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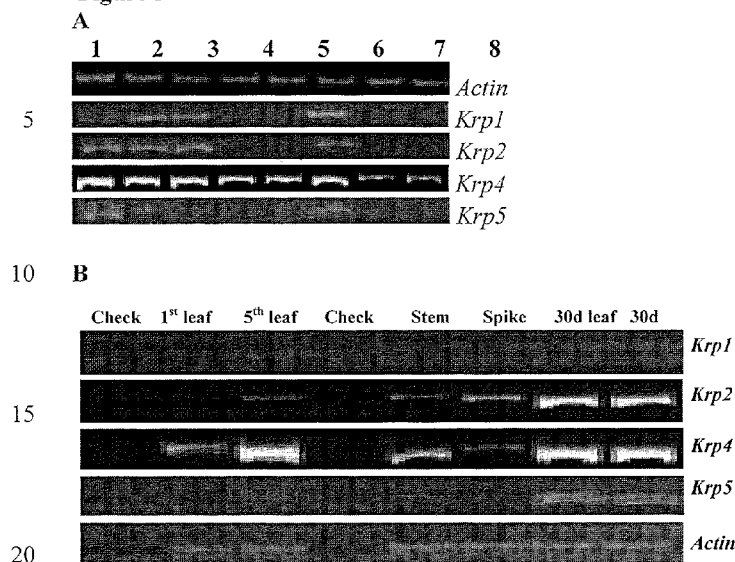
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[Continued on next page]

(54) Title: IDENTIFICATION AND USE OF KRP MUTANTS IN WHEAT

Figure 3



(57) Abstract: The invention provides a wheat cell, part, tissue culture or whole plant comprising at least one disrupted KRP gene of the present invention. The present invention also provides methods of increasing weight, size, and/or number of one or more organs, and/or yield of a wheat plant by utilizing the disrupted KRP genes of the present invention. Furthermore, methods of breeding wheat plants to produce new wheat plants having increased weight, size, and/or number of one or more organs, and/or yield are provided. The present invention provides isolated Kinase Inhibitor Protein (KIP) Related Protein (KRP) polynucleotide sequences and isolated KRP polypeptide sequences and methods of their use.



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IDENTIFICATION AND USE OF KRP MUTANTS IN WHEAT

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/474,203, filed April 11, 2011, which is hereby incorporated by reference in their entireties for all purposes.

TECHNICAL FIELD

The invention generally relates to identifying and using compositions and methods for improving the agronomic characteristics of wheat, such as by increasing wheat yield. More specifically, the present invention relates to compositions and methods for improving one or more agronomic characteristics of wheat by identifying and using one or more mutant Kinase Inhibitor Protein (KIP) Related Proteins (KRP).

BACKGROUND

The most important trait as a target for crop improvement is yield. Efforts to improve crop yields by developing new plant varieties can be divided into two approaches. One is to reduce crop yield losses by breeding or engineering crop varieties with increased resistance to abiotic stress conditions such as drought, cold, or salt or to biotic stress conditions resulting from pests or disease-causing pathogens. While this approach has value, it does not provide fundamentally improved crop yield in the absence of stress conditions and in fact, such resistance may direct plant resources that otherwise would be available for increased yield in the plant. The second approach is to breed or engineer new crop varieties in which the basic yield capacity is increased.

Classical breeding programs have initially produced substantial gains in improved yield in a variety of crops. A commonly experienced pattern though has been substantial gains in yield initially followed by incremental further improvements that become smaller and more difficult to obtain. More recently developed approaches based on molecular biology technologies have in principle offered the potential to achieve substantial improvement in crop yield by altering the timing, location, or level of expression of plant genes or heterologous genes that play a role in plant growth and/or development. Substantial progress has been made over the past twenty years in identifying plant genes and or

heterologous genes that have a role in plant growth and/or development. Despite these gains in using molecular approaches, there continues to be a large unmet need for improved agronomic and horticultural plants produced through more conventional plant breeding. Because of the complexity of plant growth regulation and how it relates in the end to yield traits, it is still not obvious which, if any, of particular genes would be clear candidates to improve crop yield through either plant breeding and/or molecular techniques.

KRP proteins belong to a class of cell cycle inhibitors that bind to and inhibit cyclin/CDK kinase complexes. Mutation of conserved residues within KRP family members are expected to modify KRP's ability to function as an inhibitor of cyclin-CDK kinase complexes. Specifically, some mutations in KRP genes would lead to expression of a non-functional KRP cell cycle inhibitor or a cell cycle inhibitor with reduced activity. This loss of or reduced cyclin/CDK kinase inhibitory activity would lead to increased cyclin-CDK kinase activity in cells when normally these cells would have reduced cyclin-CDK activity. This loss of or reduced cyclin/CDK kinase inhibitory activity would lead to increased cell divisions in tissue where the normal wild-type KRP version is expressed. This increased cell division would result in positive agronomic traits such as increased yield, increased seed size, larger plants, larger leaves, larger roots etc. For background on KRP-related technologies, see, for example, WO/2007/016319 and US20070056058, each of which is incorporated by reference in its entirety for all purposes. The present invention identifies new KRP genes and proteins in wheat as well as providing methods for their use in producing improved wheat plants through conventional plant breeding and/or molecular methodologies.

SUMMARY OF INVENTION

The present invention provides a plant comprising in its genome one or more disrupted KRP genes. The present invention in another aspect provides a plant cell, plant part, or tissue culture derived from the plants of the present invention.

The present invention provides mutants in *KRP* genes, for example, KRP1, KRP2, KRP4, KRP5, including but not limited to those as listed in Tables 2 to 3.

In another aspect, the present invention provides methods for increasing weight, size, and/or number of one or more organs in a plant. The organ can be any part of a plant, for example, organs that contribute to yield in a plant. In some embodiments, the organ is seed, leaf, branch, root, shoot, stigma, ovule, pollen, seed pods, seed heads, or tiller. In some

embodiments, said methods comprise disturbing one or more KRPs in the plant. In some embodiments, methods for increasing seed weight, seed size, seed number and/or yield in a plant are provided. In one embodiment, the plant is a monocotyledon plant. In some embodiments, the plant can be a monocotyledon plant selected from the *Triticeae* tribe, for example, wheat. Methods of disrupting a gene function include but are not limited to mutagenesis (e.g., chemical mutagenesis, radiation mutagenesis, transposon mutagenesis, insertional mutagenesis, signature tagged mutagenesis, site-directed mutagenesis, and natural mutagenesis), antisense, knock-outs, and/or RNA interference.

In some embodiments, mutations described in the Tables 2 and 3 can be integrated into species closely related to the plants in the *Triticeae* tribe, or plants closely related to wheat. In some embodiments, amino acids in conserved domains or sites compared to KRP orthologs in other species can be substituted or deleted to make mutants with reduced or abolished activity, mutants that lead to loss-of-function (e.g., protein instability), and/or mutants that lead to gain-of-function (e.g., more stable or more active protein). In some embodiments, one or more KRPs in a wheat plant are knocked down or knocked out by one or more methods available to one skilled in the art.

In some embodiments, in a tetraploid wheat plant, one or two copies of a KRP gene are disrupted (e.g., KRP1A, KRP1B; KRP2A, KRP2B; KRP4A, KRP4B; and KRP5A, KRP5B); in a hexaploid wheat plant, one or more copies of one, two, or three copies of a KRP gene are disrupted (e.g., KRP1A, KRP1B, KRP1D; KRP2A, KRP2B, KRP2D; KRP4A, KRP4B, KRP4D; and KRP5A, KRP5B, KRP5D).

In another aspect, the present invention provides methods of producing a plant having increased weight, size, and/or number of one or more organs, for example, a plant with increased seed size, seed number, and/or seed yield compared to a wild type reference plant. Such methods comprising utilizing mutations in the KRP genes as described herein.

The present invention also provides a plant having increased seed size, seed number, and/or seed yield compared to a wild type reference plant, wherein the plant has one or more mutations in one or more KRP genes. In some embodiments, said plant is a monocot plant. In some embodiments, said monocot plant is a plant from the *Triticeae* tribe. In some embodiments, said plant is wheat.

The present invention further provides a seed, a fruit, a plant cell or a plant part of the transgenic plants as described herein. For example, the present invention provides a pollen of the plant, an ovule of the plant, a genetically related plant population comprising the plant, a

tissue culture of regenerable cells of the plant. In some embodiments, the regenerable cells are derived from embryos, protoplasts, meristematic cells, callus, pollen, leaves, anthers, stems, petioles, roots, root tips, fruits, seeds, flowers, cotyledons, and/or hypocotyls.

The present invention also provides methods of breeding a crop species having improved agronomic and horticultural characteristics, such as new plant types having increased weight, size, and/or number of one or more organs, for example, a plant with increased seed size, seed number, seed weight and/or seed yield compared to a wild type reference plant.

In some embodiments, such methods comprise making a cross between a *Triticum sp.* mutant with one or more mutations listed in Tables 2 and 3 with a second *Triticum sp.* to produce an F1 plant, or with a species in the *Triticeae* tribe which can intercross with said *Triticum sp.* The method may further comprise backcrossing the F1 plant to the second *Triticum sp.* or species in the *Triticeae* tribe; and repeating the backcrossing step to generate an near isogenic line, wherein the one or more mutations are integrated into the genome of said second *Triticum sp.* or the species in the *Triticeae* tribe; wherein the near isogenic line derived from the second *Triticum sp.* or the species in the *Triticeae* tribe with the integrated mutations has altered weight, size, and/or number of one or more organs, for example, altered seed weight, seed size, seed number, and/or seed yield. Optionally, such methods can be facilitated by molecular markers or TILLING®.

The present invention also provides methods of decreasing the activity of one or more KRP proteins in a plant cell, plant part, tissue culture or whole plant comprising contacting the plant cell, plant part, tissue culture or whole plant with an inhibitory nucleic acid having complementarity to a gene encoding said KRP protein. In some embodiments, the plant is a plant from the *Triticeae* tribe. In some embodiments, said plant is wheat.

The present invention provides isolated wheat KRPs, including by way of example, KRP1A, KRP1B, KRP1D, KRP2A, KRP2B, KRP2D, KRP4B, KRP4D, KRP5A, KRP5D, and mutations in these genes. Based on our findings, it appears that hexaploid wheat has been naturally selected to have what appear to be knock-out mutations in KRP4A and KRP5B. This was discovered during the course of TILLING®. For KRP4A, the gene appears to be completely missing from the hexaploid wheat genome, although it is still present in the tetraploid genome. For KRP5B, there appears to be one missing nucleotide in the gene, which would shift the translational frame and produce a predicted truncation of a few amino acids further on.

The present invention provides an isolated nucleic acid sequence comprising a sequence selected from the group consisting of SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, and fragments and variations derived from thereof, which encode a KRP gene.

5 In one embodiment, the present invention provides an isolated polynucleotide encoding plant KRP protein, comprising a nucleic acid sequence that shares at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, or at least 99.9% identity to SEQ ID
10 NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77.

The present invention further provides an isolated amino acid sequence (e.g., a peptide, polypeptide and the like) comprising a sequence selected from the group consisting of SEQ ID NOs: 42, 45, 48, 51, 54, 57, 61, 65, 69, 72, 75, and 78 and fragments and
15 variations derived from thereof, which form a KRP protein.

In some embodiments, the present invention provides an isolated amino acid sequence which forms a protein that shares an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at
20 least 99.6%, at least 99.7%, at least 99.8%, or at least 99.9% identity to SEQ ID NOs: 42, 45, 48, 51, 54, 57, 61, 65, 69, 72, 75, and 78.

In one embodiment, isolated polynucleotides of the present invention comprise a sequence selected from the group consisting of: (a) sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or
25 portions thereof; (b) complements of the sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or portions thereof; (c) reverse complements of the sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or portions thereof; (d) reverse sequences of the sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47,
30 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or portions thereof; and (e) sequences having at least 50%, 75%, 90%, 95% or 98% identity, as defined herein, to a sequence of (a)-(d) or a specified region of a sequence of (a)-(d).

The present invention also provides a chimeric gene comprising the isolated nucleic acid sequence of any one of the polynucleotides described above operably linked to suitable regulatory sequences.

The present invention also provides recombinant constructs comprising the chimeric gene as described above.

The present invention further provides interfering RNA (RNAi) constructs based on nucleic acid sequences of the present invention. In some embodiments, the RNAi constructs are can be transformed into a wheat plant to down-regulate one or more KRPs. The RNAi construct can be, but is not limited to antisense oligonucleotide construct, double-strand oligonucleotide construct, siRNA construct, or inverted repeat construct. In some embodiment, the RNAi constructs comprise a plant promoter, such as a constitutive promoter, an inducible promoter, or a tissue-specific promoter. In some embodiments, the promoter is embryonic specific or seed specific.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a phylogenetic tree of rice (Os), corn (Zm) and wheat (Ta) KRPs.

Figure 2 depicts PCR using indicated genome-specific primers on wheat deleted for a given genome. N1A=A genome deleted. N1B=B genome deleted. N1D=D genome deleted. T4=tetraploid TILLING® line. T6=hexaploid TILLING® line.

Figure 3 depicts RT-PCR on wheat KRP transcripts. RNA was extracted using a QIAGEN® kit, and cDNAs were produced with the INVITROGEN™ reverse transcription system. **A**, expression in young seeds from three developmental stages. Lanes 1-3: 2-6 days after anthesis (pools 1, 2 and 3). Lanes 4-6: 8-12 days after anthesis (pools 1, 2 and 3). Lanes 7-8: 14-18 days after anthesis (pools 1 and 2). **B**, expression in indicated tissues. Actin served as an internal transcript control.

SEQUENCES

Sequence listings for SEQ ID No: 1 – SEQ ID No: 87 are part of this application and are incorporated by reference herein. Sequence listings are provided at the end of this document.

DETAILED DESCRIPTION

All publications, patents and patent applications, including any drawings and appendices, and all nucleic acid sequences and polypeptide sequences identified by GenBank Accession numbers, herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

Definitions

As used herein, the verb “comprise” as is used in this description and in the claims and its conjugations are used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded.

As used herein, the term “plant” refers to any living organism belonging to the kingdom Plantae (i.e., any genus/species in the Plant Kingdom). This includes familiar organisms such as but not limited to trees, herbs, bushes, grasses, vines, ferns, mosses and green algae. The term refers to both monocotyledonous plants, also called monocots, and dicotyledonous plants, also called dicots. Examples of particular plants include but are not limited to plants in the *Triticeae* tribe (e.g., plants in the *Triticum* genus), plants in the tribe of *Oryzeae* (e.g., plants in *Oryza* genus), plants in the *Andropogoneae* tribe (e.g., plants in the *Zea* genus, such as corn). Other non-limiting examples of plant include, potatoes, roses, apple trees, sunflowers, bananas, tomatoes, opo, pumpkins, squash, lettuce, cabbage, oak trees, guzmania, geraniums, hibiscus, clematis, poinsettias, sugarcane, taro, duck weed, pine trees, Kentucky blue grass, zoysia, coconut trees, brassica leafy vegetables (e.g. broccoli, broccoli raab, Brussels sprouts, cabbage, Chinese cabbage (Bok Choy and Napa), cauliflower, cavalo, collards, kale, kohlrabi, mustard greens, rape greens, and other brassica leafy vegetable crops), bulb vegetables (e.g. garlic, leek, onion (dry bulb, green, and Welch), shallot, and other bulb vegetable crops), citrus fruits (e.g. grapefruit, lemon, lime, orange, tangerine, citrus hybrids, pummelo, and other citrus fruit crops), cucurbit vegetables (e.g. cucumber, citron melon, edible gourds, gherkin, muskmelons (including hybrids and/or cultivars of cucumis melons), water-melon, cantaloupe, and other cucurbit vegetable crops),

fruiting vegetables (including eggplant, ground cherry, pepino, pepper, tomato, tomatillo, and other fruiting vegetable crops), grape, leafy vegetables (e.g. romaine), root/tuber and corm vegetables (e.g. potato), and tree nuts (almond, pecan, pistachio, and walnut), berries (e.g., tomatoes, barberries, currants, elderberries, gooseberries, honeysuckles, mayapples, 5 nannyberries, Oregon-grapes, see-buckthorns, hackberries, bearberries, lingonberries, strawberries, sea grapes, lackberries, cloudberry, loganberries, raspberries, salmonberries, thimbleberries, and wineberries), cereal crops (e.g., corn, rice, wheat, barley, sorghum, millets, oats, ryes, triticales, buckwheats, fonio, quinoa, oil palm), pome fruit (e.g., apples, pears), stone fruits (e.g., coffees, jujubes, mangos, olives, coconuts, oil palms, pistachios, 10 almonds, apricots, cherries, damsons, nectarines, peaches and plums), vine (e.g., table grapes, wine grapes), fiber crops (e.g. hemp, cotton), ornamentals, and the like.

As used herein, the term "plant part" refers to any part of a plant including but not limited to the shoot, root, stem, seeds, stipules, leaves, petals, flowers, ovules, bracts, branches, petioles, internodes, bark, pubescence, tillers, rhizomes, fronds, blades, pollen, 15 stamen, and the like. The two main parts of plants grown in some sort of media, such as soil, are often referred to as the "above-ground" part, also often referred to as the "shoots", and the "below-ground" part, also often referred to as the "roots".

The term "a" or "an" refers to one or more of that entity; for example, "a gene" refers to one or more genes or at least one gene. As such, the terms "a" (or "an"), "one or more" and 20 "at least one" are used interchangeably herein. In addition, reference to "an element" by the indefinite article "a" or "an" does not exclude the possibility that more than one of the elements are present, unless the context clearly requires that there is one and only one of the elements.

As used herein, the term "nucleic acid" refers to a polymeric form of nucleotides of 25 any length, either ribonucleotides or deoxyribonucleotides, or analogs thereof. This term refers to the primary structure of the molecule, and thus includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modified nucleic acids such as methylated and/or capped nucleic acids, nucleic acids containing modified bases, backbone modifications, and the like. The terms "nucleic acid" and "nucleotide sequence" 30 are used interchangeably.

As used herein, the terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. These terms also

include proteins that are post-translationally modified through reactions that include glycosylation, acetylation and phosphorylation.

As used herein, the term "homologous" or "homologue" or "ortholog" is known in the art and refers to related sequences that share a common ancestor or family member and are determined based on the degree of sequence identity. The terms "homology", "homologous", "substantially similar" and "corresponding substantially" are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences. These terms describe the relationship between a gene found in one species, subspecies, variety, cultivar or strain and the corresponding or equivalent gene in another species, subspecies, variety, cultivar or strain. For purposes of this invention homologous sequences are compared. "Homologous sequences" or "homologues" or "orthologs" are thought, believed, or known to be functionally related. A functional relationship may be indicated in any one of a number of ways, including, but not limited to: (a) degree of sequence identity and/or (b) the same or similar biological function. Preferably, both (a) and (b) are indicated. The degree of sequence identity may vary, but in one embodiment, is at least 50% (when using standard sequence alignment programs known in the art), at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least 98.5%, or at least about 99%, or at least 99.5%, or at least 99.8%, or at least 99.9%. Homology can be determined using software programs readily available in the art, such as those discussed in *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987) Supplement 30, section 7.718, Table 7.71. Some alignment programs are MacVector (Oxford Molecular Ltd, Oxford, U.K.), ALIGN Plus (Scientific and Educational Software, Pennsylvania) and AlignX (Vector NTI, Invitrogen, Carlsbad, CA). Another alignment program is Sequencher (Gene Codes, Ann Arbor, Michigan), using default parameters.

As used herein, the term "nucleotide change" refers to, e.g., nucleotide substitution, deletion, and/or insertion, as is well understood in the art. For example, mutations contain alterations that produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded protein or how the proteins are made.

5 As used herein, the term "protein modification" refers to, e.g., amino acid substitution, amino acid modification, deletion, and/or insertion, as is well understood in the art,

As used herein, the term "derived from" refers to the origin or source, and may include naturally occurring, recombinant, unpurified, or purified molecules. A nucleic acid
10 or an amino acid derived from an origin or source may have all kinds of nucleotide changes or protein modification as defined elsewhere herein.

As used herein, the term "at least a portion" or "fragment" of a nucleic acid or polypeptide means a portion having the minimal size characteristics of such sequences, or any larger fragment of the full length molecule, up to and including the full length molecule. For
15 example, a portion of a nucleic acid may be 12 nucleotides, 13 nucleotides, 14 nucleotides, 15 nucleotides, 16 nucleotides, 17 nucleotides, 18 nucleotides, 19 nucleotides, 20 nucleotides, 22 nucleotides, 24 nucleotides, 26 nucleotides, 28 nucleotides, 30 nucleotides, 32 nucleotides, 34 nucleotides, 36 nucleotides, 38 nucleotides, 40 nucleotides, 45 nucleotides, 50 nucleotides, 55 nucleotides, and so on, going up to the full length nucleic acid. Similarly, a portion of a
20 polypeptide may be 4 amino acids, 5 amino acids, 6 amino acids, 7 amino acids, and so on, going up to the full length polypeptide. The length of the portion to be used will depend on the particular application. A portion of a nucleic acid useful as hybridization probe may be as short as 12 nucleotides; in one embodiment, it is 20 nucleotides. A portion of a polypeptide useful as an epitope may be as short as 4 amino acids. A portion of a polypeptide that
25 performs the function of the full-length polypeptide would generally be longer than 4 amino acids.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window.
30 When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the

functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences which differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4:11-17 (1988).

As used herein, the term "substantially complementary" means that two nucleic acid sequences have at least about 65%, preferably about 70% or 75%, more preferably about 80% or 85%, even more preferably 90% or 95%, and most preferably about 98% or 99%, sequence complementarities to each other. This means that primers and probes must exhibit sufficient complementarity to their template and target nucleic acid, respectively, to hybridize under stringent conditions. Therefore, the primer and probe sequences need not reflect the exact complementary sequence of the binding region on the template and degenerate primers can be used. For example, a non-complementary nucleotide fragment may be attached to the 5'-end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer has sufficient complementarity with the sequence of one of the strands to be amplified to hybridize therewith, and to thereby form a duplex structure which can be extended by polymerizing means. The non-complementary nucleotide sequences of the primers may include restriction enzyme sites. Appending a restriction enzyme site to the end(s) of the target sequence would be particularly helpful for cloning of the target sequence. A substantially complementary primer sequence is one that has sufficient sequence complementarity to the amplification template to result in primer binding and second-strand synthesis. The skilled person is familiar with the requirements of primers to have sufficient sequence complementarity to the amplification template.

As used herein, the terms "polynucleotide", "polynucleotide sequence", "nucleic acid sequence", "nucleic acid fragment", and "isolated nucleic acid fragment" are used interchangeably herein. These terms encompass nucleotide sequences and the like. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that

optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. Nucleotides (usually found in their 5'-monophosphate form) are referred to by a single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

As used herein, the phrase "a biologically active variant" or "functional variant" with respect to a protein refers to an amino acid sequence that is altered by one or more amino acids with respect to a reference sequence, while still maintains substantial biological activity of the reference sequence. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. Alternatively, a variant can have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations can also include amino acid deletion or insertion, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software.

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target allowing a DNA polymerase to attach, thereby serving as a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The (amplification) primer is preferably single stranded for maximum efficiency in amplification. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and composition (A/T vs. G/C content) of primer. A pair of bi-directional primers consists of one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

The terms "stringency" or "stringent hybridization conditions" refer to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH,

formamide concentration and the like. These conditions are empirically optimized to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The terms as used include reference to conditions under which a probe or primer will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g. at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe or primer. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na⁺ ion, typically about 0.01 to 1.0 M Na⁺ ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30° C for short probes or primers (e.g. 10 to 50 nucleotides) and at least about 60° C for long probes or primers (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringent conditions or “conditions of reduced stringency” include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37° C and a wash in 2×SSC at 40° C. Exemplary high stringency conditions include hybridization in 50% formamide, 1M NaCl, 1% SDS at 37° C, and a wash in 0.1×SSC at 60° C. Hybridization procedures are well known in the art and are described by e.g. Ausubel et al., 1998 and Sambrook et al., 2001.

As used herein, “coding sequence” refers to a DNA sequence that codes for a specific amino acid sequence. “Regulatory sequences” refer to nucleotide sequences located upstream (5′ non-coding sequences), within, or downstream (3′ non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

As used herein, “regulatory sequences” may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

As used herein, “promoter” refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a DNA sequence that can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to

enhance the level or tissue specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity.

As used herein, a "plant promoter" is a promoter capable of initiating transcription in plant cells whether or not its origin is a plant cell, e.g. it is well known that *Agrobacterium* promoters are functional in plant cells. Thus, plant promoters include promoter DNA obtained from plants, plant viruses and bacteria such as *Agrobacterium* and *Bradyrhizobium* bacteria. A plant promoter can be a constitutive promoter or a non-constitutive promoter.

As used herein, a "constitutive promoter" is a promoter which is active under most conditions and/or during most development stages. There are several advantages to using constitutive promoters in expression vectors used in plant biotechnology, such as: high level of production of proteins used to select transgenic cells or plants; high level of expression of reporter proteins or scorable markers, allowing easy detection and quantification; high level of production of a transcription factor that is part of a regulatory transcription system; production of compounds that requires ubiquitous activity in the plant; and production of compounds that are required during all stages of plant development. Non-limiting exemplary constitutive promoters include, CaMV 35S promoter, opine promoters, ubiquitin promoter, actin promoter, alcohol dehydrogenase promoter, etc.

As used herein, a "non-constitutive promoter" is a promoter which is active under certain conditions, in certain types of cells, and/or during certain development stages. For example, tissue specific, tissue preferred, cell type specific, cell type preferred, inducible promoters, and promoters under development control are non-constitutive promoters. Examples of promoters under developmental control include promoters that preferentially initiate transcription in certain tissues, such as stems, leaves, roots, or seeds.

As used herein, "inducible" or "repressible" promoter is a promoter which is under chemical or environmental factors control. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, or certain chemicals, or the presence of light.

As used herein, a "tissue specific" promoter is a promoter that initiates transcription only in certain tissues. Unlike constitutive expression of genes, tissue-specific expression is the result of several interacting levels of gene regulation. As such, in the art sometimes it is preferable to use promoters from homologous or closely related plant species to achieve efficient and reliable expression of transgenes in particular tissues. This is one of the main reasons for the large amount of tissue-specific promoters isolated from particular plants and tissues found in both scientific and patent literature. Non-limiting tissue specific promoters include, beta-amylase gene or barley hordein gene promoters (for seed gene expression), tomato p_{z7} and p_{z130} gene promoters (for ovary gene expression), tobacco RD2 gene promoter (for root gene expression), banana TRX promoter and melon actin promoter (for fruit gene expression), and embryo specific promoters, e.g., a promoter associated with an amino acid permease gene (AAP1), an oleate 12-hydroxylase:desaturase gene from *Lesquerella fendleri* (LFAH12), an 2S2 albumin gene (2S2), a fatty acid elongase gene (FAEL), or a leafy cotyledon gene (LEC2).

As used herein, a "tissue preferred" promoter is a promoter that initiates transcription mostly, but not necessarily entirely or solely in certain tissues.

As used herein, a "cell type specific" promoter is a promoter that primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots, leaves, stalk cells, and stem cells.

As used herein, a "cell type preferred" promoter is a promoter that primarily drives expression mostly, but not necessarily entirely or solely in certain cell types in one or more organs, for example, vascular cells in roots, leaves, stalk cells, and stem cells.

As used herein, the "3' non-coding sequences" or "3' untranslated regions" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht, I. L., et al. (1989) *Plant Cell* 1:671-680.

As used herein, "RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript. An RNA transcript is referred to as the mature RNA when it is an RNA sequence derived from

post-transcriptional processing of the primary transcript. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a DNA that is complementary to and synthesized from an mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into the double-stranded form using the Klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or in vitro. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA, and that blocks the expression of a target gene (U.S. Pat. No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes. The terms "complement" and "reverse complement" are used interchangeably herein with respect to mRNA transcripts, and are meant to define the antisense RNA of the message.

As used herein, the term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is regulated by the other. For example, a promoter is operably linked with a coding sequence when it is capable of regulating the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in a sense or antisense orientation. In another example, the complementary RNA regions of the invention can be operably linked, either directly or indirectly, 5' to the target mRNA, or 3' to the target mRNA, or within the target mRNA, or a first complementary region is 5' and its complement is 3' to the target mRNA.

As used herein, the term "recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques.

As used herein, the phrases "recombinant construct", "expression construct", "chimeric construct", "construct", and "recombinant DNA construct" are used interchangeably herein. A recombinant construct comprises an artificial combination of nucleic acid fragments, e.g., regulatory and coding sequences that are not found together in nature. For example, a chimeric construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found

in nature. Such construct may be used by itself or may be used in conjunction with a vector. If a vector is used then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) EMBO J. 4:2411-2418; De Almeida et al., (1989) Mol. Gen. Genetics 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, immunoblotting analysis of protein expression, or phenotypic analysis, among others. Vectors can be plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating.

The term “expression”, as used herein, refers to the production of a functional end-product e.g., an mRNA or a protein (precursor or mature).

As used herein, the phrase “plant selectable or screenable marker” refers to a genetic marker functional in a plant cell. A selectable marker allows cells containing and expressing that marker to grow under conditions unfavorable to growth of cells not expressing that marker. A screenable marker facilitates identification of cells which express that marker.

As used herein, the term “inbred”, “inbred plant” is used in the context of the present invention. This also includes any single gene conversions of that inbred. The term single allele converted plant as used herein refers to those plants which are developed by a plant breeding technique called backcrossing wherein essentially all of the desired morphological and physiological characteristics of an inbred are recovered in addition to the single allele transferred into the inbred via the backcrossing technique.

As used herein, the term “sample” includes a sample from a plant, a plant part, a plant cell, or from a transmission vector, or a soil, water or air sample.

As used herein, the term “offspring” refers to any plant resulting as progeny from a vegetative or sexual reproduction from one or more parent plants or descendants thereof. For instance an offspring plant may be obtained by cloning or selfing of a parent plant or by crossing two parent plants and include selfings as well as the F1 or F2 or still further generations. An F1 is a first-generation offspring produced from parents at least one of which is used for the first time as donor of a trait, while offspring of second generation (F2) or subsequent generations (F3, F4, etc.) are specimens produced from selfings of F1's, F2's etc. An F1 may thus be (and usually is) a hybrid resulting from a cross between two true breeding parents (true-breeding is homozygous for a trait), while an F2 may be (and usually is) an offspring resulting from self-pollination of said F1 hybrids.

As used herein, the term “cross”, “crossing”, “cross pollination” or “cross-breeding” refer to the process by which the pollen of one flower on one plant is applied (artificially or naturally) to the ovule (stigma) of a flower on another plant.

As used herein, the term “cultivar” refers to a variety, strain or race of plant that has been produced by horticultural or agronomic techniques and is not normally found in wild populations.

As used herein, the terms “dicotyledon” and “dicot” refer to a flowering plant having an embryo containing two seed halves or cotyledons. Dicotyledon plants at least include the Eudicot, Magnoliid, Amborella, Nymphaeales, Austrobaileyales, Chloranthales, and Ceratophyllum groups. Eudicots include these clades: *Ranunculales*, *sabiales*, *Proteales*, *Trochodendrales*, *Buxales*, and *Core Eudicots* (e.g., *Berberidopsidales*, *Dilleniales*, *Gunnerales*, *Caryophyllales*, *Santalales*, *Saxifragales*, *Vitales*, *Rosids* and *Asterids*). Non-limiting examples of dicotyledon plants include tobacco, tomato, pea, alfalfa, clover, bean, soybean, peanut, members of the Brassicaceae family (e.g., camelina, Canola, oilseed rape, etc.), amaranth, sunflower, sugarbeet, cotton, oaks, maples, roses, mints, squashes, daisies, nuts; cacti, violets and buttercups.

As used herein, the term “monocotyledon” or “monocot” refer to any of a subclass (Monocotyledoneae) of flowering plants having an embryo containing only one seed leaf and usually having parallel-veined leaves, flower parts in multiples of three, and no secondary growth in stems and roots. Non-limiting examples of monocotyledon plants include lilies, orchids, corn, rice, wheat, barley, sorghum, millets, oats, ryes, triticales, buckwheats, fonio, quinoa, grasses, such as tall fescue, goat grass, and Kentucky bluegrass; grains, such as wheat, oats and barley, irises, onions, palms.

As used herein, the term “gene” refers to any segment of DNA associated with a biological function. Thus, genes include, but are not limited to, coding sequences and/or the regulatory sequences required for their expression. Genes can also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be
5 obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

As used herein, the term “genotype” refers to the genetic makeup of an individual cell, cell culture, tissue, organism (e.g., a plant), or group of organisms.

10 As used herein, the term “hemizygous” refers to a cell, tissue or organism in which a gene is present only once in a genotype, as a gene in a haploid cell or organism, a sex-linked gene in the heterogametic sex, or a gene in a segment of chromosome in a diploid cell or organism where its partner segment has been deleted.

As used herein, the terms “heterologous polynucleotide” or a “heterologous nucleic
15 acid” or an “exogenous DNA segment” refer to a polynucleotide, nucleic acid or DNA segment that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell, but has been modified. Thus, the terms refer to a DNA segment which is foreign or heterologous to the cell, or homologous to the
20 cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

As used herein, the term “heterologous trait” refers to a phenotype imparted to a transformed host cell or transgenic organism by an exogenous DNA segment, heterologous polynucleotide or heterologous nucleic acid.

25 As used herein, the term “heterozygote” refers to a diploid or polyploid individual cell or plant having different alleles (forms of a given gene) present at least at one locus.

As used herein, the term “heterozygous” refers to the presence of different alleles (forms of a given gene) at a particular gene locus.

As used herein, the terms “homolog” or “homologue” refer to a nucleic acid or
30 peptide sequence which has a common origin and functions similarly to a nucleic acid or peptide sequence from another species.

As used herein, the term “homozygote” refers to an individual cell or plant having the same alleles at one or more or all loci. When the term is used with reference to a specific locus or gene, it means at least that locus or gene has the same alleles.

As used herein, the terms “homozygous” or “HOMO” refer to the presence of identical alleles at one or more or all loci in homologous chromosomal segments. When the terms are used with reference to a specific locus or gene, it means at least that locus or gene has the same alleles.

As used herein, the term “hybrid” refers to any individual cell, tissue or plant resulting from a cross between parents that differ in one or more genes.

As used herein, the term “inbred” or “inbred line” refers to a relatively true-breeding strain.

As used herein, the term “line” is used broadly to include, but is not limited to, a group of plants vegetatively propagated from a single parent plant, via tissue culture techniques or a group of inbred plants which are genetically very similar due to descent from a common parent(s). A plant is said to “belong” to a particular line if it (a) is a primary transformant (T0) plant regenerated from material of that line; (b) has a pedigree comprised of a T0 plant of that line; or (c) is genetically very similar due to common ancestry (e.g., via inbreeding or selfing). In this context, the term “pedigree” denotes the lineage of a plant, e.g. in terms of the sexual crosses affected such that a gene or a combination of genes, in heterozygous (hemizygous) or homozygous condition, imparts a desired trait to the plant.

As used herein, the terms “mutant” or “mutation” refer to a gene, cell, or organism with an abnormal genetic constitution that may result in a variant phenotype.

As used herein, the term “open pollination” refers to a plant population that is freely exposed to some gene flow, as opposed to a closed one in which there is an effective barrier to gene flow.

As used herein, the terms “open-pollinated population” or “open-pollinated variety” refer to plants normally capable of at least some cross-fertilization, selected to a standard, that may show variation but that also have one or more genotypic or phenotypic characteristics by which the population or the variety can be differentiated from others. A hybrid, which has no barriers to cross-pollination, is an open-pollinated population or an open-pollinated variety.

As used herein when discussing plants, the term “ovule” refers to the female gametophyte, whereas the term “pollen” means the male gametophyte.

As used herein, the term “phenotype” refers to the observable characters of an individual cell, cell culture, organism (e.g., a plant), or group of organisms which results from the interaction between that individual’s genetic makeup (i.e., genotype) and the environment.

5 As used herein, the term “plant tissue” refers to any part of a plant. Examples of plant organs include, but are not limited to the leaf, stem, root, tuber, seed, branch, pubescence, nodule, leaf axil, flower, pollen, stamen, pistil, petal, peduncle, stalk, stigma, style, bract, fruit, trunk, carpel, sepal, anther, ovule, pedicel, needle, cone, rhizome, stolon, shoot, pericarp, endosperm, placenta, berry, stamen, and leaf sheath.

10 As used herein, the term “self-crossing”, “self pollinated” or “self-pollination” means the pollen of one flower on one plant is applied (artificially or naturally) to the ovule (stigma) of the same or a different flower on the same plant.

As used herein, the term “transformation” refers to the transfer of nucleic acid (*i.e.*, a nucleotide polymer) into a cell. As used herein, the term “genetic transformation” refers to
15 the transfer and incorporation of DNA, especially recombinant DNA, into a cell.

As used herein, the term “transformant” refers to a cell, tissue or organism that has undergone transformation. The original transformant is designated as “T0” or “T₀.” Selfing the T0 produces a first transformed generation designated as “T1” or “T₁.”

20 As used herein, the term “transgene” refers to a nucleic acid that is inserted into an organism, host cell or vector in a manner that ensures its function.

As used herein, the term “transgenic” refers to cells, cell cultures, organisms (e.g., plants), and progeny which have received a foreign or modified gene by one of the various methods of transformation, wherein the foreign or modified gene is from the same or different species than the species of the organism receiving the foreign or modified gene.

25 As used herein, the term “transposition event” refers to the movement of a transposon from a donor site to a target site.

As used herein, the term “variety” refers to a subdivision of a species, consisting of a group of individuals within the species that are distinct in form or function from other similar arrays of individuals.

30 As used herein, the term “vector”, “plasmid”, or “construct” refers broadly to any plasmid or virus encoding an exogenous nucleic acid. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into virions or cells, such as, for example, polylysine compounds and the like. The vector may be

a viral vector that is suitable as a delivery vehicle for delivery of the nucleic acid, or mutant thereof, to a cell, or the vector may be a non-viral vector which is suitable for the same purpose. Examples of viral and non-viral vectors for delivery of DNA to cells and tissues are well known in the art and are described, for example, in Ma et al. (1997, Proc. Natl. Acad. Sci. U.S.A. 94:12744-12746).

As used herein, the phrase “seed size” refers to the volume of the seed material itself, which is the space occupied by the constituents of the seed.

As used herein, the phrase “seed number” refers to the average number of seeds produced from each fruit, each plant, or each predetermined area (e.g., 1 acre).

As used herein, the phrase “Test Weight” or “Grain Test Weight” is a determination of bulk density (mass/volume), measured for commerce under specific conditions defined in the U.S. by the USDA-FGIS. Test weight is a general indicator of grain quality and higher test weight normally means higher quality grain. Grain test weight in units of pounds per bushel specifies the weight of a “volume” bushel, which is 32 quarts (30,283 cubic centimeters) of grain. When grain is traded, samples are usually tested for quality, and test weight is one of the tests carried out. Test weights have been a part of U.S. grain grades since the United States Grain Standards Act was passed by Congress in 1916. U.S. grades for most grains specify test weight minimums for each grade level. For instance, the official minimum allowable test weight in the U.S. for No. 1 yellow corn is 56 lbs/bu and for No. 2 yellow corn is 54 lbs/bu (USDA-GIPSA, 1996). By law, a “weight” bushel of corn is exactly 56 pounds, a soybean bushel is 60 pounds, and a wheat bushel is 60 pounds, regardless of the test weight. The “weight” bushel is used for the basis of payment for grain, but price discounts are often tied to shipments of lower grade grain possessing low test weight.

As used herein, the phrase “Grain Apparent Density” refers to grain density determined in a fashion wherein the bulk density (mass/volume) of cereal seed is sometimes measured with the aid of a gas pycnometer, which typically uses helium and measures the volume of the sample. Grain kernels contain internal void spaces and intercellular spaces and are not completely porous to helium. Since the gas cannot reach all internal spaces, the volume of material comprising the kernel can be overestimated with gas pycnometry and a density lower than the “true density” of grain material is determined (Chang, CS (1988) Cereal Chem:65:13-15).

As used herein, the phrase “Grain True Density” refers to the bulk density of grain, expressed as the quotient of mass divided by volume, whereby all void space not comprising

solid materials of the seed has been eliminated before, or discounted in, determination of the volume used in the calculation (Chang, CS (1988) Cereal Chem:65:13-15).

As used herein, the term “cyclin dependent kinase inhibitor” (also referred to herein as “CDK inhibitor” or “CKI”) refers to a class of proteins that negatively regulate cyclin dependent kinases (CDKs). CKIs amenable to the present invention are those having separate polypeptide regions capable of independently binding a cyclin and a CDK. Such CKIs include, for example, identified families of plant CKIs (the seven identified Arabidopsis CKIs), having homology to Kinase Inhibitor Proteins (KIPs) in animals, referred to as KIP-related proteins (KIPs) (also known as Inhibitors of “CDKs,” or “ICKs”).

The term “naturally occurring,” in the context of CKI polypeptides and nucleic acids, means a polypeptide or nucleic acid having an amino acid or nucleotide sequence that is found in nature, i.e., an amino acid or nucleotide sequence that can be isolated from a source in nature (an organism) and which has not been intentionally modified by human intervention. As used herein, laboratory strains of plants which may have been selectively bred according to classical genetics are considered naturally-occurring plants.

As used herein, “wild-type CKI gene” or “wild-type CKI nucleic acid” refers to a sequence of nucleic acid, corresponding to a CKI genetic locus in the genome of an organism, that encodes a gene product performing the normal function of the CKI protein encoded by a naturally-occurring nucleotide sequence corresponding to the genetic locus. A genetic locus can have more than one sequence or allele in a population of individuals, and the term “wild-type” encompasses all such naturally-occurring alleles that encode a gene product performing the normal function. “Wild-type” also encompasses gene sequences that are not necessarily naturally occurring, but that still encode a gene product with normal function (e.g., genes having silent mutations or encoding proteins with conservative substitutions).

As used herein, the term “wild-type CKI polypeptide” or “wild-type CKI protein” refers to a CKI polypeptide encoded by a wild-type gene. A genetic locus can have more than one sequence or allele in a population of individuals, and the term “wild-type” encompasses all such naturally-occurring alleles that encode a gene product performing the normal function.

Breeding Methods

Classic breeding methods can be included in the present invention to introduce one or more recombinant KRPs of the present invention into other plant varieties, or other close-related species that are compatible to be crossed with the transgenic plant of the present invention.

5 Open-Pollinated Populations. The improvement of open-pollinated populations of such crops as rye, many maizes and sugar beets, herbage grasses, legumes such as alfalfa and clover, and tropical tree crops such as cacao, coconuts, oil palm and some rubber, depends essentially upon changing gene-frequencies towards fixation of favorable alleles while maintaining a high (but far from maximal) degree of heterozygosity. Uniformity in such
10 populations is impossible and trueness-to-type in an open-pollinated variety is a statistical feature of the population as a whole, not a characteristic of individual plants. Thus, the heterogeneity of open-pollinated populations contrasts with the homogeneity (or virtually so) of inbred lines, clones and hybrids.

Population improvement methods fall naturally into two groups, those based on
15 purely phenotypic selection, normally called mass selection, and those based on selection with progeny testing. Interpopulation improvement utilizes the concept of open breeding populations; allowing genes to flow from one population to another. Plants in one population (cultivar, strain, ecotype, or any germplasm source) are crossed either naturally (e.g., by wind) or by hand or by bees (commonly *Apis mellifera* L. or *Megachile rotundata* F.) with
20 plants from other populations. Selection is applied to improve one (or sometimes both) population(s) by isolating plants with desirable traits from both sources.

There are basically two primary methods of open-pollinated population improvement. First, there is the situation in which a population is changed en masse by a chosen selection procedure. The outcome is an improved population that is indefinitely propagable by
25 random-mating within itself in isolation. Second, the synthetic variety attains the same end result as population improvement but is not itself propagable as such; it has to be reconstructed from parental lines or clones. These plant breeding procedures for improving open-pollinated populations are well known to those skilled in the art and comprehensive reviews of breeding procedures routinely used for improving cross-pollinated plants are
30 provided in numerous texts and articles, including: Allard, *Principles of Plant Breeding*, John Wiley & Sons, Inc. (1960); Simmonds, *Principles of Crop Improvement*, Longman Group Limited (1979); Hallauer and Miranda, *Quantitative Genetics in Maize Breeding*, Iowa State

University Press (1981); and, Jensen, *Plant Breeding Methodology*, John Wiley & Sons, Inc. (1988).

Mass Selection. In mass selection, desirable individual plants are chosen, harvested, and the seed composited without progeny testing to produce the following generation. Since selection is based on the maternal parent only, and there is no control over pollination, mass selection amounts to a form of random mating with selection. As stated herein, the purpose of mass selection is to increase the proportion of superior genotypes in the population.

Synthetics. A synthetic variety is produced by crossing inter se a number of genotypes selected for good combining ability in all possible hybrid combinations, with subsequent maintenance of the variety by open pollination. Whether parents are (more or less inbred) seed-propagated lines, as in some sugar beet and beans (*Vicia*) or clones, as in herbage grasses, clovers and alfalfa, makes no difference in principle. Parents are selected on general combining ability, sometimes by test crosses or topcrosses, more generally by polycrosses. Parental seed lines may be deliberately inbred (e.g. by selfing or sib crossing). However, even if the parents are not deliberately inbred, selection within lines during line maintenance will ensure that some inbreeding occurs. Clonal parents will, of course, remain unchanged and highly heterozygous.

Whether a synthetic can go straight from the parental seed production plot to the farmer or must first undergo one or two cycles of multiplication depends on seed production and the scale of demand for seed. In practice, grasses and clovers are generally multiplied once or twice and are thus considerably removed from the original synthetic.

While mass selection is sometimes used, progeny testing is generally preferred for polycrosses, because of their operational simplicity and obvious relevance to the objective, namely exploitation of general combining ability in a synthetic.

The number of parental lines or clones that enter a synthetic varies widely. In practice, numbers of parental lines range from 10 to several hundred, with 100-200 being the average. Broad based synthetics formed from 100 or more clones would be expected to be more stable during seed multiplication than narrow based synthetics.

Pedigreed varieties. A pedigreed variety is a superior genotype developed from selection of individual plants out of a segregating population followed by propagation and seed increase of self pollinated offspring and careful testing of the genotype over several generations. This is an open pollinated method that works well with naturally self pollinating species. This method can be used in combination with mass selection in variety development.

Variations in pedigree and mass selection in combination are the most common methods for generating varieties in self pollinated crops.

Hybrids. A hybrid is an individual plant resulting from a cross between parents of differing genotypes. Commercial hybrids are now used extensively in many crops, including corn (maize), sorghum, sugarbeet, sunflower and broccoli. Hybrids can be formed in a number of different ways, including by crossing two parents directly (single cross hybrids), by crossing a single cross hybrid with another parent (three-way or triple cross hybrids), or by crossing two different hybrids (four-way or double cross hybrids).

Strictly speaking, most individuals in an out breeding (i.e., open-pollinated) population are hybrids, but the term is usually reserved for cases in which the parents are individuals whose genomes are sufficiently distinct for them to be recognized as different species or subspecies. Hybrids may be fertile or sterile depending on qualitative and/or quantitative differences in the genomes of the two parents. Heterosis, or hybrid vigor, is usually associated with increased heterozygosity that results in increased vigor of growth, survival, and fertility of hybrids as compared with the parental lines that were used to form the hybrid. Maximum heterosis is usually achieved by crossing two genetically different, highly inbred lines.

The production of hybrids is a well-developed industry, involving the isolated production of both the parental lines and the hybrids which result from crossing those lines. For a detailed discussion of the hybrid production process, see, e.g., Wright, *Commercial Hybrid Seed Production* 8:161-176, In Hybridization of Crop Plants.

Targeting Induced Local Lesions in Genomes (TILLING®)

TILLING® (Targeting Induced Local Lesions in Genomes) is a method in molecular biology that allows directed identification of mutations in a specific gene. TILLING® was introduced in 2000, using the model plant *Arabidopsis thaliana*. TILLING® has since been used as a reverse genetics method in other organisms such as zebrafish, corn, wheat, rice, soybean, tomato and lettuce.

The method combines a standard and efficient technique of mutagenesis with a chemical mutagen (e.g., Ethyl methanesulfonate (EMS)) with a sensitive DNA screening-technique that identifies single base mutations (also called point mutations) in a target gene. EcoTILLING is a method that uses TILLING® techniques to look for natural mutations in individuals, usually for population genetics analysis. See Comai, et al., 2003, Efficient

discovery of DNA polymorphisms in natural populations by EcoTILLING. The Plant Journal 37, 778-786. Gilchrist et al. 2006. Use of EcoTILLING as an efficient SNP discovery tool to survey genetic variation in wild populations of *Populus trichocarpa*. Mol. Ecol. 15, 1367-1378. Mejlhede et al. 2006. EcoTILLING for the identification of allelic variation within the powdery mildew resistance genes *mlo* and *Mla* of barley. Plant Breeding 125, 461-467. Nieto et al. 2007, EcoTILLING for the identification of allelic variants of melon *eIF4E*, a factor that controls virus susceptibility. BMC Plant Biology 7, 34-42, each of which is incorporated by reference hereby for all purposes. DEcoTILLING is a modification of TILLING® and EcoTILLING which uses an inexpensive method to identify fragments (Garvin et al., 2007, DEco-TILLING: An inexpensive method for SNP discovery that reduces ascertainment bias. Molecular Ecology Notes 7, 735-746).

The TILLING® method relies on the formation of heteroduplexes that are formed when multiple alleles (which could be from a heterozygote, or a pool of multiple homozygotes and heterozygotes) are amplified in a PCR, heated, and then slowly cooled. A “bubble” forms at the mismatch of the two DNA strands (the induced mutation in TILLING® or the natural mutation or SNP in EcoTILLING), which is then cleaved by single stranded nucleases. The products are then separated by size on several different platforms.

Several TILLING® centers exist over the world that focus on agriculturally important species: UC Davis (USA), focusing on Rice; Purdue University (USA), focusing on Maize; University of British Columbia (CA), focusing on *Brassica napus*; John Innes Centre (UK), focusing on *Brassica rapa*; Fred Hutchinson Cancer Research, focusing on Arabidopsis; Southern Illinois University (USA), focusing on Soybean; John Innes Centre (UK), focusing on Lotus and Medicago; and INRA (France), focusing on Pea and Tomato.

More detailed description on methods and compositions on TILLING® can be found in references Nos. 1 – 35b, US 5994075, US 2004/0053236 A1, WO 2005/055704, and WO 2005/048692, each of which is hereby incorporated by reference for all purposes.

The inventors used TILLING® in both tetraploid (4x) wheat (containing A and B genomes) and hexaploid (6x) wheat (containing A, B and D genomes) for the following wheat KRP genes: *KRP1*, *KRP2*, *KRP4* and *KRP5*. The numbering of the KRPs in wheat does not necessarily correspond to the numbering of the KRPs in Arabidopsis (e.g. wheat KRP1 is not necessarily equivalent to Arabidopsis KRP1).

Triticeae tribe

Intense use of wild Triticeae can be seen in the Levant as early as 23,000 years ago. Triticeae is a tribe within the Pooideae subfamily of grasses that includes genera with many domesticated species. Major crop genera are found in this tribe including wheat (See Wheat taxonomy), barley, and rye; crops in other genera include some for human consumption and others used for animal feed or rangeland protection. Among the world's cultivated species, this tribe has some of the most complex genetic histories. An example is bread wheat, which contains the genomes of three species, only one of them originally a wheat *Triticum* species.

Genera in the *Triticeae* tribe include, but are not limited to, Aegilops (goat grasses - jointed goatgrass, Tausch goatgrass, etc.); Agropyron (crested wheatgrasses - Desert wheatgrass, quackgrass, etc.); Amblyopyrum (Slim wheat grass – amblyopyrum, etc.); Australopyrum (Australian wheatgrasses - velvet wheatgrass, pectinated wheatgrass, etc.); Cockayne (See Stenostachys; Cockayne is a younger, and hence invalid, name for Stenostachys, etc.); Crithopsis (delileana grass etc.); Dasypyrum (Mosquito grass; etc.); Elymus (Elymus (wild ryes - blue wildrye, Texas ryegrass, etc.); Elytrigia; Eremium (Argentine desert ryegrass, etc.); Eremopyrum (false wheatgrasses - tapertip false wheatgrass, annual wheatgrass, etc.); Festucopsis; Haynaldia; Henrardia; Heteranthelium; Hordelymus; Hordeum (barleys - common barley, foxtail barley, etc.); Hystrix (porcupine grass- bottlebrush grass, etc.); Kengyilia; Leymus (wild rye- American dune grass, lyme grass, etc.); Lophopyrum (tall wheatgrass); Malacurus Pascopyrum (western wheatgrass etc.); Peridictyon; Psathyrostachys (Russian wildrye, etc.); Pseudoroegneria (bluebunch wheatgrasses - beardless wheatgrass, etc.); Secale (Ryes - Cereal rye, Himalayan Rye, etc.); Sitanion; Stenostachys (New Zealand wheatgrasses, etc); Taeniatherum (medusahead etc.); Thinopyrum (intermediate wheatgrass, Russian wheatgrass, thick quackgrass, etc.); Triticum (Wheats - common wheat, durum wheat, etc.).

Triticeae and its sister tribe Bromeae (possible cultivars: Bromus mango S. America) when joined form a sister clade with Poeae and Aveneae (oats). Inter-generic gene flow characterized these taxa from the early stages. For example, Poeae and Aveneae share a genetic marker with barley and 10 other members of Triticeae, whereas all 19 genera of Triticeae bear a wheat marker along with Bromeae. Genera within Triticeae contain diploid, allotetraploid and/or allohexaploid genomes, the capacity to form allopolyploid genomes varies within the tribe. In this tribe, the majority of diploid species tested are closely related

to *Aegilops*, the more distal members (earliest branch points) include *Hordeum* (Barley), *Eremian*, *Psathyrostachys*.

Many genera and species of *Triticeae* are exemplary of allopolyploids, having more chromosomes than seen in typical diploids. Typically allopolyploids are tetraploid or hexaploid, AABB or AABBDD. The creation of polyploid species results from natural random events tolerated by polyploid capable plants. Likewise natural allopolyploid plants may have selective benefits and may allow the recombination of distantly related genetic material facilitating at a later time a reversion back to diploid. Poulard wheat is an example of a stable allotetraploid wheat.

Aegilops appears to be basal to several taxa such as *Triticum*, *Ambylopyrum*, and *Crithopsis*. Certain species such as *Aegilops speltoides* could potentially represent core variants of the taxa. The generic placement may be more a matter of nomenclature. *Aegilops* and *Triticum* genera are very closely related; the *Aegilops* species occupy most of the basal branch points in bread wheat evolution indicating that *Triticum* genus evolved from *Aegilops* after an estimated 4 million years ago. The divergence of the genomes is followed by allotetraploidization of a speltoid goatgrass x basal wheat species *Triticum boeoticum* with strains in the middle eastern region giving rise to cultivated emmer wheat.

Triticum spp.

Triticum sp. is a grass cultivated worldwide. In 2007 world production of wheat was 607 million tons, making it the third most-produced cereal after maize (784 million tons) and rice (651 million tons). Globally, wheat is the leading source of vegetable protein in human food, having a higher protein content than either maize (corn) or rice, the other major cereals. In terms of total production tonnages used for food, it is currently second to rice as the main human food.

Wheat is planted to a limited extent as a forage crop for livestock, and its straw can be used as a construction material for roofing thatch. The husk of the grain, separated when milling white flour, is bran. Wheat germ is the embryo portion of the wheat kernel. It is a concentrated source of vitamins, minerals, and protein, and is sustained by the larger, starch storage region of the kernel—the endosperm.

Non-limiting examples of *Triticum* species include, *T. aestivum* (e.g., common wheat, or bread wheat, a.k.a. *Triticum aestivum* L. subsp. *Aestivum*; Club wheat, a.k.a. *Triticum aestivum* subspecies *compactum* (Host) MacKey; Macha wheat, a.k.a. *Triticum aestivum*

subsp. *macha* (Dek. and Men.) MacKey; Vavilovi wheat, a.k.a. *Triticum aestivum* subsp. *vavilovi* (Tuman) Sears; Shot wheat, a.k.a. *Triticum aestivum* subsp. *sphacrococcum* (Perc.) MacKey), *T. aethiopicum*, *T. araraticum*, *T. boeoticum* (e.g., wild Einkorn, a.k.a. *Triticum boeotictim* Boiss), *T. carthlicum*, *T. compactum*, *T. dimitrium*, *T. dicoccoides* (e.g., wild emmer, a.k.a. *Triticum dicoccoides* (Koern. ex Ascb. & Graebn.) Aaronsohn.), *T. dicoccum* (e.g., Emmer), *T. durum* (e.g., durum wheat), *T. ispahanicum*, *T. karamyshevii*, *T. macha*, *T. militinae*, *T. monococcum* (e.g., Einkorn, a.k.a. *Triticum monococcum* L.), *T. polonicum*, *T. spelta*, *T. sphaerococcum*, *T. timopheevii* (e.g. timopheevi wheat, a.k.a. *Triticum timopheevii* (Zbuk.) Zbuk.), *T. turanicum* (e.g., oriental wheat, a.k.a. *Triticum turanicum* jakubz), *T. turgidum* (e.g., poulard wheat, a.k.a. *Triticum turgidum* L.), *T. urartu*, *T. vavilovii*, and *T. zhukovskiyi*.

Wheat genetics is more complicated than that of most other domesticated species. Some wheat species are diploid, with two sets of chromosomes, but many are stable polyploids, with four sets of chromosomes (tetraploid) or six (hexaploid). Most tetraploid wheats (e.g. emmer and durum wheat) are derived from wild emmer, *T. dicoccoides*. Wild emmer is itself the result of a hybridization between two diploid wild grasses, *T. urartu* and a wild goatgrass such as *Aegilops searsii* or *Ae. speltoides*. The unknown grass has never been identified among now surviving wild grasses, but the closest living relative is *Aegilops speltoides*. The hybridization that formed wild emmer (AABB) occurred in the wild, long before domestication, and was driven by natural selection. Hexaploid wheats evolved in farmers' fields. Common wheat (*Triticum aestivum*, $2n = 42$, AABBDD) is one of the most important cereal crops in the world. Either domesticated emmer or durum wheat hybridized with yet another wild diploid grass (*Aegilops cylindrica*) to make the hexaploid wheats, spelt wheat and bread wheat. These have three sets of paired chromosomes, three times as many as in diploid wheat. Synthetic hexaploids made by crossing the wild goatgrass wheat ancestor *Aegilops tauschii* and various durum wheats are now being deployed, and these increase the genetic diversity of cultivated wheats.

Plant breeding methods for *Triticum spp.* are well known. Non-limiting methods for *Triticum spp.* breeding and agriculturally important traits (e.g., improving wheat yield, biotic stress tolerance, and abiotic stress tolerance etc.) are described in references Nos. 36 – 51, US 7652204, US 6197518, US 7034208, US 7528297, US 6407311, US20080040826, US20090300783, US20060223707, US20110027233, US20080028480, US20090320152,

US20090320151, WO/2001/029237A2, WO/2008/025097A1, and WO/2003/057848A2, each of which is incorporated by reference in its entirety for all purposes.

Genetic materials may be transferred between *Triticum spp.* and other species, for example, some plant species in the *Triticeae* tribe. Xiang et al., describe somatic hybrids between wheat and *Setaria italica* (Genome 47: 680–688 (2004)); Ge et al. describe protoplast electrofusion between common wheat and Italian ryegrass (In Vitro Cellular and Developmental Biology - Plant 42(2):179-187. 2006); Yue et al. describe asymmetric somatic hybridization between *Aeluropus littorulis sinensis* and wheat (Plant Science, Volume 161, Issue 2, July 2001, Pages 259-266); Cai et al. describe somatic hybrids between *Festuca arundinacea* Schreb. and wheat (*Triticum aestivum* L.); Xiang et al. describe asymmetric somatic hybridization between wheat and *Avena sativa* L. (Science in China, Vol 46(3), 243-252); Zhou et al. describe asymmetric somatic hybridization between wheat and asymmetric somatic hybridization between wheat and *Avena sativa Haynaldia villosa* (Science in China, 44(3): 294-304); Xia et al. describe asymmetric somatic hybridization between wheat and *Agropyron elongatum* (Host) Nevishi (Theor Appl Genet. 2003 Jul;107(2):299-305. Epub 2003 Mar 19); Li et al. describe symmetric somatic hybridization between wheat and *Psathyrostachys juncea* (Sheng Wu Gong Cheng Xue Bao. 2004 Jul;20(4):610-4). More hybridization between *Triticum spp.* and other species are described in reference Nos. 56 – 64.

Kinase Inhibitor Protein (KIP) Related Protein (KRP)

Plants have cyclin dependent kinases (CDK) that regulate the transitions between different phases of the cell cycle (Verkest et al., 2005, Switching the Cell Cycle. Kip-Related Proteins in Plant Cell Cycle Control, Plant Physiology, November 2005, Vol. 139, pp. 1099–1106, incorporated by reference in its entirety herein).

In *Arabidopsis (Arabidopsis thaliana)*, at least two classes of CDKs are involved in cell cycle regulation: the A-type CDKs that are represented by only one gene in the model species *Arabidopsis* (designated Arath;CDKA;1) and the B-type CDK family that has four members, grouped into the B1 (Arath;CDKB1;1 and Arath;CDKB1;2) and B2 (Arath;CDKB2;1 and Arath;CDKB2;2) subclasses (Vandepoele et al., 2002, Genome-wide analysis of core cell cycle genes in *Arabidopsis*. Plant Cell 14: 903–916). A-type CDKs display kinase activity from late G1 phase until the end of mitosis, suggesting a role for this particular CDK at both the G1-to-S and G2-to-M transition points (Magyar et al., 1997;

Porceddu et al., 2001; Sorrell et al., 2001). A central role for CDKA;1 in controlling cell number has been demonstrated using transgenic tobacco (*Nicotiana tabacum*) plants with reduced A-type CDK activity (Hemerly et al., 1995). The requirement for Arath;CKDA;1 at least for entry into mitosis has been demonstrated as well by *cdka;1* null mutants that fail to progress through the second mitosis during male gametophytic development (Nowack et al., 2005). The group of B-type CDKs displays a peak of activity at the G2-to-M phase transition only (Magyar et al., 1997; Porceddu et al., 2001; Sorrell et al., 2001), suggesting that they play a role at the onset of, or progression through, mitosis. Correspondingly, cells of plants with reduced B-type CDK activity arrest in the G2 phase of the cell cycle (Porceddu et al., 2001; Boudolf et al., 2004).

CDK is regulated by cyclins. Plant cyclins are very complicated. There are at least 49 different cyclins in Arabidopsis, which were classified into seven subclasses (A, B, C, D, H, P, and T) (Vandepoele et al., 2002; Wang et al., 2004). CDK are also regulated by docking of small proteins, generally known as CDK inhibitors (CKIs). CKIs have been identified in many organisms, e.g., budding yeast (*Saccharomyces cerevisiae*), fission yeast (*Schizosaccharomyces pombe*), mammals, and plants, see, Mendenhall, 1998; Kwon T.K. et al. 1998; Vlach J. et al. 1997; Russo et al., 1996; Wang et al., 1997, 1998 and 2000; Lui et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002a, 2002b; Coelho et al., 2005; Jasinski S. et al., 2002, each of which is incorporated by reference in its entirety).

Plant CKIs are also known as KIP Related Proteins (KRPs). They have cyclin binding and CDK binding domains at their C-terminal, however the mechanism regulating this protein stability and function remains unknown (Zhou et al., 2003a; Weinl et al. 2005). KRP activity can be both regulated at the transcriptional level or at the posttranslational level (Wang et al., 1998; De Veylder et al., 2001; Jasinski et al., 2002b; Ormenese et al., 2004; Coqueret, 2003; Hengst, 2004; Verkest et al., 2005; Coelho et al., 2005, each of which is incorporated by reference in its entirety). KRPs in plant normally localize in nucleus (Jasinski et al., 2002b; Zhou et al., 2003a; Weinl et al., 2005).

KRP can function as an integrator of developmental signals, and control endocycle onset, in different cell cycle programs (e.g., proliferation, endoreduplication, and cell cycle exit). See Wang et al., 1998; Richard et al., 2001; Himanen et al., 2002; Grafi and Larkins, 1995; Joubert et al., 1999; Verkest et al., 2005; Weinl et al., 2005; Boudolf et al., 2004b.

KRP Mutations

The present invention further provides mutated *KRP* polynucleotides and mutated *KRP* amino acid sequences compared to a wild type *KRP* gene or a wild type *KRP* protein. In some embodiments, the present invention provides mutations in one or more *KRP* genes that can be used to increase weight, size, and/or number of one or more organs, for example, to increase seed size, seed number, seed weight, and/or seed yield in a plant.

The mutations in a mutated *KRP* gene of the present invention can be in the coding region or the non-coding region of the *KRP* genes. The mutations can either lead to, or not lead to amino acid changes in the encoded *KRP* polypeptides. In some embodiments, the mutations can be missense, severe missense, silent, nonsense mutations. For example, the mutation can be nucleotide substitution, insertion, deletion, or genome re-arrangement, which in turn may lead to reading frame shift, amino acid substitution, insertion, deletion, and/or polypeptides truncation. As a result, the mutant *KRP* gene encodes a *KRP* polypeptide having less inhibition activity on a cyclin/CDK complex compared to a polypeptide encoded by its corresponding wild-type *KRP* gene.

As used herein, a nonsense mutation is a point mutation, e.g., a single-nucleotide polymorphism (SNP), in a sequence of DNA that results in a premature stop codon, or a nonsense codon in the transcribed mRNA, and in a truncated, incomplete, and usually nonfunctional protein product. A missense mutation (a type of nonsynonymous mutation) is a point mutation in which a single nucleotide is changed, resulting in a codon that codes for a different amino acid (mutations that change an amino acid to a stop codon are considered nonsense mutations, rather than missense mutations). This can render the resulting protein nonfunctional. Silent mutations are DNA mutations that do not result in a change to the amino acid sequence of a protein. They may occur in a non-coding region (outside of a gene or within an intron), or they may occur within an exon in a manner that does not alter the final amino acid sequence. A severe missense mutation changes the amino acid, which lead to dramatic changes in conformation, charge status etc.

The mutations can be located at any portion of a *KRP* gene, for example, at the 5', the middle, or the 3' of a *KRP* gene, resulting mutations in any portions of the encoded *KRP* protein, for example, in the CDK binding domain or the cyclin binding domain, so long as the mutated gene encodes a mutant *KRP* polypeptide partially or completely lose the ability to inhibit one or more cyclin/CDK complexes, compared to the protein encoded by the corresponding wild type *KRP* gene. The *KRP* and the cyclin/CDK complexes can belong to

the same plant species, different plant species in the same genus, or different plant species in different genus.

Mutant KRP protein of the present invention can have one or more modifications to the wild-type KRP, or biologically active variant, or fragment thereof. Particularly suitable modifications include amino acid substitutions, insertions, deletions, or truncation. For example, amino acid substitutions can be generated as modifications in the CDK or the cyclin-binding region that reduce or eliminate binding. Similarly, amino acid substitutions can be generated as modifications in the CDK or the cyclin-binding region of the KRP that reduce or eliminate the inhibitory activity of the KRP towards the Cyclin/CDK complex. In typical embodiments, at least one non-conservative amino acid substitution, insertion, or deletion in the CDK binding region or the cyclin binding region is made to disrupt or modify binding of the CKI polypeptide to a CDK or cyclin protein. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule. Insertional KRP mutants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in the wild-type KRP protein molecule, biologically active variant, or fragment thereof. The insertion can be one or more amino acids. The insertion can consist, e.g., of one or two conservative amino acids. Amino acids similar in charge and/or structure to the amino acids adjacent to the site of insertion are defined as conservative. Alternatively, mutant KRP protein includes the insertion of an amino acid with a charge and/or structure that is substantially different from the amino acids adjacent to the site of insertion. In some other embodiments, the mutant KRP is a truncated protein losing one or more domains compared to the corresponding wild type KRP protein.

Methods of Increasing Organ Weight, Organ Size, Organ Number and/or Yield

The present invention further provides methods of increasing weight, size, and/or number of one or more organs, for example, methods of increasing seed weight, seed size, seed number, and/or yield in a plant. In some embodiments, the plant is a monocot plant. In some embodiments, the plant is a plant species in the *Triticeae* tribe, for example, a wheat plant. In some embodiments, the methods comprise disrupting one or more KRPs in the plant. The disruption can be at genomic level, transcriptional level, post-transcriptional level, translational level, and/or post translational level. In some embodiments, the methods comprise introducing one or more mutations into one or more *KRP* genes in the plant. In

some embodiments, the methods comprise knocking-down expression of one or more *KRP* genes in the plant. In some embodiments, the methods comprise knocking-down *KRP* mRNAs stability in the plant. In some embodiments, the methods comprise down-regulating one or more *KRP* proteins activity in the plant.

5 For example, in some embodiments, the methods comprise introducing one or more *KRP* mutations of the present invention into the genome of the plant. In some embodiments, the methods comprise hybridizing a first plant having one or more mutated *KRPs* of the present invention with a second plant. In some embodiments, the hybridizing step comprises crossing the first plant with the second plant. In some embodiments, the hybridizing step
10 comprises transferring the genetic materials in the first plant to the second plant through in vitro breeding, e.g., somatic hybridization.

Alternatively, the methods comprise mutating one or more *KRPs* in a plant. Methods of mutating a target gene have been known to one skilled in the art. These methods include, but are not limited to, mutagenesis (e.g., chemical mutagenesis, radiation mutagenesis,
15 transposon mutagenesis, insertional mutagenesis, signature tagged mutagenesis, site-directed mutagenesis, and natural mutagenesis), TILLING®, homologous recombination, knock-outs/knock-ins, antisense and RNA interference. Various types of mutagenesis can be used to produce and/or isolate variant nucleic acids that encode for protein molecules and/or to further modify/mutate the proteins of the present invention. They include but are not limited
20 to site-directed, random point mutagenesis, homologous recombination, DNA shuffling, mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like. Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis,
25 mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, e.g., involving chimeric constructs, is also included in the present invention. In one embodiment, mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, e.g., sequence, sequence comparisons, physical properties, crystal structure or the like. For more information of
30 mutagenesis in plants, such as agents, protocols, see reference Nos. 66 to 70, each of which is herein incorporated by reference in its entirety).

In some embodiments, random mutations in *KRP* genes are created *in vitro*. For example, a library of *KRP* genes with one or more random mutations can be generated, and

the produced mutant KRP genes are subjected to the in vitro KRP-Cylin-CDK kinase assay described herein to determine if the mutant KRP genes can be used for increasing weight, size, and/or number of one or more organs, for example, for increasing seed size, seed number, seed weight and/or yield. Methods for in vitro mutagenesis include, but are not limited to error-prone PCR, Rolling circle error-prone PCR, mutator strains, temporary mutator strains, insertion mutagenesis, chemical mutagenesis (e.g., EMS, nitrous acid etc.), DNA shuffling, and site directed random mutagenesis. More methods are described in Chusacultanachai et al, Fujii et al., Braman, and Trower. Commercial random mutagenesis kits are available, such as Random Mutagenesis Kits from Jena Bioscience.cat. No. PP-101, Diversify® PCR random mutagenesis kit from Clontech.

In some embodiments, mutated KRPs of the present invention are generated *in vivo* by methods such as TILLING®, site-directed mutagenesis, homologous recombination, etc. The produced mutant KRP genes are screened and subjected to the in vitro KRP-Cylin-CDK kinase assay described herein to determine if the mutant KRP genes can be used for increasing weight, size, and/or number of one or more organs, for example, for increasing seed size, seed number, seed weight and/or yield.

In some embodiments, the methods comprise knocking down expression of one or more KRPs in the plant. Techniques which can be employed in accordance with the present invention to knock down gene expression, include, but are not limited to: (1) disrupting a gene's transcript, such as disrupting a gene's mRNA transcript; (2) disrupting the function of a polypeptide encoded by a gene, or (3) disrupting the gene itself.

For example, antisense RNA, ribozyme, dsRNAi, RNA interference (RNAi) technologies can be used in the present invention to target RNA transcripts of one or more *KRP* genes. Antisense RNA technology involves expressing in, or introducing into, a cell an RNA molecule (or RNA derivative) that is complementary to, or antisense to, sequences found in a particular mRNA in a cell. By associating with the mRNA, the antisense RNA can inhibit translation of the encoded gene product. The use of antisense technology to reduce or inhibit the expression of specific plant genes has been described, for example in European Patent Publication No. 271988, Smith et al., Nature, 334:724-726 (1988); Smith et. al., Plant Mol. Biol., 14:369-379 (1990)).

A ribozyme is an RNA that has both a catalytic domain and a sequence that is complementary to a particular mRNA. The ribozyme functions by associating with the

mRNA (through the complementary domain of the ribozyme) and then cleaving (degrading) the message using the catalytic domain.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing or transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The RNAi technique is discussed, for example, in Elbashir, et al., *Methods Enzymol.* 26:199 (2002); McManus & Sharp, *Nature Rev. Genetics* 3:737 (2002); PCT application WO 01/75164; Martinez et al., *Cell* 110:563 (2002); Elbashir et al., *supra*; Lagos-Quintana et al., *Curr. Biol.* 12:735 (2002); Tuschl et al., *Nature Biotechnol.* 20:446 (2002); Tuschl, *Chembiochem.* 2:239 (2001); Harborth et al., *J. Cell Sci.* 114:4557 (2001); et al., *EMBO J.* 20:6877 (2001); Lagos-Quintana et al., *Science* 294:8538 (2001); Hutvagner et al., *loc cit*, 834; Elbashir et al., *Nature* 411:494 (2001).

The term “dsRNA” or “dsRNA molecule” or “double-strand RNA effector molecule” refers to an at least partially double-strand ribonucleic acid molecule containing a region of at least about 19 or more nucleotides that are in a double-strand conformation. The double-stranded RNA effector molecule may be a duplex double-stranded RNA formed from two separate RNA strands or it may be a single RNA strand with regions of self-complementarity capable of assuming an at least partially double-stranded hairpin conformation (i.e., a hairpin dsRNA or stem-loop dsRNA). In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as RNA/DNA hybrids. The dsRNA may be a single molecule with regions of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In one aspect, the regions of self-complementarity are linked by a region of at least about 3-4 nucleotides, or about 5, 6, 7, 9 to 15 nucleotides or more, which lacks complementarity to another part of the molecule and thus remains single-stranded (i.e., the “loop region”). Such a molecule will assume a partially double-stranded stem-loop structure, optionally, with short single stranded 5' and/or 3' ends. In one aspect the regions of self-complementarity of the hairpin dsRNA or the double-stranded region of a duplex dsRNA will comprise an Effector Sequence and an Effector Complement (e.g., linked by a single-stranded loop region in a hairpin dsRNA). The Effector Sequence or Effector Strand is that strand of the double-stranded region or duplex which is incorporated in or associates with RISC. In one aspect the double-stranded RNA effector molecule will comprise an at least 19 contiguous nucleotide effector sequence,

preferably 19 to 29, 19 to 27, or 19 to 21 nucleotides, which is a reverse complement to the RNA of KRP, or an opposite strand replication intermediate, or the anti-genomic plus strand or non-mRNA plus strand sequences of KRP. In one embodiment, said double-stranded RNA effector molecules are provided by providing to a plant, plant tissue, or plant cell an expression construct comprising one or more double-stranded RNA effector molecules. In one embodiment, the expression construct comprise a double-strand RNA derived from any one of SEQ ID NOs 1-5. One skilled in the art will be able to design suitable double-strand RNA effector molecule based on the nucleotide sequences of KRPs in the present invention.

In some embodiments, the dsRNA effector molecule of the invention is a “hairpin dsRNA”, a “dsRNA hairpin”, “short-hairpin RNA” or “shRNA”, i.e., an RNA molecule of less than approximately 400 to 500 nucleotides (nt), or less than 100 to 200 nt, in which at least one stretch of at least 15 to 100 nucleotides (e.g., 17 to 50 nt, 19 to 29 nt) is based paired with a complementary sequence located on the same RNA molecule (single RNA strand), and where said sequence and complementary sequence are separated by an unpaired region of at least about 4 to 7 nucleotides (or about 9 to about 15 nt, about 15 to about 100 nt, about 100 to about 1000 nt) which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. The shRNA molecules comprise at least one stem-loop structure comprising a double-stranded stem region of about 17 to about 100 bp; about 17 to about 50 bp; about 40 to about 100 bp; about 18 to about 40 bp; or from about 19 to about 29 bp; homologous and complementary to a target sequence to be inhibited; and an unpaired loop region of at least about 4 to 7 nucleotides, or about 9 to about 15 nucleotides, about 15 to about 100 nt, about 100 to about 1000 nt, which forms a single-stranded loop above the stem structure created by the two regions of base complementarity. It will be recognized, however, that it is not strictly necessary to include a “loop region” or “loop sequence” because an RNA molecule comprising a sequence followed immediately by its reverse complement will tend to assume a stem-loop conformation even when not separated by an irrelevant “stuffer” sequence.

The plants with disrupted one or more KRPs of the present invention can be used for many purposes. In one embodiment, a plant of the present invention is used as a donor plant of genetic material which can be transferred to a recipient plant to produce a plant with desired agronomic traits which has the transferred genetic material and having increased weight, size, and/or number of one or more organs, for example, a plant with increased seed weight, seed size, seed number and/or yield. Any suitable method known in the art can be

applied to transfer genetic material from a donor plant to a recipient plant. In most cases, such genetic material is genomic material.

Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several reference books (e.g., R. W. Allard, 1960, Principles of Plant Breeding, John Wiley and Son, pp. 115-161; N.W. Simmonds, 1979, Principles of Crop Improvement, Longman Group Limited; W. R. Fehr, 1987, Principles of Crop Development, Macmillan Publishing Co.; N. F. Jensen, 1988, Plant Breeding Methodology, John Wiley & Sons).

In some embodiments, a backcross breeding process is used. The backcross breeding process comprises the following steps: (a) crossing a first wheat plant having one or more disrupted *KRP* genes with a second plant that comprises the desired trait(s); (b) selecting the F_1 progeny plants that have the desired trait(s); (c) crossing the selected F_1 progeny plants with the first wheat plant or the second wheat plant to produce backcross progeny plants; (d) selecting for backcross progeny plants that have the desired trait(s) and one or more disrupted *KRP* genes to produce selected backcross progeny plants; and (e) repeating steps (c) – (d) one, two, three, four, five six, seven, eight, nine, or more times in succession to produce selected, second, third, fourth, fifth, sixth, seventh, eighth, ninth, or higher backcross progeny plants that comprise said disrupted *KRP* genes, and/or the desired trait(s).

The invention further provides methods for developing wheat varieties in a wheat breeding program using plant breeding techniques including recurrent selection, backcrossing, pedigree breeding, molecular markers (Isozyme Electrophoresis, Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), DNA Amplification Fingerprinting (DAF), Sequence Characterized Amplified Regions (SCARs). Amplified Fragment Length Polymorphisms (AFLPs), and Simple Sequence Repeats (SSRs) which are also referred to as Microsatellites, etc.) enhanced selection, genetic marker enhanced selection, and transformation. Seeds, plants, and part(s) thereof produced by such breeding methods are also part of the invention.

In one embodiment, the whole genome of the plants of the present invention with disrupted *KRP*(s) is transferred into a recipient plant. This can be done by conventional breeding such as crossing, or somatic hybridization. In another embodiment, at least the parts having the disrupted *KRP*(s) of the donor plant's genome are transferred. This can be done by crossing donor plants to a recipient plant to create a F_1 plant, followed with one or more

backcrosses to one of the parent plants to give plants with the desired genetic background. Molecular marker assisted breeding can be utilized to monitor the transfer of the genetic material. The produced offsprings can be selected for having increased weight, size, and/or number of one or more organs, for example, having increased seed weight, seed size, seed number and/or yield.

In one embodiment, the recipient plant is an elite line having one or more certain agronomically important traits. As used herein, “agronomically important traits” include any phenotype in a plant or plant part that is useful or advantageous for human use. Examples of agronomically important traits include but are not limited to those that result in increased biomass production, increased food production, improved food quality, decrease in cracking, quicker color change when the fruit matures etc. Additional examples of agronomically important traits includes pest resistance, vigor, development time (time to harvest), enhanced nutrient content, increased seed oil content, novel growth patterns, flavors or colors, salt, heat, drought and cold tolerance, and the like.

Other agronomically important traits include resistance to biotic and/or abiotic stresses. As used herein, the phrase “biotic stress” or “biotic pressure” refers to a situation where damage is done to plants by other living organisms, such as bacteria, viruses, fungi, parasites, insects, weeds, animals and human. As used herein, the phrase “abiotic stress” or “abiotic pressure” refers to the negative impact of non-living factors on plants in a specific environment. The non-living variable must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of plants in a significant way. Non-limiting examples of stressors are high winds, extreme temperatures, drought, flood, and other natural disasters, such as tornados and wildfires.

In some embodiments, the method comprises i) making a cross between a plant of the present invention to a second plant to produce a F1 plant, for example, a wheat plant with one or more disrupted *KRP* genes. Optionally, the method further comprises ii) backcrossing the F1 plant to the first or the second wheat plant; and iii) repeating the backcrossing step to generate a near isogenic line, wherein the one or more disrupted *KRPs* in the first wheat plant are integrated into the genome of the near isogenic line.

In some embodiments, the disrupted *KRP* gene is selected from the group consisting of *TaKRP1A*, *TaKRP1B*, *TaKRP1D*, *TaKRP2A*, *TaKRP2B*, *TaKRP2D*, *TaKRP4A*, *TaKRP4B*, *TaKRP4D*, *TaKRP5A*, *TaKRP5B*, or *TaKRP5D*. In some embodiments, the first wheat plant

comprises one or more mutations selected from any one of mutations listed in Tables 2 and 3 for a particular *KRP* gene.

In some embodiments, the methods of the present invention can increase the average weight, size, and/or number of one or more organs, for example, increase the average seed weight, seed size, seed number and/or yield of a plant by at least 5%, at least 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 210%, 220%, 230%, 240%, 250%, 260%, 270%, 280%, 290%, 300%, 310%, 320%, 330%, 340%, 350%, 360%, 370%, 380%, 390%, 400%, or greater when compared to a control plant not having disrupted *KRP*(s).

The mutated *KRPs* in a plant genome can be viewed as quantitative trait loci (QTLs) related to organ weight, organ size, organ number and/or yield. A QTL is a region of DNA that is associated with a particular phenotypic trait - these QTLs are often found on different chromosomes. Knowing the number of QTLs that explains variation in a particular phenotypic trait informs about the genetic architecture of the trait. It may tell that plant with preferred phenotype is controlled by many genes of small effect, or by a few genes of large effect. Therefore, QTL mapping can be applied to determine the parts of the donor plant's genome comprising the mutated *KRPs*, and facilitate the breeding methods.

One or more of such QTLs of mutated *KRPs* in a donor can be transferred to a recipient plant, confirming the phenotype of having increased weight, size, and/or number of one or more organs, for example, increased seed weight, seed size, seed number, and/or yield. In some further embodiments, the QTLs related to mutated *KRPs* can be combined with one or more other QTLs that contribute to agriculturally important phenotypes, such as yield enhancement, resistance to biotic and abiotic stresses, etc. The primers in the present invention used for genotyping the mutated *KRPs* can be used as molecular markers indicating the presence or absence of the mutated *KRPs*. Instead, molecular markers closely linked to the mutated *KRPs* can be also used. Methods of developing molecular markers and their applications are described by Avise (Molecular markers, natural history, and evolution, Publisher: Sinauer Associates, 2004, ISBN 0878930418, 9780878930418), Srivastava et al.

(Plant biotechnology and molecular markers, Publisher: Springer, 2004, ISBN1402019114, 9781402019111), and Vienne (Molecular markers in plant genetics and biotechnology, Publisher: Science Publishers, 2003), each of which is incorporated by reference in its entirety.

5 Without wishing to be bound by any theory, besides increased seed size, seed number, seed weight and/or yield, a plant having one or more disrupted KRPs may have one or more other phenotypes that are agriculturally or industrially important, which include, but are not limited to, increased plant vigor, organ size, increased adaptability to the environment, increased oil production, increased biomass production, and traits that allow a plant to grow
10 better under certain environments with specific temperatures, soil conditions and levels of sunlight and precipitation compared to a wild type control plant.

Tissue Culture and Grafting

Modern plant tissue culture is performed under aseptic conditions under filtered air.
15 Living plant materials from the environment are naturally contaminated on their surfaces (and sometimes interiors) with microorganisms, so surface sterilization of starting materials (explants) in chemical solutions (usually alcohol or bleach) is required. Explants are then usually placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired. Solid and liquid
20 media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar.

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the
25 morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots. A balance of both auxin and cytokinin will often produce an unorganized growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition. As cultures grow, pieces are typically sliced off and transferred to new
30 media (subcultured) to allow for growth or to alter the morphology of the culture. The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard. As shoots emerge from a culture, they may be sliced off and rooted with

auxin to produce plantlets which, when mature, can be transferred to potting soil for further growth in the greenhouse as normal plants.

The tissue obtained from the plant to culture is called an explant. Based on work with certain model systems, particularly tobacco, it has often been claimed that a totipotent explant can be grown from any part of the plant. However, this concept has been vitiated in practice. In many species explants of various organs vary in their rates of growth and regeneration, while some do not grow at all. The choice of explant material also determines if the plantlets developed via tissue culture are haploid or diploid. Also the risk of microbial contamination is increased with inappropriate explants. Thus it is very important that an appropriate choice of explant be made prior to tissue culture.

The specific differences in the regeneration potential of different organs and explants have various explanations. The significant factors include differences in the stage of the cells in the cell cycle, the availability of or ability to transport endogenous growth regulators, and the metabolic capabilities of the cells. The most commonly used tissue explants are the meristematic ends of the plants like the stem tip, auxiliary bud tip and root tip. These tissues have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and cytokinins. Some explants, like the root tip, are hard to isolate and are contaminated with soil microflora that become problematic during the tissue culture process. Certain soil microflora can form tight associations with the root systems, or even grow within the root. Soil particles bound to roots are difficult to remove without injury to the roots that then allows microbial attack. These associated microflora will generally overgrow the tissue culture medium before there is significant growth of plant tissue. Aerial (above soil) explants are also rich in undesirable microflora. However, they are more easily removed from the explant by gentle rinsing, and the remainder usually can be killed by surface sterilization. Most of the surface microflora do not form tight associations with the plant tissue. Such associations can usually be found by visual inspection as a mosaic, decolorization or localized necrosis on the surface of the explant.

An alternative for obtaining uncontaminated explants is to take explants from seedlings which are aseptically grown from surface-sterilized seeds. The hard surface of the seed is less permeable to penetration of harsh surface sterilizing agents, such as hypochlorite, so the acceptable conditions of sterilization used for seeds can be much more stringent than for vegetative tissues.

Tissue cultured plants are clones, if the original mother plant used to produce the first explants is susceptible to a pathogen or environmental condition, the entire crop would be susceptible to the same problem, conversely any positive traits would remain within the line also. Plant tissue culture is used widely in plant science; it also has a number of commercial

5 applications. Applications include:

1. Micropropagation is widely used in forestry and in floriculture. Micropropagation can also be used to conserve rare or endangered plant species.

2. A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. pathogen resistance/tolerance.

10 3. Large-scale growth of plant cells in liquid culture inside bioreactors as a source of secondary products, like recombinant proteins used as biopharmaceuticals.

4. To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.

5. To cross-pollinate distantly related species and then tissue culture the resulting
15 embryo which would otherwise normally die (Embryo Rescue).

6. For production of doubled monoploid (dihaploid) plants from haploid cultures to achieve homozygous lines more rapidly in breeding programs, usually by treatment with colchicine which causes doubling of the chromosome number.

7. As a tissue for transformation, followed by either short-term testing of genetic
20 constructs or regeneration of transgenic plants.

8. Certain techniques such as meristem tip culture can be used to produce clean plant material from infected stock, such as potatoes and many species of soft fruit.

9. Micropropagation using meristem and shoot culture to produce large numbers of identical individuals.

25 Non-limiting exemplary tissue culture methods for wheat, rice, maize have been described by Trione et al., Dodig, et al., O'Hara et al., Zaidi et al., Wang et al., Ting et al., Hawes et al., and Sheridan, each of which is incorporated by reference in its entirety.

The present invention also provides a cutting, a rootstock, a scion, or an explant from the plants as described above for grafting.

30 Grafting is a method of asexual plant propagation widely used in agriculture and horticulture where the tissues of one plant are encouraged to fuse with those of another. It is most commonly used for the propagation of trees and shrubs grown commercially. In most cases, one plant is selected for its roots, and this is called the stock or rootstock. The other

plant is selected for its stems, leaves, flowers, or fruits and is called the scion. The scion contains the desired genes to be duplicated in future production by the stock/scion plant. In stem grafting, a common grafting method, a shoot of a selected, desired plant cultivar is grafted onto the stock of another type. In another common form called budding, a dormant side bud is grafted on the stem of another stock plant, and when it has fused successfully, it is encouraged to grow by cutting out the stem above the new bud.

For successful grafting to take place, the vascular cambium tissues of the stock and scion plants must be placed in contact with each other. Both tissues must be kept alive until the graft has taken, usually a period of a few weeks. Successful grafting only requires that a vascular connection takes place between the two tissues. A physical weak point often still occurs at the graft, because the structural tissue of the two distinct plants, such as wood, may not fuse.

Exemplary grafting techniques include, approach grafting, budding grafting (patch budding, chip budding, T-budding), cleft grafting, side grafting, whip grafting, stub grafting, awl grafting, veneer grafting, bark grafting, tongue grafting, et al. Detailed non-limiting grafting methods for wheat and maize are described in Lacadena, 1968, and Katsumi et al., each of which is incorporated by reference in its entirety.

Plant Transformation

The isolated polynucleotides of the present invention comprise a sequence selected from the group consisting of: (a) sequences recited in SEQ ID NO: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or portions thereof; (b) complements of the sequences recited in SEQ ID NO: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or portions thereof; (c) reverse complements of the sequences recited in SEQ ID NO: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or portions thereof; (d) reverse sequences of the sequences recited in SEQ ID NO: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77, or portions thereof; and (e) sequences having at least 50%, 75%, 90%, 95% or 98% identity, as defined herein, to a sequence of (a)-(d) or a specified region of a sequence of (a)-(d).

The present invention also provides recombinant polynucleotide sequences comprising the isolated nucleic acid sequence of any one of the polynucleotides described above operably linked to suitable regulatory sequences.

The present invention also provides recombinant constructs comprising the recombinant polynucleotide sequences as described above. The present invention further comprises interfering RNA (RNAi) constructs based on the nucleic acid sequences of the present invention, targeting one or more KRPs in a *Triticum spp.*

5 The polynucleotides of the present invention can be transformed into a plant. The most common method for the introduction of new genetic material into a plant genome involves the use of living cells of the bacterial pathogen *Agrobacterium tumefaciens* to literally inject a piece of DNA, called transfer or T-DNA, into individual plant cells (usually following wounding of the tissue) where it is targeted to the plant nucleus for chromosomal
10 integration. There are numerous patents governing *Agrobacterium* mediated transformation and particular DNA delivery plasmids designed specifically for use with *Agrobacterium*---for example, US4536475, EP0265556, EP0270822, WO8504899, WO8603516, US5591616, EP0604662, EP0672752, WO8603776, WO9209696, WO9419930, WO9967357, US4399216, WO8303259, US5731179, EP068730, WO9516031, US5693512, US6051757
15 and EP904362A1. *Agrobacterium*-mediated plant transformation involves as a first step the placement of DNA fragments cloned on plasmids into living *Agrobacterium* cells, which are then subsequently used for transformation into individual plant cells. *Agrobacterium*-mediated plant transformation is thus an indirect plant transformation method. Methods of *Agrobacterium*-mediated plant transformation that involve using vectors with no T-DNA are
20 also well known to those skilled in the art and can have applicability in the present invention. See, for example, U.S. Patent No. 7,250,554, which utilizes P-DNA instead of T-DNA in the transformation vector.

Direct plant transformation methods using DNA have also been reported. The first of these to be reported historically is electroporation, which utilizes an electrical current applied
25 to a solution containing plant cells (M. E. Fromm et al., *Nature*, 319, 791 (1986); H. Jones et al., *Plant Mol. Biol.*, 13, 501 (1989) and H. Yang et al., *Plant Cell Reports*, 7, 421 (1988). Another direct method, called “biolistic bombardment”, uses ultrafine particles, usually tungsten or gold, that are coated with DNA and then sprayed onto the surface of a plant tissue with sufficient force to cause the particles to penetrate plant cells, including the thick cell
30 wall, membrane and nuclear envelope, but without killing at least some of them (US 5,204,253, US 5,015,580). A third direct method uses fibrous forms of metal or ceramic consisting of sharp, porous or hollow needle-like projections that literally impale the cells, and also the nuclear envelope of cells. Both silicon carbide and aluminum borate whiskers

have been used for plant transformation (Mizuno et al., 2004; Petolino et al., 2000; US5302523 US Application 20040197909) and also for bacterial and animal transformation (Kaepler et al., 1992; Raloff, 1990; Wang, 1995). There are other methods reported, and undoubtedly, additional methods will be developed. However, the efficiencies of each of these indirect or direct methods in introducing foreign DNA into plant cells are invariably extremely low, making it necessary to use some method for selection of only those cells that have been transformed, and further, allowing growth and regeneration into plants of only those cells that have been transformed.

For efficient plant transformation, a selection method must be employed such that whole plants are regenerated from a single transformed cell and every cell of the transformed plant carries the DNA of interest. These methods can employ positive selection, whereby a foreign gene is supplied to a plant cell that allows it to utilize a substrate present in the medium that it otherwise could not use, such as mannose or xylose (for example, refer US 5767378; US 5994629). More typically, however, negative selection is used because it is more efficient, utilizing selective agents such as herbicides or antibiotics that either kill or inhibit the growth of nontransformed plant cells and reducing the possibility of chimeras. Resistance genes that are effective against negative selective agents are provided on the introduced foreign DNA used for the plant transformation. For example, one of the most popular selective agents used is the antibiotic kanamycin, together with the resistance gene neomycin phosphotransferase (nptII), which confers resistance to kanamycin and related antibiotics (see, for example, Messing & Vierra, *Gene* 19: 259-268 (1982); Bevan et al., *Nature* 304:184-187 (1983)). However, many different antibiotics and antibiotic resistance genes can be used for transformation purposes (refer US 5034322, US 6174724 and US 6255560). In addition, several herbicides and herbicide resistance genes have been used for transformation purposes, including the bar gene, which confers resistance to the herbicide phosphinothricin (White et al., *Nucl Acids Res* 18: 1062 (1990), Spencer et al., *Theor Appl Genet* 79: 625-631(1990), US 4795855, US 5378824 and US 6107549). In addition, the dhfr gene, which confers resistance to the anticancer agent methotrexate, has been used for selection (Bourouis et al., *EMBO J.* 2(7): 1099-1104 (1983).

The expression control elements used to regulate the expression of a given protein can either be the expression control element that is normally found associated with the coding sequence (homologous expression element) or can be a heterologous expression control element. A variety of homologous and heterologous expression control elements are known

in the art and can readily be used to make expression units for use in the present invention. Transcription initiation regions, for example, can include any of the various opine initiation regions, such as octopine, mannopine, nopaline and the like that are found in the Ti plasmids of *Agrobacterium tumefaciens*. Alternatively, plant viral promoters can also be used, such as the cauliflower mosaic virus 19S and 35S promoters (CaMV 19S and CaMV 35S promoters, respectively) to control gene expression in a plant (U.S. Patent Nos. 5,352,605; 5,530,196 and 5,858,742 for example). Enhancer sequences derived from the CaMV can also be utilized (U.S. Patent Nos. 5,164,316; 5,196,525; 5,322,938; 5,530,196; 5,352,605; 5,359,142; and 5,858,742 for example). Lastly, plant promoters such as prolifera promoter, fruit specific promoters, Ap3 promoter, heat shock promoters, seed specific promoters, etc. can also be used.

Either a gamete specific promoter, a constitutive promoter (such as the CaMV or Nos promoter), an organ specific promoter (e.g., stem specific promoter), or an inducible promoter is typically ligated to the protein or antisense encoding region using standard techniques known in the art. The expression unit may be further optimized by employing supplemental elements such as transcription terminators and/or enhancer elements. The expression cassette can comprise, for example, a seed specific promoter (e.g. the phaseolin promoter (U.S. Pat. No. 5,504,200). The term "seed specific promoter", means that a gene expressed under the control of the promoter is predominantly expressed in plant seeds with no or no substantial expression, typically less than 10% of the overall expression level, in other plant tissues. Seed specific promoters have been well known in the art, for example, US Patent Nos. 5,623,067, 5,717,129, 6,403,371, 6,566,584, 6,642,437, 6,777,591, 7,081,565, 7,157,629, 7,192,774, 7,405,345, 7,554,006, 7,589,252, 7,595,384, 7,619,135, 7,642,346, and US Application Publication Nos. 20030005485, 20030172403, 20040088754, 20040255350, 20050125861, 20050229273, 20060191044, 20070022502, 20070118933, 20070199098, 20080313771, and 20090100551.

Thus, for expression in plants, the expression units will typically contain, in addition to the protein sequence, a plant promoter region, a transcription initiation site and a transcription termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the expression unit are typically included to allow for easy insertion into a preexisting vector.

In the construction of heterologous promoter/structural gene or antisense combinations, the promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural

setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

In addition to a promoter sequence, the expression cassette can also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes. If the mRNA encoded by the structural gene is to be efficiently processed, DNA sequences which direct polyadenylation of the RNA are also commonly added to the vector construct. Polyadenylation sequences include, but are not limited to the *Agrobacterium* octopine synthase signal (Gielen *et al.*, EMBO J 3:835-846 (1984)) or the nopaline synthase signal (Depicker *et al.*, Mol. and Appl. Genet. 1:561-573 (1982)). The resulting expression unit is ligated into or otherwise constructed to be included in a vector that is appropriate for higher plant transformation. One or more expression units may be included in the same vector. The vector will typically contain a selectable marker gene expression unit by which transformed plant cells can be identified in culture. Usually, the marker gene will encode resistance to an antibiotic, such as G418, hygromycin, bleomycin, kanamycin, or gentamicin or to an herbicide, such as glyphosate (Round-Up) or glufosinate (BASTA) or atrazine. Replication sequences, of bacterial or viral origin, are generally also included to allow the vector to be cloned in a bacterial or phage host, preferably a broad host range prokaryotic origin of replication is included. A selectable marker for bacteria may also be included to allow selection of bacterial cells bearing the desired construct. Suitable prokaryotic selectable markers include resistance to antibiotics such as ampicillin, kanamycin or tetracycline. Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art. For instance, in the case of *Agrobacterium* transformations, T-DNA sequences will also be included for subsequent transfer to plant chromosomes.

To introduce a desired gene or set of genes by conventional methods requires a sexual cross between two lines, and then repeated back-crossing between hybrid offspring and one of the parents until a plant with the desired characteristics is obtained. This process, however, is restricted to plants that can sexually hybridize, and genes in addition to the desired gene will be transferred.

Recombinant DNA techniques allow plant researchers to circumvent these limitations by enabling plant geneticists to identify and clone specific genes for desirable traits, such as resistance to an insect pest, and to introduce these genes into already useful varieties of

plants. Once the foreign genes have been introduced into a plant, that plant can then be used in conventional plant breeding schemes (e.g., pedigree breeding, single-seed-descent breeding schemes, reciprocal recurrent selection) to produce progeny which also contain the gene of interest.

5 Genes can be introduced in a site directed fashion using homologous recombination. Homologous recombination permits site specific modifications in endogenous genes and thus inherited or acquired mutations may be corrected, and/or novel alterations may be engineered into the genome. Homologous recombination and site-directed integration in plants are discussed in, for example, U.S. Patent Nos. 5,451,513; 5,501,967 and 5,527,695.

10 Methods of producing transgenic plants are well known to those of ordinary skill in the art. Transgenic plants can now be produced by a variety of different transformation methods including, but not limited to, electroporation; microinjection; microprojectile bombardment, also known as particle acceleration or biolistic bombardment; viral-mediated transformation; and Agrobacterium-mediated transformation. See, for example, U.S. Patent
15 Nos. 5,405,765; 5,472,869; 5,538,877; 5,538,880; 5,550,318; 5,641,664; 5,736,369 and 5,736,369; International Patent Application Publication Nos. WO2002/038779 and WO/2009/117555; Lu et al., (Plant Cell Reports, 2008, 27:273-278); Watson et al., Recombinant DNA, Scientific American Books (1992); Hinchey et al., Bio/Tech. 6:915-922 (1988); McCabe et al., Bio/Tech. 6:923-926 (1988); Toriyama et al., Bio/Tech. 6:1072-1074
20 (1988); Fromm et al., Bio/Tech. 8:833-839 (1990); Mullins et al., Bio/Tech. 8:833-839 (1990); Hiei et al., Plant Molecular Biology 35:205-218 (1997); Ishida et al., Nature Biotechnology 14:745-750 (1996); Zhang et al., Molecular Biotechnology 8:223-231 (1997); Ku et al., Nature Biotechnology 17:76-80 (1999); and, Raineri et al., Bio/Tech. 8:33-38 (1990)), each of which is expressly incorporated herein by reference in their entirety.

25 *Agrobacterium tumefaciens* is a naturally occurring bacterium that is capable of inserting its DNA (genetic information) into plants, resulting in a type of injury to the plant known as crown gall. Most species of plants can now be transformed using this method, including cucurbitaceous species.

30 Microprojectile bombardment is also known as particle acceleration, biolistic bombardment, and the gene gun (Biolistic® Gene Gun). The gene gun is used to shoot pellets that are coated with genes (e.g., for desired traits) into plant seeds or plant tissues in order to get the plant cells to then express the new genes. The gene gun uses an actual explosive (.22 caliber blank) to propel the material. Compressed air or steam may also be

used as the propellant. The Biolistic® Gene Gun was invented in 1983-1984 at Cornell University by John Sanford, Edward Wolf, and Nelson Allen. It and its registered trademark are now owned by E. I. du Pont de Nemours and Company. Most species of plants have been transformed using this method.

5 A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single gene on one chromosome, although multiple copies are possible. Such transgenic plants can be referred to as being hemizygous for the added gene. A more accurate name for such a plant is an independent segregant, because each transformed plant represents a unique T-DNA integration event (U.S. Patent No. 6,156,953). A transgene locus
10 is generally characterized by the presence and/or absence of the transgene. A heterozygous genotype in which one allele corresponds to the absence of the transgene is also designated hemizygous (U.S. Patent No. 6,008,437).

General transformation methods, and specific methods for transforming certain plant species (e.g., maize, rice, wheat, barley, soybean) are described in U.S. Patent Nos. 4940838,
15 5464763, 5149645, 5501967, 6265638, 4693976, 5635381, 5731179, 5693512, 6162965, 5693512, 5981840, 6420630, 6919494, 6329571, 6215051, 6369298, 5169770, 5376543, 5416011, 5569834, 5824877, 5959179, 5563055, and 5968830, each of which is incorporated by reference in its entirety.

20 This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference.

25 EXAMPLES

Example 1

Identification of *KRP* homologues from wheat

The inventors used available EST collections and proprietary sequences to generate partial predicted proteins for the different wheat *KRP* genes (SEQ ID Nos 1 – 5).

30 A multiple sequence analysis was generated using the protein sequences for rice, maize, and wheat *KRP* genes. Alignment of sequences was done by using online Multiple Sequence Alignment (MSA) service provided by European Bioinformatics Institute and the bootstrap values were calculated by using online software TreeTop-Phylogenetic Tree

Prediction provided by Moscow State University (Figure 1). Values in the branches in Figure 1 are bootstrap values.

Based on our phylogenetic tree construction, we discerned a correspondence between the wheat, rice, and maize *KRP* genes. The relationships we figured out between the different *KRP* genes appear similar to the one reported by Barroco et al. (2006), The cyclin-dependent kinase inhibitor Orysa;KRP1 plays an important role in seed development of rice, Plant Physiology, 142: 1053-1064, except for a more external position of *OsKRP3*. This demonstrates a clear correspondence between the wheat and rice/maize genes.

The wheat KRP homologues *TaKRP1*, *TaKRP2*, *TaKRP4*, *TaKRP5* were named according to the rice *KRP* nomenclature. *OsKRP1*, *OsKRP4*, and *OsKRP5* genes are expressed in the seeds. *OsKRP4* is the only one that carries a consensus CDK phosphorylation site.

The sequence of *KRPs* from the A, B and D genomes (SEQ ID Nos 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 and 77) were obtained using wheat BAC libraries.

Example 2

Genome-specific primers for TILLING® of wheat KRPs

The genome specificity of the wheat KRP primers using Chinese-Spring (CS) nulli-tetrasomic lines and TILLING® cultivars were validated. The validation for wheat KRP4 primers is shown in Figure 2. Table 1 lists the TILLING® primer sets for each wheat KRP.

Table 1. TILLING® primers for wheat KRPs

Gene/Genome	Primer name	Primer sequence (5' to 3')	SEQ ID NO
KRP1A	Krp-A1_AF1	GTCTGTAGAACGGCGTTACG	6
	Krp-A1_R8	CCCCAGCTCTACAGTGAGTAACTT	7
KRP1B	Krp-B1_F5	GGAGAGAATCCAAAGAGCAAC	8
	Krp-B1_R7	CCAGCTCTACAGTGAGTAAC	9
KRP1D	Krp-D1_F4	CGTGTTATATGTGTACGCACAC	10
	Krp-D1_DR2	GGCTATAATGCTTCTTTCTGGAGC	11
KRP2A	Krp-A2_BF3	CAAACGGCCAAAGCGACGG	12
	Krp-A2_AR3	CTAGTGTTGCATAGTTAGCTC	13
KRP2B	Krp-B2_BF3	CAAACGGCCAAAGCGACGG	14
	Krp-B2_BR2	TCTCGCCTCCGAGTCTAAGT	15
KRP2D	Krp-D2_BF3	CAAACGGCCAAAGCGACGG	16
	Krp-D2_DR1	CAGATTTGAACAAGGTGGATC	17

KRP4A_exon1	WKP4_AF1	TACCCGCGCCTCGCTTAAATCCGCCAAA	18
	WKP4_AR1	GTCAACTCGTGAAAGAAGAGTTGGGACAGA	19
KRP4A_exons 2-3	WKP4_AF2	CCTTAGGCAAGTTCGGTAAGAAATGTGTA	20
	WKP4_AR2	GTGGTCATTACAGAATGAGTTGCTAACCGTC	21
KRP4B_exon1	WKP4_BF1	TTACCCGCGCCTCGCTTAAATCCGCAAG	22
	WKP4_BR1	GCTCAAACAGCGAAAGAAGAGTTAGACGGA	23
KRP4B_exons 2-3	WKP4_BF3	CTGGCCTACTCATGTGAGACTGAGAGATTA	24
	WKP4_BR2	GTGGTCATTACAGAATGAGCTGCTAACCGTT	25
KRP4D_exon1	WKP4_DF1	TACCCGCCCCCTCGCTTAAATCCGCCAAA	26
	WKP4_DR1	ACTCAAATAGCGAAAGAAGAGTTAGCCAGGA	27
KRP4D_exons 2-3	WKP4_DF3	TGGCCTACTCATGTGACACTGAGAGATTG	28
	WKP4_BR2	GTGGTCATTACAGAATGAGCTGCTAACCGTT	29
KRP5A_exon1	Krp-A5_AF7	GTAAGCACAGGAAGCAGAGC	30
	Krp-A5_AR6	CTCAGTCGTATTCGTATCGG	31
KRP5A_exons 2-3	Krp-A5_AF4	CACACCTCACATTGTGTGATG	32
	Krp-A5_R1	ACAGAGATCAATGGAGGAGC	33
KRP5B	Krp-B5_BF4	TGCGCCTCACATTGTCTAGC	34
	Krp-A5_R1	ACAGAGATCAATGGAGGAGC	35
KRP5D	Krp-D5_DF4	ATGCTAGAACATGAGCTGTCTG	36
	Krp-D5_DR3	GCTGATGGTGGTGGTCATTC	37

Example 3

Discovery of a natural KRP-A4 deletion in hexaploid wheat

Both sets of A genome KRP4 primers did not amplify the KRP4 gene in the hexaploid TILLING® line (Figure 2), suggesting a possible natural deletion in the KRP4 A genome of the hexaploid TILLING® line. This was confirmed with the use of 3 gene-specific primers.

The B and D copies of KRP4 in hexaploid wheat were TILL'ed. The hexaploid mutations can easily be moved between tetraploid and hexaploid wheat by crosses.

Example 4

Wheat KRP expression studies

To determine which KRP genes were expressed in early seed development, the transcript levels of the KRP genes were assessed by RT-PCR (Figure 3A). These results showed that KRP4 transcript levels were higher than those of the other genes and that its transcripts accumulated over a longer developmental period than the transcripts from *KRP1*, *KRP2*, and *KRP5*. These last three genes were detected in the different pools from the 2-6 days after anthesis (DAA) samples and only in one out of the three pooled samples collected

8-12 DAA. No transcripts from *KRP1*, *KRP2*, and *KRP5* were detected in the 14-18 DAA samples, whereas *KRP4* transcripts were still abundant during this developmental stage. This data suggest that *KRP4* has a different transcription profile than the other *KRP* genes during seed development.

5 The transcript levels of these four *KRP* genes were determined in other tissues (Figure 3B). Transcripts from *KRP2*, *KRP4*, and *KRP5* were detected in cDNAs from 30-day old leaves. *KRP1* was the only one not detected at that stage. *KRP1* and *KRP5* were not detected in cDNA samples from the first leaf and were very faint at the 5th leaf-stage. Both were absent from stems and spikes. Transcripts from *KRP2* and *KRP4* were detected in stems and
10 spikes.

Example 5

Mutations of *Triticum KRP* Genes identified in TILLING®

Screening of the TILLING® population for *KRP* mutants resulted in plants with
15 silent, splice, nonsense (premature stop codons) and/or missense (severe or non-severe) mutations in *KRP1*, *KRP2*, *KRP4* and *KRP5* (*A*, *B*, and *D* genomes) genes. TILLING® was conducted according to Uauy, C., F. Paraiso, et al. (2009) "A modified TILLING® approach to detect induced mutations in tetraploid and hexaploid wheat" BMC Plant Biol 9: 115.

Positions and effects of mutations in *KRP1*, *KRP2*, *KRP4* and *KRP5* (*A*, *B*, and *D*
20 genomes) genes are provided in Tables 2-3 below (* indicates the mutation results in a stop codon, = indicates the mutation is silent).

Table 2. Summary of *Triticum durum* (tetraploid) *KRP4A* and *KRP4B* mutants

KRP mutant	Nucleotide Change [^]	Effect	Mutation Score
KRP4A-242	G688A	W186*	nonsense
KRP4A-296	C248T	T104I	missense
KRP4A-1220	C335T	S133F	Type II [§]
KRP4A-1031	C994T	N176=	silent
KRP4B-842	G184A	G41E	missense
KRP4B-309	G277A	R72K	missense
KRP4B-587	G291A	E77K	missense
KRP4B-650	G407A	S111N	missense
KRP4B-112	G434A	S120N	missense
KRP4B-161	C440T	P122L	Type II
KRP4B-1280	G461A	S129N	missense

[^]Nucleotide numbering is dependent upon the location of TILLING® primers.

[§]Type I and Type II mutations are defined in the wheat breeding program section.

Table 3. Summary of *Triticum aestivum* (hexaploid) KRP mutants

KRP mutant	Nucleotide Change [^]	Effect	Mutation Score
KRP1A-2887	G484A	splice	splice
KRP1B-2371	C293T	S98L	missense
KRP1B-2201	G585A	S144N	missense
KRP1B-2199	C657T	S156F	Type II [§]
KRP1D-2259	C181T	Q61*	nonsense
KRP2A-2241	G758A	splice	splice
KRP2B-3004	C775T	S179L	missense
KRP2D-0905	C29T	A10V	missense
KRP4B-2023	G321A	E87K	missense
KRP4B-149	C401T	P109L	Type II
KRP4B-491	G496A	A141T	missense
KRP4B-823	G390A	R105=	silent
KRP4B-566	G399A	T108=	silent
KRP4D-586	G387A	splice	splice
KRP4D-404	C407T	P109S	Type II
KRP4D-557	C814T	L180S	Type I
KRP4D-558	C563T	P161S	missense
KRP5A-2327	G1910A	G141R	Type II
KRP5A-802		P43S	missense
KRP5A-2506		E89K	missense
KRP5D-425	C1770T	S138L	missense
KRP5D-2194	C2044T	P194L	Type I

[^]Nucleotide numbering is dependent upon the location of TILLING® primers.

[§]Type I and Type II mutations are defined in the wheat breeding program section.

5

Example 6

Wheat breeding program

The wheat KRP TILLING® mutants are prioritized for the breeding program from most important to less important in the following manner: 1) Nonsense and splice mutants, 2) Type I severe missense, 3) Type II severe missense. Type I severe missense mutations are non-conservative amino acid substitutions in regions of the KRP protein known to be

10

essential for binding to cyclin or cyclin-dependent kinase (CDK) and are predicted by SIFT (Sorting Intolerant From Tolerant) analysis (Ng and Henikoff, SIFT: predicting amino acid changes that affect protein function, *Nucl. Acids Res.* (2003) 31 (13): 3812-3814) to be deleterious to protein function. Type II severe missense mutations are non-conservative amino acid substitutions outside of the cyclin and CDK binding domains but which satisfy two additional criteria. First, they are in regions of the protein determined by BLOCKS analysis (Henikoff, S. and Henikoff, J.G. (1991) *Nucleic Acids Res.*, 19, 6565-6572) to be evolutionarily conserved and therefore possibly of functional significance. Secondly, they have a SIFT (Ng, P.C. and Henikoff, S. (2003) *Nucleic Acids Res.* July 1; 31(13): 3812-3814) score of less than 0.05, and are therefore predicted to be deleterious to protein function.

M3 seed homozygous or heterozygous for a given KRP TILLING® mutation is grown. Backcrosses with the hexaploid spring wheat background parent are performed, ideally through several rounds (to the BC3 or BC4 level), to eliminate deleterious background mutations. Background mutations could contribute to undesirable traits such as delayed maturity, premature senescence, increased susceptibility to wheat pathogens, slow germination, and/or sterility. The progeny of each backcross (F1, BC1, BC2, etc.) are also selfed to produce F2 lines. F2 lines are genotyped to identify ones that are homozygous for the wild type or for the *krr* mutant allele. Homozygote wild type and mutant siblings are seed expanded to F3 for field trials.

Wheat *krr* mutant alleles are introgressed into other spring and winter wheat lines to transfer the yield enhancement to commercial varieties.

Crosses between mutants are done to generate multiple stack mutants within a given *KRP* gene (e.g. *KRP1A/1B*, *KRP1B/KRP1D*, *KRP1A/1B/1D*, etc., all possible combinations) or across different *KRP* genes (e.g. *KRP1A/2A*, *KRP2B/ KRP4B*, *KRP4D/KRP5A*, *KRP1B/KRP2A/ KRP5D*, etc., all possible combinations).

The overall grain yield per unit area is determined (e.g. lbs/acre) and yield components such as seed count, seed size/weight (thousand kernel weight), seed per spike, head (spike) number, spike length, awn length, and/or tiller number, are measured. Agronomic characteristics such as stand rate, maturity rate and peduncle rate are also measured.

Example 7**Characterization of hexaploid TILLING® mutant KRP4B-149**

Hexaploid mutant line KRP4B-149 was backcrossed to the non-mutagenized recurrent parent to reduce the mutation load. BC₁F₂ lines (backcrossed once and selfed) homozygous for the presence of the mutation were selected. In addition, a KRP4B-149 sibling line homozygous for the lack of the mutation was used as a control.

Plants were grown in outside field plots in California. The experiment was organized in a Complete Randomized Block Design (RCBD) with four replications. Differences between the individual mutants and the single control line were tested using the Dunnett test.

The following parameters were measured: heading time, height, number of spikelets per spike, grains per spike, thousand kernel weight (TKW) and yield.

While early reports indicate that the KRP4B-149 mutant grew faster and flowered earlier, later reports for the KRP4B-149 mutant showed no-significant differences for most of the parameters except for a 10 days delay in flowering time ($P=0.0003$) and a slight increase in two spikelets per spike ($P=0.006$) which was not reflected in an increase in the number of grains or in grain yield. However, the delay in flowering time was not seen in the BC₂F₂ generation in the greenhouse.

Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patents, and patent publications cited are incorporated by reference herein in their entirety for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

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Claims

1. A plant cell, plant part, plant tissue culture or whole plant of a *Triticum* species comprising a mutated *KRP* gene selected from the group consisting of *TaKRP1*, *TaKRP2*, *TaKRP4*, *TaKRP5*, and combinations thereof.
2. The plant cell, plant part, plant tissue culture or whole plant of claim 1, wherein the mutated *KRP* is selected from the group consisting of *TaKRP1A*, *TaKRP1B*, *TaKRP1D*, *TaKRP2A*, *TaKRP2B*, *TaKRP2D*, *TaKRP4A*, *TaKRP4B*, *TaKRP4D*, *TaKRP5A*, *TaKRP5B*, or *TaKRP5D*.
3. The plant cell, plant part, plant tissue culture or whole plant of claim 1, wherein the mutated *KRP* comprises at least one mutation.
4. The plant cell, plant part, plant tissue culture or whole plant of claim 1, wherein the mutated *KRP* gene comprises one or more mutations selected from any one of the mutations listed in Tables 2 and 3 for a particular *KRP* gene
5. A method of increasing organ weight, organ size, organ number and/or yield of a wheat plant comprising disrupting one or more *KRPs* in the plant.
6. The method of claim 5, wherein the *KRP* in the wheat is *TaKRP1*, *TaKRP2*, *TaKRP4*, or *TaKRP5*.
7. The method of claim 6, wherein the *KRP* in a tetraploid wheat plant is *TaKRP1A*, *TaKRP1B*, *TaKRP2A*, *TaKRP2B*, *TaKRP4A*, *TaKRP4B*, *TaKRP5A*, or *TaKRP5B*, and wherein the *KRP* in a hexaploid wheat is *TaKRP1A*, *TaKRP1B*, *TaKRP1D*, *TaKRP2A*, *TaKRP2B*, *TaKRP2D*, *TaKRP4A*, *TaKRP4B*, *TaKRP4D*, *TaKRP5A*, *TaKRP5B*, or *TaKRP5D*.
8. The method of claim 5, wherein the *KRP* gene function is disrupted by nucleotide substitution, deletion, insertion, T-DNA, transposon, or homologous recombination.

9. The method of claim 5, wherein the wheat plant comprises one or more mutations selected from any one of mutations listed in Tables 2 and 3 for a particular *KRP* gene.
10. A method of producing a wheat plant with increased organ size, organ weight, organ number and/or yield compared to a wild type plant, comprising
- i) making a cross between a first wheat plant to a second wheat plant to produce a F1 plant, wherein the first wheat plant comprises one or more disrupted *KRP* genes.
11. The method of claim 10, wherein the method further comprises
- ii) backcrossing the F1 plant to the first or the second wheat plant; and
- iii) repeating the backcrossing step to generate a near isogenic line, wherein the one or more disrupted *KRPs* in the first wheat plant are integrated into the genome of the near isogenic line.
12. The method of claim 10 or claim 11, wherein the *KRP* gene is selected from the group consisting of *TaKRP1A*, *TaKRP1B*, *TaKRP1D*, *TaKRP2A*, *TaKRP2B*, *TaKRP2D*, *TaKRP4A*, *TaKRP4B*, *TaKRP4D*, *TaKRP5A*, *TaKRP5B*, or *TaKRP5D*.
13. The method of claim 10 or claim 11, wherein the first wheat plant comprises one or more mutations selected from any one of mutations listed in Tables 2 and 3 for a particular *KRP* gene.
14. A recombinant RNAi construct for down-regulating one or more genes in a plant cell, plant part, plant tissue culture or whole plant, wherein the recombinant vector comprising a polynucleotide comprising at least 15 consecutive nucleotides of the complement of SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 or 77.
15. A plant cell, plant part, plant tissue culture or whole plant, wherein the plant cell, plant part, plant tissue culture or whole plant comprising the recombinant RNAi construct of claim 14.

16. The plant cell, plant part, plant tissue culture or whole plant of claim 15, wherein the plant is a plant of the *Triticeae* tribe.
17. The plant cell, plant part, plant tissue culture of whole plant of claim 16, wherein the plant is a plant of the *Triticum* genus.
18. The plant cell, plant part, plant tissue culture or whole plant of claim 17, wherein the plant in the *Triticum* genus is a wheat plant.
19. The plant cell, plant part, plant tissue culture or whole plant of claim 18, wherein the wheat plant is tetraploid or hexaploid.
20. A method for down-regulating one or more genes in a plant cell, plant part, plant tissue culture or whole plant, comprising incorporating a polynucleotide comprising at least 15 consecutive nucleotides of the complement of SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76 or 77.
21. An isolated polynucleotide comprising a sequence selected from the group consisting of: (a) sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76, 77, and portions thereof; (b) complements of the sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76, 77 and portions thereof; (c) reverse complements of the sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76, 77, and portions thereof; (d) reverse sequences of the sequences recited in SEQ ID NOs: 40, 41, 43, 44, 46, 47, 49, 50, 52, 53, 55, 56, 58, 59, 60, 62, 63, 64, 66, 67, 68, 70, 71, 73, 74, 76, 77, and portions thereof; and (e) sequences having at least 90% identity to a sequence of (a)-(d) or a specified region of a sequence of (a)-(d).
22. An isolated polypeptide comprising an amino acid sequence selected from the group consisting of (a) sequences recited in SEQ ID NOs: 42, 45, 48, 51, 54, 57, 61, 65, 69, 72, 75, 78, and portions thereof; (b) sequences having at least 90% identity to a sequence of (a).

Figure 1

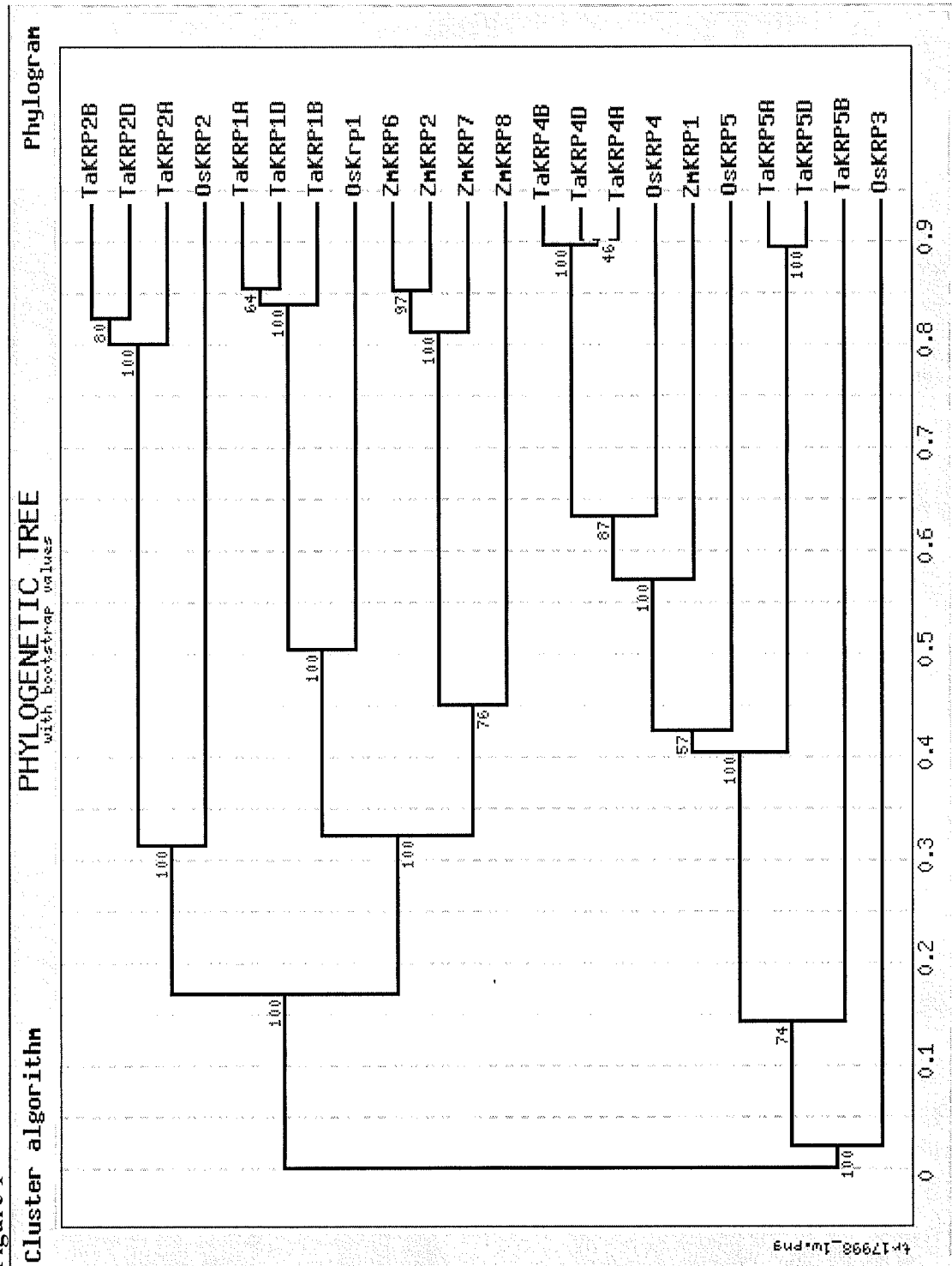


Figure 2

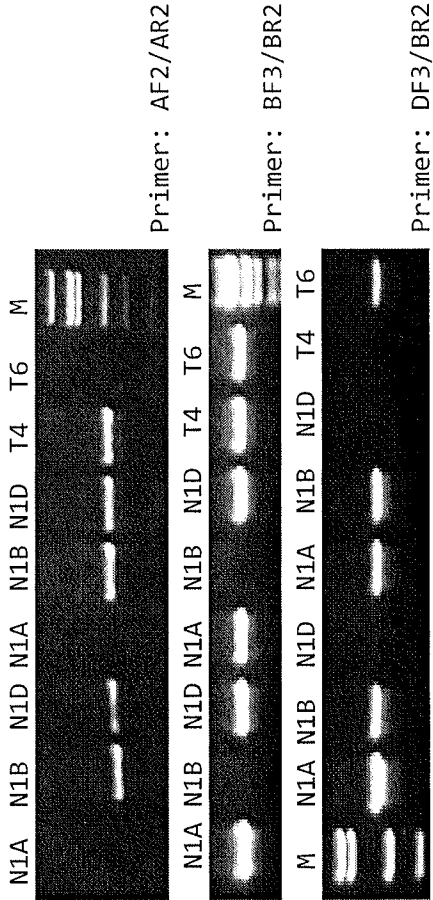


Figure 3

