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(54) **GENE REGLANT LE CYCLE DE DIVISION CELLULAIRE DES
PLANTES**

(54) **GENE REGULATING CELL DIVISION CYCLE IN PLANTS**

(57) A method of modifying the development of a plant comprising transforming a plant cell with a nucleic acid encoding a cyclin dependent kinase inhibitor to produce a transformed plant cell; and, regenerating the plant from the transformed plant cell under conditions wherein the cyclin dependent kinase inhibitor is expressed during regeneration of the plant to modify the development of the plant, wherein the nucleic acid encoding the cyclin dependent kinase inhibitor is a gene selected from the group consisting of ICK2, ICN2, ICN6, ICN7 and homologs thereof.

ABSTRACT

A method of modifying the development of a plant comprising transforming a plant cell with a nucleic acid encoding a cyclin dependent kinase inhibitor to produce a transformed plant cell; and, regenerating the plant from the transformed plant cell under conditions wherein the cyclin dependent kinase inhibitor is expressed during regeneration of the plant to modify the development of the plant, wherein the nucleic acid encoding the cyclin dependent kinase inhibitor is a gene selected from the group consisting of ICK2, ICN2, ICN6, ICN7 and homologs thereof.

CYCLIN-DEPENDENT KINASE INHIBITORS AS PLANT GROWTH REGULATORS

FIELD OF THE INVENTION

The invention relates to the modification of growth and development of plants
5 through transgenic sense or anti-sense expression of cyclin-dependent kinase inhibitor genes.

BACKGROUND OF THE INVENTION

In eucaryotes including plants, the progression of cell cycle events is regulated by a
network of gene products and factors to ensure that this crucial process is initiated as an
integral part of the growth and the developmental program, and in response to the external
10 environment. These factors exert their influences on the cell cycle machinery via various
pathways. At the center of the machinery lies an enzyme complex consisting of a catalytic
subunit, cyclin-dependent protein kinase (CDK), and a regulatory subunit, cyclin. CDKs are a
group of related serine/threonine kinases and their activity generally depends on their
association with cyclins (Pines, 1995).

15 Early work disclosed the existence of CDKs in yeast. A CDK called Cdc2 (p34^{cdc2}, or
CDK1) was identified in fission yeast *Schizosaccharomyces pombe* (Hindley and Phear,
1984) and a Cdc2 homolog called CDC28 was identified in budding yeast *Saccharomyces
cerevisiae* (Lőrincz and Reed, 1984). In yeast, Cdc2 (or CDC28) kinase appears to be solely
responsible for regulating the progression of the cell cycle.

20 Animal cells have evolved several Cdc2-related CDKs in order to achieve more
complex regulation at multiple levels. In mammalian cells, seven distinct CDKs and eight
types of cyclins have been identified (see review by Pines, 1995). Complexes of these CDKs
and cyclins appear to act sequentially at different checkpoints during the cell cycle, while
incorporating the input of different developmental and environmental cues.

25 Plants, like higher animals, have multiple CDKs (Francis and Halford, 1995; Jacobs,
1995) and cyclins (Renaudin et al., 1996). In *Arabidopsis thaliana*, at least two Cdc2
homologues, Cdc2a and Cdc2b (Ferreira et al., 1991; Hirahama et al., 1991) and as many as
twelve cyclins belonging to three groups (Renaudin et al., 1996) have been documented. Of
the two Cdc2 homologues in *A. thaliana*, Cdc2a resembles more closely Cdc2 homologues

from other species because it has a conserved PSTAIRE motif and is able to genetically complement yeast *cdc2* or *CDC28* mutants (Ferreira et al., 1991; Hirahama et al., 1991), indicating some functional homology of *A. thaliana* Cdc2a with the yeast Cdc2 kinase. Expression analyses showed that *A. thaliana cdc2a* expression was correlated with the
 5 “competence” of a cell to divide and preceded the re-entry of differentiated cells into the cell division cycle (Martinez et al., 1992; Hemerly et al., 1993), and expression of a dominant negative *cdc2a* mutant resulted in cell cycle arrest (Hemerly et al., 1995). *A. thaliana* Cdc2b is atypical in that it has a PPTALRE motif in place of the PSTAIRE motif. Like *cdc2a*, *cdc2b* is also expressed in dividing plant cells. While *cdc2a* is expressed constitutively
 10 throughout the cell cycle, *cdc2b* is reportedly expressed preferably in S and G2 phases (Segers et al., 1996).

Relatively little is known about the cyclins and other proteins and factors which regulate the activity of CDK-cyclin complexes in plant cells. Results from yeast and mammalian studies have demonstrated multiple pathways, both positive and negative, by
 15 which CDK activity can be modulated (Lees, 1995). In addition to binding by a cyclin, for example, activation of CDKs may also involve a CDK-activating kinase (CAK) which itself is a CDK, and CDC25 protein phosphatase.

A new aspect of regulating CDK activity was discovered with the identification of CDK inhibitors (see reviews by Pines, 1995; Sherr and Roberts, 1995; Harper and Elledge, 1996). These small proteins are understood to bind stoichiometrically to negatively regulate
 20 the activity of CDKs. It has been suggested that these inhibitors may be involved in animal development and cancer, in addition to their role in cell cycle regulation (Harper and Elledge, 1996). A plant CDK inhibitor activity was observed and was suggested to be involved in endosperm development in maize (Grafi and Larkins, 1995).

The activity of CDK inhibitors has been studied in animals. Transgenic mice have been generated lacking p21, p27 and p57 CDK inhibitor genes. The p21 knockout mice are reported to develop normally but are deficient in G1 checkpoint control, such as cell cycle
 25 arrest in response to DNA damage (Deng et al., 1995). Analysis of p27 knockout mice from three independent studies show that transgenic mice lacking p27 display larger body size than
 30 control mice (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). The enhanced

growth is reportedly due to an increase in cell number (Kiyokawa et al., 1996) and is gene dose-dependent (Fero et al., 1996). In comparison, none of p21 or p57 knockout display enhanced growth. The transgenic mice lacking p57 show a range of developmental defects such as defective abdominal muscles, cleft palate and renal medullary dysplasia (Yan et al., 1997; Zhang et al., 1997). A few developmental defects were observed in p27^{-/-} mice. They include impaired ovarian follicles (thus female sterility), impaired luteal cell differentiation and a disordered estrus cycle. These results reflect a disturbance of the hypothalamic-pituitary-ovarian axis. In comparison, transgenic mice lacking p21 appear to develop normally at both gross anatomic and histologic levels (Deng et al., 1995). In addition, an increase in apoptosis is observed in mice lacking p57. The CDK inhibitor p27 was over-expressed in mouse hepatocytes (Wu et al., 1996), resulting in a general decrease in overall number of adult hepatocytes which result in aberrant tissue organization, body growth and mortality.

Despite the general conservation of basic cell cycle machinery in eucaryotes, the role of plant cell division during plant growth and development is characteristically different from other eucaryotic cells. In many respects, the regulation of plant cell division and growth can be regarded as distinct from other eucaryotic cells. For example, plant cells are not mobile during morphogenesis. Different sets of hormones are involved in modulating plant growth and development. Plant cells are remarkable for their ability to re-enter the cell cycle following differentiation. Also, cell division in plants is continuous, along with organ formation, and plant body size (the number of total cells and size of the cells) can vary dramatically under different conditions. Plants also have an inherent ability to incorporate additional growth into normal developmental patterns, as is illustrated by a study showing that ectopic expression of a mitotic cyclin driven by the *cdc2a* promoter resulted in a larger but normal root system (Doerner et al., 1996). However, relatively little is known about the interaction of the regulatory genes controlling cell division patterns in plants (Meyerowitz, 1997).

A few studies of transgenic expression of cell cycle genes in plants are documented using various cell cycle genes other than CDK inhibitors. A heterologous yeast *cdc25*, a mitotic inducer gene, was introduced into tobacco plants under the control of a constitutive

CaMV 35s promoter (Bell et al., 1993). Transgenic tobacco plants showed abnormal leaves (lengthened and twisted lamina, pocketed interveinal regions), abnormal flowers, and also precocious flowering. Analysis of cell size in the root meristem revealed that transgenic plants expressing the yeast *cdc25* had much smaller cells (Bell et al., 1993). The wild type *cdc2a* gene and variants of dominant negative mutations under the control of CaMV 35s promoter have been used to transform tobacco and Arabidopsis plants (Hemerly et al., 1995). Constitutive expression of wild-type and mutant Cdc2a did not significantly alter the development of the transgenic plants. For the dominant negative Cdc2a mutant, it was not possible to regenerate Arabidopsis plants. Some tobacco plants expressing this construct were obtained and they had considerably fewer but much larger cells. These cells, however, underwent normal differentiation. Morphogenesis, histogenesis and developmental timing were unaffected (Hemerly et al., 1995). As mentioned above, ectopic expression of an Arabidopsis mitotic cyclin gene, *cyc1At*, under the control of the *cdc2a* promoter increases growth without altering the pattern of lateral root development in Arabidopsis plants (Doerner et al., 1996).

The yeast two-hybrid system has been used to identify the cyclin-dependent kinase inhibitor gene *ICK1* from a plant (Wang et al., 1997). ICK1 is different in sequence, structure and inhibitory properties from known mammalian CDK inhibitors. It has been shown that recombinant protein produced from this gene in bacteria is able to inhibit plant Cdc2-like kinase activity *in vitro* (Wang et al., 1997).

Cytotoxin genes, *i.e.* genes encoding a protein which will cause cell death, have been tested in transgenic plants for genetic ablation of specific cells or cell lines during development, including RNase (Mariani et al., 1990), DTT chain A (Thorsness et al., 1991; Czako et al., 1992), Exotoxin A (Koning et al., 1992) and ribosomal inhibitor proteins (United States Patent No. 5,723,765 issued 3 March 1998 to Oliver *et al.*). Several disadvantages may be associated with the use of cytotoxin genes for modification of transgenic plants, particularly plants of agronomic importance. The action of the cytotoxin may not be specific and may result in non-specific destruction of plant cells. This effect may be the result of diffusion of the cytotoxin, or of non-specific expression of the cytotoxin gene in non-target tissues. Non-specific low-level expression of the cytotoxin may be a difficult

problem to overcome, since most tissue-specific promoters have some levels of expression in other tissues in addition to a high level of expression in a particular tissue. Expression of a potent cytotoxin gene even at a low concentration may have a negative impact on growth and development in non-target tissues. The presence of cytotoxic proteins of transgenic origin
5 may also have a negative effect on the marketability of an edible plant, or plant product, even if the cytotoxin is demonstrably benign to consumers.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides methods for using CDK inhibitor genes to
10 modify the growth and development of plant cells and organs. In particular, the invention provides a method of modifying the development of a plant comprising (*i.e.* having or including, but not limited to) transforming a plant cell with a nucleic acid encoding a cyclin dependent kinase inhibitor to produce a transformed plant cell. A plant may then be regenerated from the transformed plant cell under conditions wherein the cyclin dependent
15 kinase inhibitor is expressed during regeneration of the plant to modify the development of the plant. The nucleic acid encoding the cyclin dependent kinase inhibitor may be homologous to *ICK1*, or may be *ICK1*, respectively encoding a cyclin-dependent kinase inhibitor homologous to *ICK1* or *ICK1* itself. In particular embodiments, the plant may be *A. thaliana*, or a member of the *Brassica* genus, or a canola variety. The nucleic acid encoding
20 the cyclin-dependent kinase inhibitor may be operably linked to a tissue-specific promoter, such as *AP3* or a promoter homologous to *AP3*. In particular embodiments, the tissue-specific promoter may mediate expression of the nucleic acid encoding the cyclin-dependent kinase inhibitor in petal and stamen primordia, and the development of the plant may be modified so that the plant is male sterile.

25 Another aspect of the invention provides transgenic plants comprising (*i.e.* having or including, but not limited to) an expressible heterologous nucleic acid encoding a cyclin dependent kinase inhibitor, wherein the heterologous nucleic acid is introduced into the plant, or an ancestor of the plant, by the foregoing method. Alternatively, the plants may comprise a nucleic acid encoding a cyclin dependent kinase inhibitor, and the plant cells may be
30 transformed with an anti-sense nucleic acid complementary to the nucleic acid encoding the

cyclin dependent kinase inhibitor, to produce a transformed plant cell. So that regenerating the plant from the transformed plant cell under conditions wherein the anti-sense nucleic acid is transcribed during regeneration of the plant inhibits the expression of the cyclin dependent kinase inhibitor and modifies the development of the plant. Plant of the invention may have a recombinant genome and the heterologous nucleic acid may be integrated into the recombinant genome. The invention encompasses plant tissues, such as seeds, comprising a heterologous nucleic acid encoding a cyclin dependent kinase inhibitor, or an anti-sense construct, that is expressed during the development of a plant from the tissue to modify the development of the plant.

The invention also provides methods of identifying nucleic acids that encode cyclin-dependent kinase inhibitors, such as nucleic acids homologous to *ICK1*, that are active in plants to modify the development of the plants.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 to 4 show the cDNA sequences of *ICK2*, *ICN2*, *ICN6* and *ICN7*, respectively; and

Figure 5 shows the alignment of deduced amino acid sequences of *ICK1*, *ICK2*, *ICN2*, *ICN6* and *ICN7*.

DETAILED DESCRIPTION OF THE INVENTION

In the following detailed description, various examples are set out of particular embodiments of the invention, together with experimental procedures that may be used to implement a wide variety of modifications and variations in the practice of the present invention.

In the context of the present invention, "promoter" means a sequence sufficient to direct transcription of a gene when the promoter is operably linked to the gene. The promoter is accordingly the portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not

universally, located in the 5' non-coding regions of a gene. A promoter and a gene are "operably linked" when such sequences are functionally connected so as to permit gene expression mediated by the promoter. The term "operably linked" accordingly indicates that DNA segments are arranged so that they function in concert for their intended purposes, such as initiating transcription in the promoter to proceed through the coding segment of a gene to a terminator portion of the gene. Gene expression may occur in some instances when appropriate molecules (such as transcriptional activator proteins) are bound to the promoter. Expression is the process of conversion of the information of a coding sequence of a gene into mRNA by transcription and subsequently into polypeptide (protein) by translation, as a result of which the protein is said to be expressed. As the term is used herein, a gene or nucleic acid is "expressible" if it is capable of expression under appropriate conditions in a particular host cell.

For the present invention, promoters may be used that provide for preferential gene expression in a specific tissue or during a specific period of development. For example, promoters may be used that are specific for leaf (Dunsmuir, *et al Nucleic Acids Res*, (1983) 11:4177-4183), root tips (Pokalsky, *et al Nucleic Acids Res*, (1989) 17:4661-4673), fruit (Peat, *et al Plant Mol. Biol*, (1989) 13:639-651; United States Patent No. 4,943,674 issued 24 July, 1990; International Patent Publication WO-A 8 809 334; United States Patent No. 5,175,095 issued 29 December, 1992; European Patent Application EP-A 0 409 629; and European Patent Application EP-A 0 409 625) or late embryogenesis (U.S. Patent No. 5,723,765 issued 3 March 1998 to Oliver *et al.*). Such promoters may, in some instances, be obtained from genomic clones of cDNAs. Depending upon the application of the present invention, those skilled in this art may choose a promoter for use in the invention which provides a desired expression pattern. Promoters demonstrating preferential transcriptional activity in plant tissues are, for example, described in European Patent Application EP-A 0 255 378 and International Patent Publication WO-A 9 113 980. Promoters may be identified from genes which have a differential pattern of expression in a specific tissue by screening a tissue of interest, for example, using methods described in United States Patent No. 4,943,674 and European Patent Application EP-A 0255378. The disclosure herein includes examples of this aspect of the invention, showing that plant tissues and organs can be modified by

transgenic expression of a plant CDK inhibitor.

Non-dividing plant cells may tolerate low level expression of CDK inhibitors, such as *ICK2*, in non-targeted tissues. Thus, the invention may be practiced in some embodiments using tissue specific promoters operably linked to CDK inhibitor encoding sequences, even
 5 when the promoter mediates a tolerable basal level of expression in other tissues.

Various aspects of the present invention encompass nucleic acid or amino acid sequences that are homologous to other sequences. As the term is used herein, an amino acid or nucleic acid sequence is "homologous" to another sequence if the two sequences are substantially identical and the functional activity of the sequences is conserved (for example,
 10 both sequences function as or encode a cyclin dependent kinase inhibitor; as used herein, sequence conservation or identity does not infer evolutionary relatedness). Nucleic acid sequences may also be homologous if they encode substantially identical amino acid sequences, even if the nucleic acid sequences are not themselves substantially identical, for example as a result of the degeneracy of the genetic code.

15 Two amino acid or nucleic acid sequences are considered substantially identical if, when optimally aligned, they share at least about 75% sequence identity, preferably at least about 90% sequence identity, and more preferably at least about 95% sequence identity. Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman (1981)
 20 *Adv. Appl. Math* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, and by computerized implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence identity may
 25 also be determined using the BLAST algorithm, described in Altschul *et al.* (1990), *J. Mol. Biol.* 215:403-10 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information (through the internet at <http://www.ncbi.nlm.nih.gov/>). The BLAST algorithm involves first
 30 identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when

aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. Initial neighborhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program may use as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci. USA* 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

An alternative indication that two nucleic acid sequences are substantially identical is that the two sequences hybridize to each other under moderately stringent, or preferably stringent, conditions. Hybridization to filter-bound sequences under moderately stringent conditions may, for example, be performed in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C (see Ausubel, *et al.* (eds), 1989, *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). Alternatively, hybridization to filter-bound sequences under stringent conditions may, for example, be performed in 0.5 M NaHPO₄, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.1 x SSC/0.1% SDS at 68°C (see Ausubel, *et al.* (eds), 1989, *supra*). Hybridization conditions may be modified in accordance with known methods depending on the sequence of interest (see Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology* --

Hybridization with Nucleic Acid Probes, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York). Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH.

5 An alternative indication that two amino acid sequences are substantially identical is that one peptide is specifically immunologically reactive with antibodies that are also specifically immunoreactive against the other peptide. Antibodies are specifically immunoreactive to a peptide if the antibodies bind preferentially to the peptide and do not bind in a significant amount to other proteins present in the sample, so that the preferential
10 binding of the antibody to the peptide is detectable in an immunoassay and distinguishable from non-specific binding to other peptides. Specific immunoreactivity of antibodies to peptides may be assessed using a variety of immunoassay formats, such as solid-phase ELISA immunoassays for selecting monoclonal antibodies specifically immunoreactive with a protein (see Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor
15 Publications, New York).

 The cyclin dependent kinase inhibitors of the present invention, and the genes encoding those inhibitors, may include non-naturally occurring sequences, such as functionally active fragments of naturally occurring sequences. For example, fragments of ICK₁, or amino acid sequences homologous to those fragments, that have cyclin dependent
20 kinase inhibitory activity may be used in some embodiments of the invention. The invention provides methods for identifying such fragments, for example by deletion mapping of active cyclin dependent kinase inhibitors. As used herein the term "cyclin dependent kinase inhibitor" therefore includes any polypeptide capable of functioning during plant development to modify the development of the plant, the invention similarly encompasses
25 nucleic acid sequences encoding such polypeptides.

 As used herein to describe nucleic acid or amino acid sequences the term "heterologous" refers to molecules or portions of molecules, such as DNA sequences, that are artificially introduced into a particular host cell. Heterologous DNA sequences may for example be introduced into a host cell by transformation. Such heterologous molecules may
30 include sequences derived from the host cell. Heterologous DNA sequences may become

integrated into the host cell genome, either as a result of the original transformation of the host cells, or as the result of subsequent recombination events.

The specificity of a CDK inhibitor may be assayed *in vivo*. For example, the *ICK1* coding sequence was fused to a known promoter which directed gene expression in pollen but not in stamen primordia. The transformants are normal and fertile. This result indicates that in specific embodiments of the invention, expression of *ICK1* is not generally toxic to tissues other than the target tissue. Phenotypes may be obtained, for example with the exemplified *AP3-ICK1* transformants, that are due to specific action of the CDK inhibitor, such as *ICK1* protein, on cell division. In such embodiments, the CDK inhibitor, such as *ICK1*, may be used as a specific tool to modify growth and development of meristematic tissues without affecting other processes.

In some embodiments, there may be important advantages to using a CDK inhibitor gene for genetic engineering in plants, particularly to ablate selected cell lineages, rather than using genes encoding cytotoxins. In accordance with the invention, the CDK inhibitor action may be made to be specific only to certain cells, avoiding the non-specific destruction of plant cells. This specificity may be achieved partly because non-dividing plant cells in non-targeted tissues may have better tolerance of low level expression of a CDK inhibitor than a cytotoxin. Thus, in accordance with the invention it may be possible to use tissue specific promoters for expressing CDK inhibitors when such promoters still have a tolerable basal level of expression in other tissues. This may usefully expand the range of promoters available for use in the invention, since most tissue-specific promoters have some levels of expression in other tissues in addition to a high level of expression in a particular tissue. In contrast, expression of a potent cytotoxic gene in one tissue, even at a low concentrations, can have a negative impact on growth and development in other tissues.

In an alternative aspect of the invention, the down-regulation of CDK inhibitors, such as *ICK1*, may be used to enhance growth during plant development. Such growth enhancement may be tissue-specific. For example, anti-sense oligonucleotides may be expressed to down-regulate expression of CDK inhibitors. The expression of such anti-sense constructs may be made to be tissue-specific by operably linking anti-sense encoding sequences to tissue-specific promoters. Anti-sense oligonucleotides, including anti-sense

RNA molecules and anti-sense DNA molecules, act to block the translation of mRNA by binding to targeted mRNA and inhibiting protein translation from the bound mRNA. For example, anti-sense oligonucleotides complementary to regions of a DNA sequence encoding a CDK inhibitor, such as ICK1, may be expressed in transformed plant cells during
5 development to down-regulate the CDK inhibitor. Alternative methods of down-regulating CDK inhibitor gene expression may include the use of ribozymes or other enzymatic RNA molecules (such as hammerhead RNA structures) that are capable of catalyzing the cleavage of RNA (as disclosed in U.S. Patent Nos. 4,987,071 and 5,591,610). The mechanism of ribozyme action generally involves sequence specific hybridization of the ribozyme molecule
10 to complementary target RNA, followed by endonucleolytic cleavage.

Arabidopsis thaliana "Columbia" may be used as a convenient model system for identifying CDK inhibitors that are useful in various embodiments of the present invention. *Arabidopsis* plants are generally grown in pots placed in growth chambers (20°C 16h/8h of day/night). Other plants may also of course be used in various embodiments of the invention
15 in accordance with known transformation and growth techniques.

Yeast two-hybrid cloning and assay techniques may be used to assess and identify CDK inhibitors useful in the present invention. For example, a cDNA library may be made using poly (A) mRNA isolated from whole plants at different stages of development and cloned in a suitable vector, such as Gal4 TA- (transcription-activation domain) vector pBI771
20 (Koholmi et al., 1997). The cDNA of the gene (such as *cdc2a*, cyclin $\delta 2$ and cyclin $\delta 3$) to be used for screening the library may be cloned in a suitable vector, such as the Gal4 DB- (DNA-binding domain) vector. The yeast strain, such as YPB2, harboring the construct may be transformed using the library DNA.

In one example, for analysis of Cdc2a interactions, a total of 1.8×10^7 transformants
25 were subjected to two-hybrid selection on supplemented synthetic dextrose medium lacking leucine, tryptophan and histidine but containing 5 mM 3-amino-1,2,4-triazole. The selected colonies were assayed for β -galactosidase activity using standard methods. DNAs were isolated from positive clones and used to transform *E. coli*. Clones harboring the TA-fusion cDNAs were identified by PCR and plasmids were then isolated for DNA sequencing.

30 Interactions in the yeast two-hybrid system may, for example, be analyzed by either

filter assay (Chevray and Nathans, 1992) using X-gal as the substrate or by a quantification assay using ONPG (ortho-nitrophenyl-beta-D-galactoside) as the substrate (Reynolds and Lundlad, 1994). Three or more independent transformants may be used for each interaction.

Standard methods are available for the preparation of constructs for use in identifying and characterizing CDK inhibitors useful in various embodiments of the invention. General molecular techniques may for example be performed by procedures generally described by Ausubel et al. (1995). Alternative equivalent methods or variations thereof may be used in accordance with the general knowledge of those skilled in this art.

In one example, the *AP3* promoter was cloned by the polymerase chain reaction (PCR) from *Arabidopsis thaliana* "Columbia" genomic DNA, on the basis of the published sequence (Irish and Yamamoto, 1995; GenBank Accession U30729). The promoter was cloned in a modified binary vector pBI121 (Clontech). *ICK1* cDNA (Wang et al., 1997) was similarly amplified by PCR and transcriptionally fused with the *AP3* promoter and the chimeric gene ends with a nopaline synthase terminator. In addition, a construct consisting of *ICK1* fused to an anther-specific promoter of *Bgp1* (Xu et al., 1993) was prepared similarly. The resulting plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 (bearing helper plasmid pMP90; Koncz and Schell 1986).

In accordance with various aspects of the invention, plant cells may be transformed with heterologous nucleic acids. Transformation techniques that may be employed include plant cell membrane disruption by electroporation, microinjection and polyethylene glycol based transformation (such as are disclosed in Paszkowski et al. *EMBO J.* 3:2717 (1984); Fromm et al., *Proc. Natl. Acad. Sci. USA* 82:5824 (1985); Rogers et al., *Methods Enzymol.* 118:627 (1986); and in U.S. Patent Nos. 4,684,611; 4,801,540; 4,743,548 and 5,231,019), ballistic transformation such as DNA particle bombardment (for example as disclosed in Klein, et al., *Nature* 327: 70 (1987); Gordon-Kamm, et al. "The Plant Cell" 2:603 (1990); and in U.S. Patent Nos. 4,945,050; 5,015,580; 5,149,655 and 5,466,587) and *Agrobacterium* mediated transformation methods (such as those disclosed in Horsch et al. *Science* 233: 496 (1984); Fraley et al., *Proc. Nat'l Acad. Sci. USA* 80:4803 (1983); and U.S. Patent Nos. 4,940,838 and 5,464,763).

Transformed plant cells may be cultured to regenerate whole plants having the

transformed genotype and display a desired phenotype, as for example modified by the expression of a heterologous CDK inhibitor during development. A variety of plant culture techniques may be used to regenerate whole plants, such as are described in Evans *et al.* "Protoplasts Isolation and Culture", Handbook of Plant Cell Culture, Macmillian Publishing Company, New York, 1983; or Binding, "Regeneration of Plants, Plant Protoplasts", CRC Press, Boca Raton, 1985; or in Klee *et al.*, *Ann. Rev. of Plant Phys.* 38:467 (1987).

Standard techniques may be used for plant transformation, such as transformation of *Arabidopsis*. In one example, the *AP3-ICK1* and *Bgp1-ICK1* constructs were tested in *A. thaliana* by *in planta* transformation techniques. Wild type (WT) *A. thaliana* seeds of ecotype "Columbia" were planted in soiled 4" pots and plants grew in controlled growth chamber or greenhouse. The vacuum infiltration method of *in planta* transformation (Bechtold *et al.*, 1993) was used to transform *A. thaliana* plants with overnight culture of *A. tumefaciens* strain GV3101 bearing both the helper noplone plasmid and the binary construct containing the described chimeric gene. pMP90 is a disarmed Ti plasmid with intact *vir* region acting *in trans*, gentamycin and kanamycin selection markers as described in Koncz and Schell (1986). Following infiltration, plants were grown to maturity and seeds (T1) were collected each pod individually. Seeds were surfaced sterilized and screened on selective medium containing 50 mg/L kanamycin with or without 200 mg/L timentin. After about four weeks on selection medium, the non-transformed seedlings died. The transformed seedlings were transferred to soil in pots. Leaf DNA was isolated (Edwards *et al.*, 1991) and analyzed by PCR for the presence of the DNA insertion. Genomic DNA was also isolated and used in Southern hybridization (Southern, 1975) to determine the copy number of the inserted sequence in a given transformant. To determine the segregation, T2 seeds were collected from T1 plants. Wherever the T1 plant was male sterile, crosses was made using the WT *A. thaliana* pollen to obtain seeds. As described, T2 seeds were surface-sterilized and screened on selective medium.

Alternative embodiments of the invention may make use of techniques for transformation of *Brassica*. Such as transformation of *B. napus* cv. Westar and *B. carinata* cv. Dorolla or breeding line C90-1088 by co-cultivation of cotyledonary petioles or hypocotyl explants with *A. tumefaciens* bearing the plasmids described herein. Transformation of *B.*

napus plants may, for example, be performed according to the method by Moloney et al. (1989). Modifications of that method may include the introduction of a 7-day explant-recovery period following co-cultivation, on MS medium with the hormone benzyladenine (BA), and the antibiotic timentin for the elimination of *Agrobacterium*. Transformation of *B. carinata* plants may be performed according to the method by Babic et al. (1998). Cotyledonary petiole explants may be dipped in suspension of *Agrobacterium* bearing the desired constructs and placed on 7-cm filter paper (Whatman no. 1) on top of the regeneration medium for 2 days. After co-cultivation, explants may be transferred onto the selection medium containing 50 mg/L kanamycin. Regenerated green shoots may first be transferred to a medium to allow elongation and then to a rooting medium all containing 50 mg/L kanamycin. Putative transformants with roots (T0) may be transferred to soil. Genomic DNA may be isolated from developing leaves for PCR and Southern analyses. Seeds (T1) from transgenic plants may then be harvested.

Transgenic plants may be observed and characterized for the presence (absence of) the desired phenotypic trait, such as petals, male sterility and ability to set seeds after pollination using wild type pollen. For example, to determine the development of floral organs, flowers at different stages of development may be dissected and examined under a stereomicroscope. Floral samples may also be examined using scanning electron microscope for more defined morphology of floral organ meristems and their development.

Genomic clones of sequences encoding putative CDK inhibitors may be cloned using standard techniques. For example, to clone a genomic *ICK1* encoding sequence, genomic DNA may be isolated from two-week old *A. thaliana* seedlings according to a procedure described (Lohdi et al., 1994). In one example, the genomic sequence spanning the *ICK1* cDNA sequence was amplified by 30 cycles of PCR (polymerase chain reaction) using sequence-specific primers with incorporated restriction sites. *Pfu* DNA polymerase (Stratagene), which has a higher replication fidelity than the *Taq* DNA polymerase, may be used. The amplified DNA fragment may be cloned into a suitable vector, such as pGEM5Zf(+) (Promega). Plasmids may then be purified and sequenced.

In one example, an *ICK1* cDNA isolated from the two hybrid screening was cloned in frame into pBI786, a modified His₆-tagged vector derived from pRSETB (Invitrogen) and the



resulting construct used to transform *E. coli* strain NM522. Recombinant His₆-ICK1 was purified using Ni-NTA agarose resin (QLAGEN) according to manufacturer's instructions except that the final washing was with buffer D, pH 6.0 and the protein was eluted with 2 ml buffer D, pH 4.0. The eluent was renatured by diluting with 10X volume of a renaturing
 5 buffer (10 mM Tris pH 7.5, 500 mM NaCl, 400 mM arginine HCl, 20 μ M MgCl₂, 20 μ M ZnAc and 0.1% Tween 20) and dialysed in the same buffer (500 ml per 2 ml sample) at 4°C overnight. The protein samples may be concentrated with Filtron 10K concentrators. The sample was then dialyzed at 4°C for 3 h against 1000 volumes of the kinase assay buffer (see below) containing 0.4 mM DTT and each (0.4 μ g/ml) of the protease inhibitors soybean
 10 trypsin inhibitor, antipain and apotinin. The protein was stored at -80°C.

Kinase assays may be useful in some aspects of the invention, for example to assay the function of CDK inhibitors on particular kinases. For example, kinases may be purified from *A. thaliana* tissues or cultured *B. napus* cells. Plant materials may be homogenized in 2 ml per gram tissue of ice cold extraction buffer consisting of 25 mM Tris pH 8.0, 100 mM
 15 NaCl, 10 mM DTT, 5 mM NaF, 1 mM Na₃VO₄, 1 mM β -glycerophosphate, 2.5 mM EDTA, 400 μ g/ml AEBSF [4-(2-aminoethyl)-enzensulfonyl fluoride], 1 μ g/ml leupeptin and 1 μ g/ml pepstatin. The homogenate was centrifuged at 12,000g at 4°C for 30 min. The supernatants may be used to purify Cdc2-like protein kinases using p13^{suc1}-conjugated agarose beads (Oncogene Sciences). The required amount of supernatant (150 μ g protein for each reaction)
 20 was added to the beads and tumbled at 4°C for 2 h. The beads may be washed twice in a washing buffer consisting of 50 mM Tris pH 7.4, 250 mM NaCl, 0.1% NP-40, 2.5 mM EDTA, 1 mM DTT and inhibitor cocktail of (in final concentrations) 10 μ g/ml apotinin, 10 μ g/ml antipain, 10 μ g/ml soybean trypsin inhibitor, 10 mM β -glycerophosphate, 1 mM NaF and 0.2 mM Na₃VO₄. Beads may then be washed twice in the kinase assay buffer (50 mM
 25 Tris pH 7.4, 10 mM MgCl₂, 2 mM EGTA, 2 mM DTT and the inhibitor cocktail). For inhibition assays, the recombinant protein was added to the reactions and incubated (tumbling slowly) for 1.5 h at 4°C. The kinase reaction was initiated by adding 1 μ g/ μ l histone H1 (Sigma), 25 μ M ATP and 0.05 μ Ci/ μ l ³²P- γ -ATP (final concentrations), and stopped after 20 min incubation by adding the sample buffer. Denatured supernatant was resolved by SDS-
 30 PAGE.

RNA isolation and northern blotting analysis may be useful in various embodiments of the invention. For example, to analyze *ICK1* expression during plant development, various tissues may be taken from *Arabidopsis* plants. To analyse the effects of ABA and low temperature, seedlings may be treated as described (Wang et al., 1995). Briefly, seedlings (12 days) grown in pots may be cleared of soil with water, then floated in 0.1X strength MS medium without sucrose and hormones. Low temperature treatment was at 5°C for 24 h. ABA treatment was carried out in a solution containing 50 µM ABA. Seedling samples may be removed after various treatment times. Total RNA was isolated using TRIzol reagent (GIBCO BRL). For northern analysis, the indicated amount of RNA was fractionated in a 1.2% agarose gel and transferred onto Hybond-N+ nylon membrane (Amersham). The RNA was crosslinked to the membrane by UV-light (Stratalinker, Stratagene) and hybridized with ³²P-labeled probes. The membranes may be wrapped and used to expose Hyperfilm MP (Amersham) film. Membranes may be stripped by treating with a boiling solution of 0.1X SSC and 0.1% SDS for 5 min. Quantitation of hybridized signal was performed using Molecular Dynamics PhosphorImager and the accompanying software.

In Vitro binding assays may be useful in various aspects of the invention, for example to assay the interaction of a CDK inhibitor, or fragments of a CDK inhibitor, and a particular kinase. As an example of such an approach, ³⁵S-Met labeled Cdc2a, CycD3 and ATMPK2 proteins may be expressed from a T7 promoter construct using an *in vitro* coupled rabbit reticulocyte transcription/translation system ('TNT', Promega). Ni²⁺-NTA beads (Qiagen) may be equilibrated and blocked in NETN buffer lacking EDTA (NTN) (Bai et al., 1996), and supplemented with 2 mg/ml BSA. Equilibrated beads may be incubated with His₆-ICK1 (5 µg for each 10 µl beads) in 1 ml of NTN buffer for 2 h with tumbling at 10°C followed by washing with 2 X 1 ml NTN buffer. Binding experiments may be carried out in a total volume of 100 µl NTN containing 10 µl beads, plus 5 µl ³⁵S-Met labeled protein. The binding reaction was incubated at 10°C for 2 h, followed by washing with 3 X 0.5 ml NTN buffer. Washed beads may be eluted with 10 µl SDS-containing denaturing buffer at 100°C for 5 min, and bound ³⁵S-Met labeled proteins analyzed by SDS-PAGE. Gels may be imbibed with a fluorography enhancer ('Amplify', Amersham) prior to drying and exposure to X-ray film.

Deletion constructs may be useful for domain mapping to determine the functional

domains of a CDK inhibitor. For example, N-terminal deletion constructs of ICK1 were made using cDNAs with deletions of various lengths from the N-terminal end. The C-terminal deletion constructs were prepared by PCR using *Pfu* DNA polymerase with sequence-specific primers and the resulting DNA fragments were cloned into the yeast two-hybrid vector pBI771. The deletion clones may be verified by DNA sequencing. The constructs may be used to transform a suitable yeast strain. In one such example, yeast strain YPB2 harboring either *cdc2a* or *CycD3* cloned in the BD- (binding domain) vector was transformed with deletion constructs. Interactions in the yeast two-hybrid system may then, for example, be analyzed by X-gal filter assay (Chevray and Nathans, 1992) and by liquid culture assays for relative β -galactosidase activity (for example using the modified procedure of Reynolds and Lundlad, 1994). Three or more independent transformants may be used for each interaction.

Sequence Analyses: Sequence analyses, including determination of sequence homology, may be performed using a variety of software, such as LASERGENE (DNASTAR). Database searches may also use a variety of software tools, such as the BLAST program (NCBI).

Analysis of CDK inhibitor cDNA Clones and Genomic Sequences: The yeast two-hybrid system (Fields and Song, 1989; Kohalmi et al., 1997) may be used to identify genes, such as *ICK1*, that encode inhibitor proteins able to interact with the plant cyclin dependent kinases, such as Cdc2 kinase, for use in the present invention. For example, among the 68 *ICK* (*I*nteractors of *C*dc2 *K*inase) clones identified using Cdc2a as the bait in a yeast two hybrid system (Wang et al., 1997), 55 represented various lengths of *ICK1*, 7 of *ICK2* and 6 of *ICK3*. A contig sequence for homologous clones disclosed by the yeast two hybrid assay may be used, as was the contig sequence for *ICK1* cDNA (Wang et al., 1997), to search genomic databases, such as an *Arabidopsis* EST database, for sequences homologous to those identified by the two-hybrid screen. Two EST clones homologous to the *ICK1* cDNA sequence have been identified in this way. A clone designated 96D15T7 possessed an extra 5' sequence to that of the contig assembled from the two-hybrid cDNA clones. Specific PCR primers may be synthesized and used to clone the genomic sequence spanning the entire

coding region of a CDK inhibitor gene. For *ICK1*, three independent clones harboring the genomic sequence were identified in this way, sequenced and found to be identical.

Alignment of *ICK1* genomic sequence with the *ICK1* cDNA sequence (GenBank U94772, Wang et al., 1997) reveals three introns. The genomic sequence in the exon regions is

5 identical to the contig of cDNA clones except at nucleotide position 318, which is a T instead of a G as in the reported cDNA sequence (Wang et al., 1997; a majority of the cDNA clones had a G, while other clones had a T at this position). The existence of a T at this position in genomic DNA was verified by sequencing additional genomic clones. The longest open
10 reading frame in the *ICK1* cDNA sequence predicts a polypeptide of 191 amino acids (Wang et al., 1997). There is an in-frame translation STOP codon 12 nucleotides upstream of the first ATG. In addition, an in-frame translation termination codon was found 30 nucleotides downstream of the predicted termination codon. Genomic southern blots may be performed to estimate the copy number of a CDK inhibitor gene and to disclose closely-related genes. For *ICK1* in
15 *A. thaliana* southern results indicate that *ICK1* exists as a single copy gene: while *BamH* I and/or *EcoR* I digested genomic DNA gave a single band, *Sal* I digestion of the DNA resulted in two bands, as was predicted by the presence of an internal *Sal* I site in the *ICK1* sequence. Hybridization at lower stringency may be used to detect non-identical homologous sequences.

CDK inhibitor in vitro assays: *In vitro* kinase assays may be used to demonstrate that a recombinant putative CDK inhibitor, such as *ICK1* protein, is an effective inhibitor of plant
20 Cdc2-like kinases. Plant CDK inhibitors may not inhibit CDK from mammalian and yeast cells (Wang et al., 1997). For example, recombinant *ICK1* is effective *in vitro* in inhibiting the histone H1 kinase activity of p13^{suc1}-associated kinases from cultured cells of heterologous *Brassica napus*. In addition, it also inhibits the activity of such kinases from *A. thaliana* seedlings, leaves and floral tissues *in vitro*.

Expression of CDK inhibitors: The expression of a CDK inhibitor in particular plant tissues may be assayed to determine, for example, whether that CDK inhibitor will have utility as a division or growth modulator when expressed in such tissues. For example, the expression of *ICK1* was analyzed in several different plant tissues. In general, the transcript abundance of *ICK1* was relatively low and showed low degrees of variation compared with the housekeeping genes such as *TUA4* (a tubulin- α gene) and *GAPDH* (glyceraldehyde

phosphate dehydrogenase) of *A. thaliana*. When leaves from plants of different ages were compared, the *ICK1* level in sample L5 (for leaves of 5-week plants) was slightly higher. To verify the functional role of a putative CDK inhibitor in such tissues, the CDK activity may also be assayed.

Assaying of CDK inhibitor activity: Putative CDK inhibitors may be assayed for suitable CDK inhibitor activity for use in the methods of the invention by a variety of tests. For example, induction of expression of the putative CDK inhibitor gene by abscisic acid (ABA), a phytohormone known to inhibit plant growth (Evans, 1984), and at low
 5 temperatures. For example, expression of the putative CDK inhibitor gene, such as *ICK1*, in seedlings, such as *A. thaliana* seedlings, may be analyzed in response to treatments with ABA. For *ICK1*, data from an example assay showed that after 24 h, ABA and low temperature treatments increased *ICK1* transcript levels to about 3 times that of the control (no ABA and at 22°C) in 2-week seedlings. The expression of the putative CDK inhibitor
 10 gene may be quantified. For *ICK1*, a correlation coefficient was obtained for the relationship of *cdc2a* level, *ICK1* level and *cdc2a/ICK1* ratio with the Cdc2-like kinase activity. The level of *cdc2a* expression was correlated with the level of Cdc2-like histone H1 kinase activity. The level of *ICK1* expression exhibited a weak negative correlation with kinase activity. The correlation coefficient for the *cdc2a/ICK1* ratio with Cdc2-like kinase activity was similar to
 15 that for *cdc2a* with Cdc2-like kinase activity. Such results are consistent with CDK (in this example Cdc2 kinase) inhibitor activity in plant cells.

Direct Interaction of ICK1 with Both Cdc2a and CycD3: CDK inhibitors for use in various aspects of the invention may be identified using a yeast two hybrid screening protocol with a variety of bait fusion protein sequences. For example, *ICK1* was independently cloned in a screen using *A. thaliana* CycD3 as the bait, indicating that *ICK1* interacts with CycD3 in the two hybrid assay. To provide evidence confirming the interaction of a CDK inhibitor with a target protein of interest, further binding assays may be conducted. For example, to test the interactions of *ICK1*, *cdc2a* and *CycD3* cDNAs were transcribed and translated in an *in vitro* system. *In vitro* expressed Cdc2a and CycD3 proteins were incubated with recombinant His₆-*ICK1* protein expressed in *E. coli*. Cdc2a and CycD3 bound to Ni-NTA beads only after they were incubated with recombinant *ICK1*. The amount of CycD3 bound to recombinant *ICK1*

protein was more than the control protein ATMPK2 which showed little binding despite the much higher input used. These results demonstrate that ICK1 is able to interact directly with both Cdc2a and CycD3. Similar assays may be used to identify CDK inhibitors capable of interacting with other cellular targets.

Mapping the Domains for ICK1 Interaction with Cdc2a and CycD3: The regions of a CDK inhibitor that are functionally involved in interactions with other proteins may be mapped by deletion mapping using a variety of techniques, such as the yeast two hybrid systems and variations thereof. Such *in vitro* assay results may be verified by *in vivo* tests, since the persistence of interactions in the two hybrid system may be affected by possible alterations in functionality of plant proteins expressed in yeast. As an example of an *in vitro* assay, to determine the functional significance of the C-terminal domain and other regions of *ICK1*, three N-terminal and three C-terminal deletion mutants were assessed for their interactions with Cdc2a and CycD3 in the two-hybrid system. Overall, β -galactosidase marker gene activation in the two hybrid system was stronger for the interaction of all ICK1 constructs with CycD3 compared to Cdc2a, indicative of a stronger or more persistent interaction between ICK1 and CycD3 in the two-hybrid system. Major shifts in β -galactosidase activity were observed when amino acid regions 3-72, 109-153 and 176-191 were deleted. An increase in activity was observed upon deletion of amino acids 3-72. In pairwise comparison, the deletion of amino acid regions 3-72, 73-108, 163-175 or 153-162 had comparable effects on the interactions of ICK1 with Cdc2a versus CycD3, as reflected by the marker gene expression, while the deletions of amino acid regions 109-153 and 176-191 had clearly differential effects. The most significant reduction in β -galactosidase activity for the interaction of ICK1 with CycD3 resulted from the deletion of amino acids 109-153, whereas the deletion of amino acids 176-191 had a more detrimental effect on the interaction with Cdc2a. The functional importance of a portion of a CDK inhibitor may also be assayed by analyzing the portion of cDNA required for the recovery of clones by each bait construct in the two hybrid system. For example, the region spanning amino acids 109-153 of ICK1 for its interaction with CycD3 was supported by the analysis of the minimum cDNA length required for the recovery of clones by each bait construct. With CycD3 as the bait, the shortest *ICK1* cDNA was N-terminal deleted for amino acids 1-129, while with Cdc2a, seven

clones with further deletions extending to amino acid 154 were also isolated. Thus, deletions extending beyond amino acid 130 rendered these clones unrecoverable by the two-hybrid screening using CycD3 as the bait. Taken together, the results indicate that, while the C-terminal domain (containing the consensus sequence with p27^{Kip1}) is most important for the interaction with Cdc2a, the amino acid region 109-153 perhaps with the C-terminal domain is important for the interaction with CycD3.

Arabidopsis transformation with ICK1 constructs: A wide variety of transformation techniques may be used in accordance with the invention to introduce CDK inhibitor genes into plants. In one aspect, the invention provides methods of assaying heterologous CDK inhibitor function in a model plant, such as *Arabidopsis*. For such assays, in one embodiment, transformation may be carried out by infiltration. For example, seeds (T1 generation) collected from infiltrated *Arabidopsis* plants may be surface-sterilized and placed onto MS medium containing 50 µg/ml kanamycin. The antibiotic timentin may also be included in the medium to prevent any bacterial growth, which could occur due to carrier-over from the infiltration. The vast majority of germinating seedlings will not be transformed, and will become pale and eventually stop growing, transformed seedlings will be green and display normal growth due to the presence of the selectable marker gene. After 4-5 weeks in the selection medium, transformants may be transferred to soil in pots. In the exemplary embodiment, the presence of the DNA insertion was confirmed by extracting the genomic DNA and then using it for PCR amplification. In one example, while the non-transformed wild-type plant gave a negative signal, all five plants selected for their resistance to kanamycin were positive for transforming DNA.

Effect of AP3-ICK1 chimeric gene on petal and stamen development: Various aspects of the invention may be used to obtain a wide variety of phenotypic variations in plant morphology or other characteristics. For example, transformed *A. thaliana* plants carrying the AP3-ICK1 construct displayed a range of phenotypes with regard to petal and stamen morphology (Table 1). Such variation may be due to the insertion in alternative embodiments of the invention of the CDK inhibitor gene into different sites of the plant genome. In the example of modified petal development using ICK1, the plants may be classed into three groups: (1) no visible petals, (2) visible petals but reduced size and (3) visible petals with no

apparent difference to those of non-transformed plants (Table 1). In terms of fertility, four out of five plants were sterile. These results demonstrate that tissue-specific expression of *ICK1* may be used to produce plants without petals and/or with male sterility. In some embodiments, the transgenic plants with male sterility may set seeds after pollination, using pollen from non-transformed plants, indicating that the female reproduction system is unaffected in these male sterile plants.

Table 1. Summary of phenotypes of *A. thaliana* plants transformed with *AP3-ICK1* chimeric gene.

Transformant	Petal	Sterility	Seed setting with WT pollen
#1	Reduced size or number	Sterile	yes
#2	No visible petals	Sterile	yes
#3	Normal	Fertile	self-fertile
#4	Reduced size or number	Sterile	yes
#5	Nearly normal	Sterile	yes

5

Co-Inheritance of the inserted gene and phenotype: T2 plants may be studied to determine the segregation of the inserted gene and also to verify whether the particular phenotype is co-inherited with the inserted gene. For example, T2 seeds of *ICK1* transformants were sterilized and placed onto the selective medium. In one such assay, T2 seeds of one transformant (#2) showed 1:1 ratio of segregation between resistant (99) versus non-resistant (102) seedlings. As transformant #2 was male sterile, the T2 seeds were obtained by crosses using wild type pollen. This ratio indicates that there is one insertion in the genome of this transformant. As expected, T2 plants displayed the same phenotype as the corresponding T1 plants.

Transgenic Brassica: In accordance with alternative embodiments of the invention, CDK inhibitors may be used to modify a wide variety of plant species. The division and growth inhibiting activity of CDK inhibitors in *Brassica* is exemplified by the following

results. The ICK1 cDNA coding region was fused in sense or anti-sense orientation to the cauliflower mosaic virus 35S promoter (a constitutive promoter). These constructs were tested in *Brassica napus* transformations. From two independent experiments, over 60 transformants were regenerated from the anti-sense construct while none was obtained from the sense construct, indicating that constitutive expression of the ICK1 CDK inhibitor terminated plant growth at a very early stage. [Were there any phenotype changes in the anti-sense plants?]

Interaction of ICK1 with other proteins: CDK inhibitors may be used in various aspects of the invention to interact with a variety of regulatory components, such as other cell cycle proteins. For example, in some embodiments, it may be desirable to target a known regulatory moiety with a CDK inhibitor. Accordingly, in one aspect of the invention, an assay is provided to determine if a CDK inhibitor interacts with a known protein. Such interactions may be analyzed by the yeast two-hybrid assay. For example, the full-length cDNA of the gene to be analyzed may be cloned in a GAL4-binding domain vector (Koholmi et al., 1997) using PCR and gene specific primers with flanking restriction sites. Such constructs may be used to transform the yeast carrying the CDK inhibitor of interest, such as ICK1 in a GAL4-activation domain vector. Using this approach, for example, the interactions of ICK1 with a number of cell cycle-related genes from *A. thaliana* were examined in accordance with the invention (Table 2). In these examples, the yeast two-hybrid assay results indicate that in particular embodiments of the invention, ICK1 protein may interact with Cdc2a but not with Cdc2b. Similarly, ICK1 may interact with D-class cyclins, CycD1, CycD2 and CycD3, while not interacting with A/B-class mitotic cyclins, CycA2, CycB1 and CycB2 (Table 2). The yeast two-hybrid assay results also indicate that ICK1 may not interact in some embodiments with PCNA, also a cell cycle protein, and ATMAP2, a kinase sharing some similarity with Cdc2 kinase. Results such as these, indicating that ICK1 interacts with the G1 cyclins and Cdc2a but not the mitotic cyclins and Cdc2b, indicate that a CDK inhibitor, such as ICK1, may in some embodiments be used in the regulation of cell cycle initiation during plant growth and differentiation.

Table 2. Analyses of ICK1 interactions with other proteins in the yeast two-hybrid

system

Gene Group Examined	Gene in DB-Vector	Old Name	Interaction with ICK1	
			Filter assay ⁽¹⁾	Quantification ⁽²⁾
Control	vector alone		-	0
Cdc2 kinase	cdc2a		+++	2.65
	cdc2b		-	0
cyclin	cycD1;1	cyclin δ 1	+++	3.13
	cycD2;1	cyclin δ 2	++++	14.80
	cycD3;1	cyclin δ 3	+++++	22.70
	cycA2;2	cyc3bAt	-	0.03
	cycB1;1	cycl1At	-	0.06
	cycB2;2	cyc2bAt	-	0.05
PCNA	PCNAAt		-	0
MAP kinase	ATMAP2		-	0

Other plant CDK inhibitors: Other plant CDK inhibitors and CDK inhibitor genes sharing functional and sequence similarity with ICK1 may be identified using an approach similar to the approach used to isolate *ICK1*, based on their interactions with either *Arabidopsis* Cdc2a or a D-class cyclin (cyclin δ 3 or cyclin δ 2). The CDK inhibitors identified in screens using Cdc2a are designated herein as *ICKs* (for *Interactors of Cdc2 Kinase*) and those identified in screens using cyclins are designated *ICNs* (for *Interactors of Cyclin*). Some CDK inhibitors may be isolated independently from both types of screens. The sequences of *ICK2*, *ICN4*, *ICN6*, and *ICN7* are shown in Figs 1 through 4. These genes share at least two functional properties with ICK1: First, all of these genes encode proteins able to

interact with either Cdc2a or a D-class cyclin or both. Such interactions may enable them to regulate the activity of plant CDKs in alternative embodiments of the invention. Second, these ICK/ICN proteins all share some sequence similarity in the region of ICK1 that is functionally important in some embodiments for its interaction with Cdc2a and cyclin $\delta 3$ (discussed above in the section on "domains for ICK1 interactions with Cdc2a and cyclin $\delta 3$ "). These homologous genes or proteins may be used in some embodiments, in a manner similar to ICK1, to modulate plant growth and development. One or more such genes or proteins may be used in some embodiments alone or in combination to provide temporal and spatial regulation of cell cycle initiation and progressing during plant development in accordance with this invention.

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. For example, additional plant cyclin-dependent kinase inhibitor genes useful in regulating morphogenesis may be disclosed using the screening methods of the invention, such genes may share functional homology with *ICK1*, while being sequence-divergent from *ICK1*. The following examples are illustrative only of various aspects or embodiments of the invention.

A set of genes and their predicted gene products have been identified in *Arabidopsis thaliana* on the basis of protein:protein interactions using the 2-hybrid system. The primary structure of these genes suggest they are components of the cell cycle regulatory machinery in this model plant species. These genes bear selected primary sequence similarity to genes and deduced gene products from other systems (notably yeast and humans) which are known to have a role in the regulation of cell division in these organisms.

A partial primary nucleic acid sequence, and deduced polypeptide sequence of the genes that have been discovered are given below:

Nucleotide sequence of *ICK2* cDNA

ick2.seq Length: 755 June 7, 1999 13:57 Type: N Check: 1650 ..

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1  GTGGAATCTA GGATAATTCT GTCTCCGTGT GTACAGGCGA CGAATCGCGG
51  TGGAAATTGTG GCGAGAAATT CAGCAGGAGC GTCGGAGACG AGTGTTGTTA
101 TAGTACGACG GCGAGATTCT CCTCCGGTTG AAGAACAGTG TCAAATCGAA
151 GAAGAAGATT CGTCGGTTTC GTGTTGTTCT ACATCGGAAG AGAAATCGAA
201 ACGGAGAATC GAATTTGTAG ATCTTGAGGA AAATAACGGT GACGATCGTG
251 AAACAGAAAC GTCGTGGATT TACGATGATT TGAATAAGAG TGAGGAATCG
301 ATGAACATGG ATTCTTCTTC GGTGGCTGTT GAAGATGTAG AGTCTCGCCG
351 CAGGTTAAGG AAGAGTCTCC ATGAGACTGT GAAGGAAGCT GAGTTAGAAG
401 ACTTTTTTCA GGTGGCGGAG AAAGATCTTC GGAATAAGTT GTTGGAATGT
451 TCTATGAAGT ATAACTTCGA TTTCGAGAAA GATGAGCCAC TTGGTGGAGG
501 AAGATACGAG TGGGTTAAAT TGAATCCATG AAGAAGACGA TGATGATAAT
551 GATGATCATT GTTTTACCA AAGTACTTAT TATTTCTCTT CTGTAATAAT
601 CTTTGCTTTG ATTTTCTTT TAACAAAATC CAAATGTAGA TATCTTTCTC
651 TCGAATAATC AATAACATGT AATTCAACTT TTGTTTGTAC TTCCTTGAGG
701 TAATTAATTA GATTCGTGTT TTTCTCGATT AATAAACTAT AAGTTTATAA
751 CTAAA

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Nucleotide sequence of *ICN2* cDNA

nucleotide.seq Length: 824 June 7, 1999 13:57 Type: N Check: 7926 ..

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1  aaaaaaaagc agagagagag agcacacaaa aatccaagag agaaaaaaat
51  gagcgagaga aagcgagagc ttgcagaaga agcttcaagc acaagcttct
101 caccactgaa gaaaacgaag cttaatgatt cttctgattc atcaccggac
151 tctcatgacg tcatcgtctt cgcggtttca tcttcttccg ttgcttcgtc
201 ggcggcttta gcgtctgatg aatgttccgt taccatcggg ggagaagaaa
251 gtgatcagtc ctcgagtatc agctccgggt gtttcaccag tgaatcgaaa
301 gaaatcgca agaacagttc gtcgtttggt gtagatctgg aggatcatca
351 aatcgaaacc gaaaccgaaa cctcaacatt catcaccagc aatttcagaa
401 aagagacgag tccagtgagt gagggtttgg gagaaacgac aacagaaatg
451 gaatcatcat cggcaacgaa gagaaaacaa ccgggggtga ggaagactcc
501 aacggcgggc gagattgagg atttgttctc ggagctagag agtcaagacg
551 ataagaagaa gcaattcata gaaaagtaca acttcgatat tgtcaatgac
601 gaaccgcttg aaggtcgcta caagtgggat cgactttaag ccatcaaaaa
651 gcaaatacca tccatgaaga agacaaaaga aaaatagggt ttgtttttcg
701 tggttaacat ttccacttgt acagctctag tctatttctc tttaaaaacc
751 tatgttacta gttcgtacaa aacaaaacaa aaaacacgac ctttataatg
801 aaatttcgga tcttggtac taaa

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Nucleotide sequence of *ICN6* cDNA

nucleotide.seq Length: 642 June 7, 1999 13:59 Type: N Check: 8341 ..

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1  ctctctccag agaaaactat aatgagcttg agagaaatga gcgaaacaaa
51  acccaagaga gattctgagt acgaaggatc aaacatcaag aggatgagac
101 tcgatgatga tgatgacgtt ttacgctcac cgacgagaac tctttcttct
151 tcttcctctt cttctctggc ttactcgggt tcagattccg gaggtttctg

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201  ctccgtcgcg ttatctgaag aagaagacga tcatctaagc tcaagcatca
251  gctctgggtg ttccagcagc gaaactaacg aaatccctac tcgtcttcca
301  ttttcagatc tggaggctca tgaaatctcc gaaaccgaaa tctcaacggt
351  actcaccaac aatttcagga aacagggaat ttcataagc gagaatctgg
401  gagaaacagc agaaatggac tccccgacga cggagatgag agatcagaga
451  aagacggaga agaagaagaa gatggaaaaa tcaccgacgc aggcagagct
501  tgatgacttt ttctcggcgg cggagagata cgaacagaaa cgattcacag
551  aaaagtacaa ctacgacatc gtcaatgata cgccgcttga aggtcggtag
601  cagtgggtta gtctgaaacc ttagaagcca tggaagaaca aa

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Nucleotide sequence of *ICN7* cDNA

nucleotide.seq Length: 533 June 7, 1999 14:00 Type: N Check: 1632 ..

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1  attaaagagt ctggttccag gtctcgcggt gactcgggtta actcggctcc
51  tgtagctcag agctctaatt aagatgaatg ttttgacaat ttcgtgagtg
101  tccaagtttc ttgtggtgaa aacagtctcg gttttgaatc aagacacagc
151  acaagggaga gcacgccttg taactttggt gacgatatgg agatcatggt
201  tacaccaggg tctagcacga ggatgatgtg cagagcaacc aaagactaca
251  caaggaaca agataacgtg atccccacca ctagtgaaat ggaggagttc
301  tttgcatatg cagagcagca gcaacagagg ctattcatgg agaagtacaa
351  cttcgacatt gtgaatgata tccccctcag cggacgttac gaatgggtgc
401  aagtcaaacc atgaagttca aaaggaaaca gtcctaaaag acatgggtgtg
451  aagttagaga attgtgatgg actttaacag aactaaccaa acatcagaaa
501  tcgtgttaat ccttaagtta ataatgtggg tta

```

(a) What is the problem?

The problem concerns the discovery of fundamental approaches for intervening and controlling plant development using molecular biotechnology. These developmental processes are known to

involve the coordinated regulation of two fundamental cell biological processes: cell division, and the acquisition of cell fate (cell-type).

- (b) How may it be accomplished according to the present knowledge?

The directed regulation of cell division could alter the pattern of plants, by altering the cell-cycle 'decision-making' process during the normal course of development. Such alternations could include alteration of the extent, plane or developmental timing of cell division events, leading to the development of altered morphologies of plant tissues and organs.

- (c) Limitations or drawbacks of present apparatus, product or process.

At this point in time, a predictive capability of the outcome of altering the expression of specific cell cycle components cannot be offered. However, the discovery of new genes and gene products implicated in the regulation of cell division offers a knowledge base from which empirical observations can be made, and predictive models developed.

- (d) What is your proposal?

We propose to protect and exploit the genes described here for potential future applications in the alteration of plant cell cycle control, with consequences for altering development.

- (e) What is thought to be novel in your proposal?

To our knowledge, these genes have not been previously described at a functional level (eg. protein:protein interaction, as described here) which would implicate them in cell cycle control.

- (f) Has the apparatus, product or process been made or tested?

The genes described have been demonstrated to interact at the protein level with selected cyclins and/or cyclin-dependent kinase proteins from *Arabidopsis*.

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Table 3

Construct Name	No. of Explants	Green Shoots recovered	Seed recovered
K1a	500	0	0
K1a	500	0	0
K2a	500	5	
K2a	500	ca. 60	9
E1a	650	4	2
E2a	600	8	6

Table 3 provides results from transgenic Arabidopsis plants

What is Claimed is:

1. A method of modifying the development of a plant comprising transforming a plant cell with a nucleic acid encoding a cyclin dependent kinase inhibitor to produce a transformed plant cell; and, regenerating the plant from the transformed plant cell under conditions wherein the cyclin dependent kinase inhibitor is expressed during regeneration of the plant to modify the development of the plant, wherein the nucleic acid encoding the cyclin dependent kinase inhibitor is a gene selected from the group consisting of ICK2, ICN2, ICN6, ICN7 and homologs thereof.
2. The method of Claim 1, wherein the plant is a member of the *Brassica* genus.
3. A method of modifying the development of a plant comprising transforming a plant cell with a nucleic acid encoding a cyclin dependent kinase inhibitor to produce a transformed plant cell; and, regenerating the plant from the transformed plant cell under conditions wherein the cyclin dependent kinase inhibitor is expressed during regeneration of the plant to modify the development of the plant, wherein the cyclin independent kinase inhibitor is a gene selected from the group consisting of ICK2, ICN2, ICN6, ICN7 and homologs thereof.
4. The method of Claim 2, wherein the plant is a member of the *Brassica* genus.
5. IK2 gene, having the cDNA nucleotide sequence of Figure 1.
6. ICN2 gene, having the cDNA nucleotide sequence of Figure 2.
7. ICN6 gene, having the cDNA nucleotide sequence of Figure 3.
8. ICN7 gene, having the cDNA nucleotide sequence of Figure 4.

GTGSAATCTAGGATAATTCTGTCTCCGTGTGTACAGGCCGACGAATCGCGGTCCAATTGTG
GCGAGAAATT CAGCAGGAGCGT CCGAGACGAGTGTTGTTATAGTACGACGGCCGAGATTCT
CCTCCGGTTGAAGAACAGTGTCAAATCGAAGAAGAAGATTCTCGGTTTCGTGTTGTTCT
ACATCGGAAGAGAAATCGAAACGGAGAATCGAATTTGTAGATCTTGAGGAAAATAACGGT
GACGATCGTGAACACAGAAACGTCGTGGATTACGATGATTTGAATAAGAGTGAGGAATCG
ATGAACATGGATTCTTCTTCGGTGGCTGTTGAAGATGTAGAGTCTCGCCGCAGGTTAAGG
AAGAGTCTCCATGAGACCGTGAAGGAAGCTGAGTTAGAAGACTTTTTTCAGGTGGCCGAG
AAAGATCTTCGAATAAGTTGTTGGAATGTTCTATGAAGTATAACTTCGATTTGAGAAA
GATGAGCCACTTGGTGGAGGAAGATACGAGTGGGTAAATTGAATCCATGAAGAAGACGA
TGATGATAATGATGATCATTGTTTTACCAAAGTACTTATTATTTCTCTTCTGTAATAAT
CTTTGCTTTGATTTTTCTTTTAACAAAATCCAAATGTAGATATCTTTCTCTCGAATAATC
AATAACATGTAATTCACCTTTTCTTTGTACTTCCTTGAGGTAATTAATTAGATTCTGTT
TTTCTCGATTAATAAACTATAAAGTTTATAACTAAA

cDNA sequence of *ICK2*

Figure 1

AAAAAAAAAGCAGAGAGAGAGAGCACAAAAATCCAAGAGAGAAAAAATGAGCGAGAGA
AAGCGAGAGCTTGCAGAAGAAGCTTCAAGCACAAGCTTCTCACCACCTGAAGAAAACGAAG
CTTAATGATTCTTCTGATTCAACCGGACTCTCATGACGTCATCGTCTTCGGGGTTTCA
TCTTCTTCCGTTGCTTCGTCCGGCGGCTTTAGCGTCTGATGAATGTTCCGTTACCATCGGT
GGAGAAGAAAAGTGATCAGTCCTCGAGTATCAGCTCCGGTTGTTTCACCAGTGAATCGAAA
CAAATCGCGAAGAACAGTTCGTCTGTTTGGTGTAGATCTGGAGGATCATCAAATCGAAACC
GAAACCGAAACCTCAACATTCAACAGCAATTCAGAAAAGAGACGAGTCCAGTGAGT
GAGGGTTTGGGAGAAACGACAACAGAAATGGAATCATCATCGGCAACGAAGAGAAAACAA
CCGGGGGTGAGGAAGACTCCAACGGCGGCGGAGATTGAGGATTTGTTCTCGGAGCTAGAG
AGTCAAGACGATAAGAAGAAGCAATTCATAGAAAAGTACAACCTTCGATATTGTCAATGAC
GAAACCGCTTGAAGGTGCTACAAGTGGGATCGACTTTAAGCCATCAAAAAGCAAATACCA
TCCATGAAGAAGACAAAAGAAAAATAGGTTTTGTTTTTCGTGGTTAACATTTCCACTTGT
ACAGCTCTAGTCTATTTCTCTTTAAAAACCTATGTTACTAGTTTCGTACAAAACAAAACAA
AAAACACGACCTTTATAATGAAATTTGGGATCTTGGCTACTAAA

cDNA sequence of *ICN2*

Figure 2

CTCTCTCCAGAGAAAATATAATGAGCTTGAGAGAAATGAGCGAAACAAAACCCAAAGAGA
GATTCTGAGTACGAAGGATCAACATCAAGAGGATGAGACTCGATGATGATGATGACGTT
TTACGCTCACCGACGAGAACTCTTTCTTCTTCTTCTTCTTCTTCTTCTTGGCTTACTCGGTT
TCAGATTCCGGAGGTTTCTGCTCCGTCCGCTTATCTGAAGAAGAAGACGATCATCTAAGC
TCAGCATCAGCTCTGTTGTTCCAGCAGCGAACTAACGAAATCCCTACTCGTCTTCCA
TTTTCAGATCTGGAGGCTCATGAATCTCCGAAACCGAAATCTCAACGTTACTCACCAAC
AATTTCAGGAAACAGGGAAATTTATCAAGCGAGAATCTGGGAGAAACAGCAGAAATGGAC
TCGGCGACGACGGAGATGAGAGATCAGAGAAAGACGGAGAAGAAGAAGATGGAAAAA
TCACCGACGCAGGCAGAGCTTGATGACTTTTTCTCGCCGCCGGAGAGATACGAACAGAAA
CGATTACAGAAAAGTACAACCTACGACATCGTCAATGATACGCCGCTTCAAGGTCGGTAC
CAGTGGGTAGTCTGAAACCTTAGAAGCCATGGAAGAACAAA

cDNA sequence of *ICN6*

Figure 3

ATTAAGAGTCTGGTTCCAGGTCTCGCGTTGACTCGGTAACTCGGCTCCTGTAGCTCAG
AGCTCTAATGAAGATGAATGTTTTGACAAATTCGTGAGTGTCCAAGTTTCTTGTCGTGAA
AACAGTCTCGGTTTTGAATCAAGACACAGCACAAGGGAGAGCACGCCTTGTAACTTTGT
GACGATATGGAGATCATGGTTACACCAGGGTCTAGCACGAGGTGGATGTGCAGAGCAACC
AAAGACTACACAAGGGAACAAGATAACGTGATCCCGACCACTAGTGAATGGAGGAGTTC
TTTGCATATGCAGAGCAGCAGCAACAGAGGCTATTGATGGAGAAGTACAACCTTCGACATT
GTGAATGATATCCCCCTCAGCGGACGTTACGAATGGGTGCAAGTCAAACCATGAAGTTCA
AAAGGAAACAGCTCCAAAAGACATGGTGTGAAGTTAGAGAATTGTGATGGAGTTTAACAG
AACTAACCAACATCAGAAATCGTGTTAATCCTTAAGTTAATAATGTGGGTTA

cDNA sequence of *ICN7*

Figure 4

[illegible]

...C..SE.K-...F..DLE...E...F.K...E..L.E...S...R...K.K...PT.AE.E Consensus

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774 TETSTYRGTH-----RKLCEHREEEKZEELSKSHENYSSE-----ESAVKSDCCCSGRKTHETV-TAEEDAAIMTEHES I ICK1.pro
775 ---CLSTH-----SKRRIE---VADJENNGDORETETSMIYDDLNSEEEM--NMDSSVAVEDVESRR-----LRSLHETVKE I ICK2.pro
776 ISSGFTSPS EIAKNSSSGV---DHQIET TETSTFITSNRUETSPVSGGTTTEHES SATK-----OPGVKTH I ICK3.pro
777 ISSGSSSTHNEIATRLP-SS---AHEI-S TEISTILLTNVRQGISSSNGGTA-EMDATTEDDQKTEHPOCKS I ICK6.pro
778 VOVSJGEN-----SLGFSRHSSTRSTPCNFV-----DH-IMVTPGSTRSHCRATKEYTREQDNVITSTH I ICK7.pro

```

DEF..AC.-.Q...F.E.--KYNFDI.VND.PLEG-RYENW.L.P.-

1154	DLKVEFK--DLKEMKK--GKIKFEKEN--GKIKFEKEN--XKE.
1134	DQVOVFK--DLRNKLLECSM--GKIKFEKEN--GKIKFEKEN--XNH.
1174	DLSELSQDUKKKGIL--GKIKFEKEN--GKIKFEKEN--DRL
1169	BESAKRYEKR--FIT--GKIKFEKEN--GKIKFEKEN--SKH.
99	EAYAL--QOORLD--GKIKFEKEN--GKIKFEKEN--OVQ

Conclusions

ICK1.pro
ICK2.pro
ICN2.pro
ICN6.pro
ICN7.pro

Consensus 'Consensus #1': When 60% (3) match the residue of the Consensus show the residue of the Consensus, otherwise show ' '.

Decoration 'Decoration 11': Shade (with black at 40% (11)) residues that match the consensus named 'Consensus 11' exactly.

Alignment of deduced amino acid sequences of *ICK1*, *ICK2*, *ICK3*, *ICK6* and *ICK7*

Figure 5