ahk2/ ahk3 35S::CKI1H405Q
1
4
CKI1/ CKI1H405Q
Actin
C
a
WT
b
ahk2/ ahk3
c
ahk2/ ahk3 35S::CKI1-14
d
ahk2/ ahk3 35S::CKI1H405Q -4
D
WT
ahk2/ ahk3