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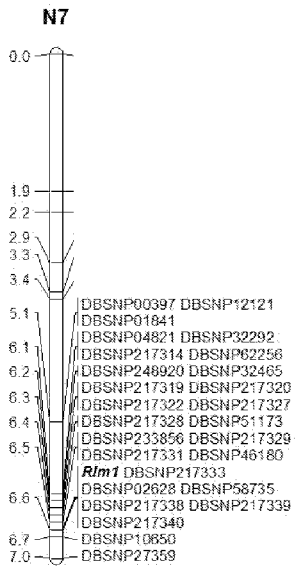
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(54) Title: MOLECULAR MARKERS FOR BLACKLEG RESISTANCE GENE RLM1 IN BRASSICA NAPUS, AND METHODS OF USING THE SAME

(57) Abstract: This disclosure concerns methods and compositions for identifying canola plants that have a blackleg resistant phenotype. Some embodiments concern molecular markers to identify, select, and/or construct blackleg resistant plants and germplasm, or to identify and counter-select plants that are susceptible or have low resistance to blackleg disease. Some embodiments concern molecular markers to identify, select, and/or construct blackleg resistant plants that carry the Rlm1 gene. This disclosure also concerns canola plants comprising a blackleg resistant phenotype that are generated by methods utilizing at least one marker described herein.

Figure 1



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[Continued on next page]

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MOLECULAR MARKERS FOR BLACKLEG RESISTANCE GENE RLM1 IN BRASSICA NAPUS, AND METHODS OF USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional application No. 62/718,029, filed August 13, 2018, entitled "MOLECULAR MARKERS FOR BLACKLEG RESISTANCE GENE RLM1 IN BRASSICA NAPUS, AND METHODS OF USING THE SAME", the disclosure of which is being incorporated by reference in its/their entirety.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates to compositions and methods for identifying canola plants that have resistance to blackleg disease, where the methods use molecular genetic markers to identify, select and/or construct blackleg resistant plants, and specifically to select or construct plants with the blackleg resistance gene Rlm1. The disclosure also relates to canola plants that are blackleg resistant that are generated by the methods of the invention.

BACKGROUND

[0003] Blackleg is a serious disease of canola that can result in significant yield loss in susceptible varieties in Canada, Europe, and Australia. Blackleg is the most common name, but stem canker or Phoma stem canker are also used for this disease. It is caused by the fungus *Leptosphaeria maculans*. In Saskatchewan a highly aggressive strain of the blackleg fungus was first detected in 1975, but now occurred in most canola-growing areas in western Canada.

[0004] Canola disease field surveys are conducted annually in the main canola production regions of western Canada. Disease surveys from recent years indicate that blackleg is commonly found in 35 to 55 per cent of crops surveyed. Average disease incidence values (percentage of plants showing blackleg symptoms) are typically one per cent for basal stem cankers and three per cent for lesions occurring elsewhere on the stem. Lesions occurring elsewhere on the stem will have less impact on seed yield and quality than will basal stem cankers. The highest incidence values are often observed in crops that had received hail damage.

[0005] As is indicated in the disease surveys, it is not unusual to observe blackleg symptoms in canola crops, even when resistant varieties are being grown. However, to prevent blackleg from negatively impacting seed yield and quality, it is important to be familiar with blackleg symptoms, the disease cycle, and disease management practices.

[0006] Blackleg is the most serious disease of canola in Australia. The severity of blackleg has risen in recent years due to increased acreage and intensity of production. Although not common, yield losses of 50 per cent and greater have been recorded in some seasons with up to 90 per cent yield loss occurring in cases where *L. maculans* has overcome major blackleg resistance genes within certain varieties.

[0007] In the autumn and winter, rainfall triggers spore release from the stubble. Within two weeks of spores landing on canola cotyledons and young leaves, clearly visible off-white colored lesions develop, within the lesion pycnidial fruiting bodies (dark colored dots) release rain-splashed spores. Blackleg infections may occur on cotyledons, leaves, stems and pods. The plant is susceptible to blackleg infection from the seedling to pod-set stages. Lesions occurring on the leaves are dirty white and are round to irregularly. On stems, blackleg lesions can be quite variable, but are usually found at the base of the stem, or at points of leaf attachment. Once a lesion has formed, the fungus grows within the plants vascular system to the crown where it causes the crown of the plant to rot, resulting in a canker. Severe canker will sever the roots from the stem, whereas a less severe infection will result in internal infection of the crown restricting water and nutrient flow within the plant. Stem lesions may be up to several inches in length and are usually white or grey with a dark border. Stem lesions may also appear as a general blackening at the base. Severe infection usually results in a dry rot or canker at the base of the stem. The stem becomes girdled and, as plants ripen prematurely, the crop is more likely to lodge. Seed may be shriveled, and pods shatter easily at harvest, resulting in seed loss.

[0008] In recent years blackleg symptoms have also been found in the plant roots, this root infection in severe cases appears to cause the entire plant to die prematurely. The root rot form of the disease is caused by the same blackleg strains that cause the stem canker and management practices to control normal blackleg are the same for the root rot form of the disease.

[0009] With increasing acres of canola and often tighter rotations, blackleg disease has again started to become an important yield and quality reducing disease.

[0010] Varietal resistance is the best defense, to date, against blackleg disease. The development of blackleg resistant varieties has led to reduced economic losses due to blackleg. As a result, many farmers are growing resistant varieties and they have been able to get away with tighter and tighter rotations. Preliminary observations from the 2010 canola disease survey show that in Manitoba the R-rated varieties are starting to show higher incidence and severity of the blackleg disease. *L. maculans* has developed new variants and these new variants are now able to infect the earlier R-rated canola varieties.

[0011] When the blackleg resistant varieties were first developed, researchers categorized the pathogen into "PG" patho-groups, which were based on the infectivity of an isolate on 3 varieties - Westar, Glacier and Quinta. With changing blackleg populations, the PG classification is insufficient to describe newer variants. This means the PG system cannot identify variability among isolates as a result of sources of resistance not found in 'Quinta' and 'Glacier'. Based on molecular markers, pathogen isolates have been able to be classified into PG groups, much quicker than the traditional plant inoculation method (Dusabenyagasani, M., and Fernando, W. G. D. 2008).

[0012] In a newer approach, by using varieties or lines of Brassica spp. carrying 14 specific resistance genes, new isolates of *L. maculans* can be differentiated or characterized into races based on the reactions observed. Rimmer (2007) had reported these 14 genes of specific resistance, designated Rlm1 to Rlm10 and LepR1 to LepR4. A study of 96 western Canadian isolates of blackleg fungus, using ten resistance genes indicated considerable variation in the pathogen population for many of these genes (Kutcher et al. 2010).

[0013] There are numerous steps in the development of any novel, desirable plant germplasm. Plant breeding programs combine desirable traits from two or more cultivars or various broad-based sources into breeding pools, from which cultivars are developed by selfing and selection of desired phenotypes. The new cultivars are evaluated to determine which have commercial potential. Plant breeding begins with the analysis and definition of problems and weaknesses of the current germplasm, the establishment of program goals, and the definition of specific breeding objectives. The next step is selection of germplasm that possess the traits to meet the program goals. The goal is to combine in a single variety an improved combination of desirable traits from the parental germplasm. These important traits may include higher seed yield, resistance to diseases and insects, better stems and roots, tolerance to drought and heat, and better agronomic quality.

[0014] Choice of breeding or selection methods depends on the mode of plant reproduction, the heritability of the trait(s) being improved, and the type of cultivar used commercially (*e.g.*, F_1 hybrid cultivar, pureline cultivar, etc.). For highly heritable traits, a choice of superior individual plants evaluated at a single location may be effective, whereas for traits with low heritability, selection should be based on mean values obtained from replicated evaluations of families of related plants. Popular selection methods commonly include pedigree selection, modified pedigree selection, mass selection, and recurrent selection.

[0015] The complexity of inheritance influences the choice of the breeding method. Backcross breeding is used to transfer favorable alleles of one or a few genes for a highly heritable trait into a desirable cultivar. This approach has been used extensively for breeding disease-resistant cultivars. Various recurrent selection techniques are used to improve quantitatively-inherited traits controlled by numerous genes. The use of recurrent selection in self-pollinating crops depends on the ease of pollination, the frequency of successful hybrids from each pollination, and the number of hybrid offspring from each successful cross.

[0016] Each breeding program should include a periodic, objective evaluation of the efficiency of the breeding procedure. Evaluation criteria vary depending on the goal and objectives, but should include gain from selection per year (based on comparisons to an appropriate standard), overall value of the advanced breeding lines, and the number of successful cultivars produced per unit of input (*e.g.*, per year, per dollar expended, etc.). Promising advanced breeding lines are then thoroughly tested and compared to appropriate standards in environments representative of the commercial target area(s) for three or more years. Candidates for new commercial cultivars are selected from among the best lines; those still deficient in a few traits may be used as parents to produce new populations for further selection. These processes, which lead to the final step of marketing and distribution, usually take from 8 to 12 years from the time the first cross is made. Therefore, development of new cultivars is a time-consuming process that requires precise forward planning, efficient use of resources, and a minimum of changes in direction.

[0017] A most difficult task in plant breeding is the identification of individuals that are genetically superior. One method of identifying a superior plant is to observe its performance relative to other experimental plants and to a widely grown standard cultivar. If a single observation is inconclusive, replicated observations provide a better estimate of its genetic worth. This task is so difficult, because (for most traits) the true genotypic value is masked by other confounding plant traits or environmental factors.

[0018] The goal of canola plant breeding is to develop new, unique, and superior canola cultivars and hybrids. The breeder initially selects and crosses two or more parental lines, followed by repeated selfing and selection, producing many new genetic combinations. The breeder can theoretically generate billions of different genetic combinations *via* crossing, selfing, and mutagenesis. Such a breeder has no direct control of the process at the cellular level. Therefore, two breeders will never develop the same line, or even very similar lines, having the same canola traits.

[0019] Each year, the plant breeder selects the germplasm to advance to the next generation. This germplasm is grown under unique and different geographical, climatic, and soil conditions. Further selections are then

made, during and at the end of the growing season. The cultivars that are developed are unpredictable. This unpredictability is due to the breeder's selection, which occurs in unique environments, and which allows no control at the DNA level (using conventional breeding procedures), with millions of different possible genetic combinations being generated. A breeder of ordinary skill in the art cannot predict the final resulting lines he develops, except possibly in a very gross and general fashion. Similarly, the same breeder cannot produce the same cultivar twice by using the exact same original parents and the same selection techniques. This unpredictability results in the expenditure of large amounts of resources, monetary and otherwise, to develop superior new canola cultivars.

[0020] The development of new canola cultivars requires the development and selection of canola varieties, crossing of these varieties, and selection of superior hybrid crosses. Hybrid seed is produced by manual crosses between selected male-fertile parents, or by using male sterility systems. These hybrids are selected for certain single gene traits (*e.g.*, pod color, flower color, pubescence color, and herbicide resistance) that indicate that the seed is truly a hybrid. Data on parental lines, as well as the phenotype of the hybrid, influence the breeder's decision regarding whether to continue with the specific hybrid cross.

[0021] Pedigree breeding is used commonly for the improvement of self-pollinating crops. In pedigree breeding, two parents that possess favorable, complementary traits are crossed to produce F_1 progeny. An F_2 population is produced by selfing one or several plants from the F_1 progeny generation. Selection of the best individuals may begin in the F_2 population; then, beginning in the F_3, the best individuals in the best families are selected. To improve the effectiveness of selection for traits with low heritability, replicated testing of families can begin in the F_4 generation. At an advanced stage of inbreeding (*e.g.*, F_6 or F_7), the best lines or mixtures of lines with similar phenotypes are tested for potential release as new cultivars. Mass and recurrent selections can be used to improve populations of either self- or cross-pollinating crops. A genetically variable population of heterozygous individuals may be either identified or created by intercrossing several different parents. The best plants may be selected based on individual superiority, outstanding progeny, or excellent combining ability. The selected plants are intercrossed to produce a new population, in which further cycles of selection may be continued.

[0022] Backcross breeding has been used to transfer genes for a simply- and highly-heritable trait into a desirable homozygous cultivar, or inbred line, which is the recurrent parent. The source of the trait to be transferred is the "donor parent." The resulting plant is expected to have the attributes of the recurrent parent (*e.g.*, cultivar), and the desirable trait transferred from the donor parent. After the initial cross, individuals possessing the phenotype of the donor parent are selected, and repeatedly crossed (backcrossed) to the recurrent

parent. The resulting plant is expected to have the attributes of the recurrent parent and the desirable trait transferred from the donor parent.

[0023] In canola breeding, the "single-seed descent procedure" refers to the planting of a segregating population, followed by harvesting a sample of one seed per resulting plant, and using the harvested one-seed sample to plant the next generation. When the population has been advanced from the F_2 generation to the desired level of inbreeding, the plants from which lines are derived will each trace to different F_2 individuals. The number of plants in a population declines each generation, due to failure of some seeds to germinate or some plants to produce at least one seed. As a result, not all of the F_2 plants originally sampled in the population will be represented by a progeny when generation advance is completed.

[0024] In a multiple-seed procedure, canola breeders commonly harvest seeds from each plant in a population and thresh them together to form a bulk. Part of the bulk is used to plant the next generation, and part is put in reserve. This procedure has been referred to as modified single-seed descent. The multiple-seed procedure has been used to save labor involved in the harvest. It is considerably faster to remove seeds with a machine, than to remove one seed from each by hand for the single-seed procedure. The multiple-seed procedure also makes it possible to plant the same number of seeds of a population for each generation of inbreeding. Enough seeds are harvested to compensate for the number of plants that did not germinate or produce seed.

[0025] Proper testing should detect any major faults and establish the level of superiority or improvement of a new cultivar over current cultivars. In addition to showing superior performance, there should be a demand for a new cultivar that is compatible with industry standards, or that creates a new market. The testing preceding release of a new cultivar should take into consideration research and development costs as well as technical superiority of the final cultivar. The introduction of a new cultivar can incur additional costs to the seed producer, the grower, the processor, and the consumer due to special required advertising and marketing, altered seed and commercial production practices, and new product utilization. For seed-propagated cultivars, it must be feasible to produce seed easily and economically.

[0026] It is the goal of the plant breeder to select plants and enrich the plant population for individuals that have desired traits, for example, disease resistance or blackleg resistance, leading ultimately to increased agricultural productivity. Consistent with the foregoing, a continuing goal of canola breeders is to develop stable, high-yielding cultivars that are agronomically sound. Current goals include maximization of the amount of grain produced on the land used, and the supply of food for both animals and humans. To accomplish these goals, the canola breeder must select and develop canola plants that have traits that result in superior cultivars,

and do so in the most cost-effective manner. Molecular markers may be used in the process of marker-assisted selection (MAS) to aid in the identification and selection of individuals or families of individuals that possess inherited attributes that are linked to the markers.

BRIEF SUMMARY OF THE DISCLOSURE

[0027] Molecular markers that are linked to blackleg resistance may be used to facilitate marker-assisted selection for the blackleg resistance trait in canola. Marker-assisted selection provides significant advantages with respect to time, cost, and labor, when compared to blackleg resistance phenotyping. Disclosed herein are particular markers identified to be within or near blackleg resistance regions in the canola genome that are polymorphic in parent genotypes and linked (*e.g.*, tightly-linked) to a blackleg resistance phenotype. These markers, offer superior utility in marker-assisted selection of canola plants and cultivars having blackleg resistance.

[0028] Described herein are methods of identifying a first canola plant that displays blackleg resistance or germplasm comprised within such a canola plant. A first canola plant or germplasm that displays blackleg resistance may in some examples be a plant or germplasm comprising blackleg resistance (or improved blackleg resistance) than is observed in a parental plant or germplasm of the first plant or germplasm. A first canola plant or germplasm that displays blackleg resistance may in some examples be a plant or germplasm comprising a better blackleg resistance than is observed in a particular conventional plant or germplasm of the same species (*e.g.*, canola) as the first plant or germplasm. Some embodiments of such methods may comprise detecting in the first canola plant or germplasm at least one marker linked to blackleg resistance, wherein the at least one marker is selected from the group consisting of: DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359.

[0029] Also described are methods of producing a canola plant or germplasm having blackleg resistance. Some embodiments of such methods may comprise introgressing at least one marker linked to blackleg resistance from a first canola plant or germplasm into a second canola plant or germplasm to produce a canola plant or germplasm that is likely to have blackleg resistance. In such examples, the at least one marker may be selected from the group consisting of: DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856,

DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359. A canola plant or germplasm produced by the foregoing methods is also included in particular embodiments.

[0030] Some embodiments include methods for producing a transgenic canola plant. Examples of such methods may comprise introducing one or more exogenous nucleic acid molecule(s) into a target canola plant or progeny thereof, wherein at least one of the one or more exogenous nucleic acid molecule(s) comprises a canola genomic nucleotide sequence that is linked to at least one marker that is linked to blackleg resistance, or wherein at least one of the one or more exogenous nucleic acid molecule(s) comprises a nucleotide sequence that is specifically hybridizable to a nucleotide sequence that is linked to at least one marker that is linked to blackleg resistance. A marker that is linked to blackleg resistance may be selected from the group consisting of: DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359. In certain examples the foregoing methods for producing a transgenic canola plant, a resulting transgenic canola plant may comprise blackleg resistance.

[0031] Some embodiments include systems and kits for identifying a canola plant that is likely to comprise blackleg resistance. Particular examples of such systems and kits may comprise a set of nucleic acid probes, each comprising a nucleotide sequence that is specifically hybridizable to a nucleotide sequence that is linked in canola to at least one marker that is linked to blackleg resistance. A marker that is linked in canola to blackleg resistance may be selected from the group consisting of: DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359. Particular examples of systems and kits for identifying a canola plant that is likely to comprise blackleg resistance may also comprise a detector that is configured to detect one or more signal outputs from the set of nucleic acid probes, or an amplicon thereof, thereby identifying the presence or absence of the at least one marker that is linked to blackleg resistance. Specific examples include instructions that correlate the presence or absence of the at least one marker with the likely resistance to blackleg.

BRIEF DESCRIPTION OF THE FIGURES

[0032] **Figure 1.** Partial map of LG N7 constructed with 700 BC1F1 progeny of Nex845CL//Nex845CL/NT152343-20 genotyped with 27 SNP markers.

SEQUENCE LISTING

[0033] **SEQ ID NOs: 1 – 27** include the SNPs and flanking sequences for the 27 markers used to genotype the BC1F1 population. These sequences were used to design the KASPar™ assays.

[0034] **SEQ ID NOs: 28 – 108** are the primer sequences for the KASPar™ assays.

DETAILED DESCRIPTION

Overview of several embodiments

[0035] It is desirable for a number of reasons to grow canola having blackleg resistance. Embodiments of the invention include, for example, compositions and methods for identifying canola plants comprising a blackleg resistance and/or germplasm carrying a genotype that is predictive and determinative of a blackleg resistant phenotype. Methods of making such canola plants and germplasm are included in some embodiments. Such methods may include, for example and without limitation, introgression of desired blackleg resistance marker alleles and/or genetic transformation methods. Canola plants and/or germplasm made by the methods such as the foregoing are included in particular embodiments. Systems and kits for selecting canola plants comprising a blackleg resistance and/or germplasm carrying a genotype that is predictive and determinative of blackleg resistance are also a feature of certain embodiments.

[0036] The identification and selection of canola plants comprising a blackleg resistance using MAS are capable of providing an effective and environmentally friendly approach for generating plants with desirable disease resistance. Embodiments of the present invention provide a number of canola marker loci and QTL chromosome intervals that demonstrate statistically significant co-segregation with (and therefore are predictive and determinative of) blackleg resistance. Detection of these markers, or additional loci linked to the markers that are therefore equivalent thereto, may be used in marker-assisted canola breeding programs to produce blackleg resistant plants and germplasm.

[0037] Some embodiments provide methods for identifying a first canola plant or germplasm (*e.g.*, a line or variety) that displays blackleg resistance. In some examples, at least one allele of one or more marker locus

(*e.g.*, a plurality of marker loci) that is linked (*e.g.*, tightly-linked) with a blackleg resistance trait is/are detected in the first canola plant or germplasm. The marker loci may be selected from the loci in **Table 2** including: DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359, and other markers that are linked to at least one of the foregoing QTL markers.

[0038] In some examples, a plurality of maker loci may be selected or identified in the same plant or germplasm. All combinations of, for example, DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359 and other markers that are linked to at least one of the foregoing QTL markers, may be included in a plurality of marker loci to be selected or identified in a plant or germplasm.

[0039] In aspects of some embodiments, the resistance to blackleg of a canola plant can be quantitated using any suitable means or method known in the art.

Abbreviations

[0040] AFLP amplified fragment length polymorphism

[0041] ASH allele specific hybridization

[0042] CCD charge coupling device

[0043] EST expressed sequence tag

[0044] FAME fatty acid methyl ester

[0045] FID flame ionization detector

[0046] GC gas chromatography

[0047] LCR ligase chain reaction

[0048] LG linkage group

[0049] LNA locked nucleic acid

[0050] LOD logarithm (base 10) of odds

[0051] MAS marker-assisted selection

[0052] NASBA nucleic acid sequence based amplification

[0053] NIR near infrared (spectroscopy)

[0054] NMR nuclear magnetic resonance (spectroscopy)

[0055] ORF open reading frame

[0056] PCR polymerase chain reaction

[0057] PNA peptide nucleic acid

[0058] QTL quantitative trait locus

[0059] RAPD randomly amplified polymorphic DNA

[0060] RFLP restriction fragment length polymorphism

[0061] RT-PCR reverse transcriptase-PCR

[0062] SNP single nucleotide polymorphism

[0063] SSCP single-strand conformation polymorphism

[0064] SSR simple sequence repeat

Terms

[0065] As used in this application, including the claims, terms in the singular and the singular forms, "a," "an," and "the," for example, include plural referents, unless the content clearly dictates otherwise. Thus, for example, a reference to "plant," "the plant," or "a plant" also refers to a plurality of plants. Furthermore, depending on the context, use of the term, "plant," may also refer to genetically-similar or identical progeny of that plant. Similarly, the term, "nucleic acid," may refer to many copies of a nucleic acid molecule. Likewise, the term, "probe," may refer to many similar or identical probe molecules.

[0066] Numeric ranges are inclusive of the numbers defining the range, and include each integer and non-integer fraction within the defined range. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

[0067] In order to facilitate review of the various embodiments described in this disclosure, the following explanation of specific terms is provided:

[0068] Isolated: An "isolated" biological component (such as a nucleic acid or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs (*i.e.*, other chromosomal and extra-chromosomal DNA and RNA, and proteins), while effecting a chemical or functional change in the component (*e.g.*, a nucleic acid may be isolated from a chromosome by breaking chemical bonds connecting the nucleic acid to the remaining DNA in the

chromosome). Nucleic acid molecules and proteins that have been "isolated" include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell, as well as chemically-synthesized nucleic acid molecules, proteins, and peptides.

[0069] Mapping population: As used herein, the term "mapping population" may refer to a plant population (*e.g.*, a canola plant population) used for gene mapping. Mapping populations are typically obtained from controlled crosses of parent genotypes, as may be provided by two inbred lines. Decisions on the selection of parents, mating design for the development of a mapping population, and the type of markers used depend upon the gene to be mapped, the availability of markers, and the molecular map. The parents of plants within a mapping population should have sufficient variation for a trait(s) of interest at both the nucleic acid sequence and phenotype level. Variation of the parents' nucleic acid sequence is used to trace recombination events in the plants of the mapping population. The availability of informative polymorphic markers is dependent upon the amount of nucleic acid sequence variation. Thus, informative markers may not be identified in particular crosses of parent genotypes, though such markers may exist.

[0070] A "genetic map" is a description of genetic linkage relationships among loci on one or more chromosomes (or linkage groups) within a given species, as may be determined by analysis of a mapping population. In some examples, a genetic map may be depicted in a diagrammatic or tabular form. The term "genetic mapping" may refer to the process of defining the linkage relationships of loci through the use of genetic markers, mapping populations segregating for the markers, and standard genetic principles of recombination frequency. A "genetic map location" refers to a location on a genetic map (relative to surrounding genetic markers on the same linkage group or chromosome) where a particular marker can be found within a given species. In contrast, a "physical map of the genome" refers to absolute distances (for example, measured in base pairs or isolated and overlapping contiguous genetic fragments) between markers within a given species. A physical map of the genome does not necessarily reflect the actual recombination frequencies observed in a test cross of a species between different points on the physical map.

[0071] Cross: As used herein, the term "cross" or "crossed" refers to the fusion of gametes *via* pollination to produce progeny (*e.g.*, cells, seeds, and plants). This term encompasses both sexual crosses (*i.e.*, the pollination of one plant by another) and selfing (*i.e.*, self-pollination, for example, using pollen and ovule from the same plant).

[0072] Backcrossing: Backcrossing methods may be used to introduce a nucleic acid sequence into plants. The backcrossing technique has been widely used for decades to introduce new traits into plants. Jensen, N., Ed. Plant Breeding Methodology, John Wiley & Sons, Inc., 1988. In a typical backcross protocol, the original variety of interest (recurrent parent) is crossed to a second variety (non-recurrent parent) that carries a gene of interest to be transferred. The resulting progeny from this cross are then crossed again to the recurrent parent, and the process is repeated until a plant is obtained wherein essentially all of the desired morphological and physiological characteristics of the recurrent plant are recovered in the converted plant, in addition to the transferred gene from the non-recurrent parent.

[0073] Introgression: As used herein, the term "introgression" refers to the transmission of an allele at a genetic locus into a genetic background. In some embodiments, introgression of a specific allele form at the locus may occur by transmitting the allele form to at least one progeny *via* a sexual cross between two parents of the same species, where at least one of the parents has the specific allele form in its genome. Progeny comprising the specific allele form may be repeatedly backcrossed to a line having a desired genetic background. Backcross progeny may be selected for the specific allele form, so as to produce a new variety wherein the specific allele form has been fixed in the genetic background. In some embodiments, introgression of a specific allele form may occur by recombination between two donor genomes (*e.g.*, in a fused protoplast), where at least one of the donor genomes has the specific allele form in its genome. Introgression may involve transmission of a specific allele form that may be, for example and without limitation, a selected allele form of a marker allele; a QTL; and/or a transgene.

[0074] Germplasm: As used herein, the term "germplasm" refers to genetic material of or from an individual plant, a group of plants (*e.g.*, a plant line, variety, and family), and a clone derived from a plant or group of plants. A germplasm may be part of an organism or cell, or it may be separate (*e.g.*, isolated) from the organism or cell. In general, germplasm provides genetic material with a specific molecular makeup that is the basis for hereditary qualities of the plant. As used herein, "germplasm" refers to cells of a specific plant; seed; tissue of the specific plant (*e.g.*, tissue from which new plants may be grown); and non-seed parts of the specific plant (*e.g.*, leaf, stem, pollen, and cells).

[0075] As used herein, the term "germplasm" is synonymous with "genetic material," and it may be used to refer to seed (or other plant material) from which a plant may be propagated. A "germplasm bank" may refer to an organized collection of different seed or other genetic material (wherein each genotype is uniquely identified) from which a known cultivar may be cultivated, and from which a new cultivar may be generated. In embodiments, a germplasm utilized in a method or plant as described herein is from a canola line or variety.

In particular examples, a germplasm is seed of the canola line or variety. In particular examples, a germplasm is a nucleic acid sample from the canola line or variety.

[0076] Gene: As used herein, the term "gene" (or "genetic element") may refer to a heritable genomic DNA sequence with functional significance. The term "gene" may also be used to refer to, for example and without limitation, a cDNA and/or an mRNA encoded by a heritable genomic DNA sequence.

[0077] Genotype: As used herein, the term "genotype" refers to the genetic constitution of an individual (or group of individuals) at one or more particular loci. The genotype of an individual or group of individuals is defined and described by the allele forms at the one or more loci that the individual has inherited from its parents. The term genotype may also be used to refer to an individual's genetic constitution at a single locus, at multiple loci, or at all the loci in its genome. A "haplotype" is the genotype of an individual at a plurality of genetic loci. In some examples, the genetic loci described by a haplotype may be physically and genetically linked; *i.e.*, the loci may be positioned on the same chromosome segment.

[0078] Quantitative trait locus: Specific chromosomal loci (or intervals) may be mapped in an organism's genome that correlates with particular quantitative phenotypes. Such loci are termed quantitative trait loci, or QTL. As used herein, the term "quantitative trait locus" (QTL) may refer to stretches of DNA that have been identified as likely DNA sequences (*e.g.*, genes, non-coding sequences, and/or intergenic sequences) that underlie a quantitative trait, or phenotype, that varies in degree, and can be attributed to the interactions between two or more DNA sequences (*e.g.*, genes, non-coding sequences, and/or intergenic sequences) or their expression products and their environment. Thus, the term "quantitative trait locus" includes polymorphic genetic loci with at least two alleles that differentially affect the expression of a phenotypic trait in at least one genetic background (*e.g.*, in at least one breeding population or progeny). In practice, QTLs can be molecularly identified to help map regions of the genome that contain sequences involved in specifying a quantitative trait, such as blackleg resistance.

[0079] As used herein, the term "QTL interval" may refer to stretches of DNA that are linked to the genes that underlie the QTL trait. A QTL interval is typically, but not necessarily, larger than the QTL itself. A QTL interval may contain stretches of DNA that are 5' and/or 3' with respect to the QTL.

[0080] Multiple experimental paradigms have been developed to identify and analyze QTLs. *See*, *e.g.*, Jansen (1996) Trends Plant Sci 1:89. The majority of published reports on QTL mapping in crop species have been based on the use of a bi-parental cross (Lynch and Walsh (1997) Genetics and Analysis of Quantitative Traits,

Sinauer Associates, Sunderland). Typically, these paradigms involve crossing one or more parental pairs that can be, for example, a single pair derived from two inbred strains, or multiple related or unrelated parents of different inbred strains or lines, which each exhibit different characteristics relative to the phenotypic trait of interest. Typically, this experimental protocol involves deriving 100 to 300 segregating progeny from a single cross of two divergent inbred lines that are, for example, selected to maximize phenotypic and molecular marker differences between the lines. The parents and segregating progeny are genotyped for multiple marker loci, and evaluated for one to several quantitative traits (*e.g.*, disease resistance). QTLs are then identified as significant statistical associations between genotypic values and phenotypic variability among the segregating progeny. The strength of this experimental protocol comes from the utilization of the inbred cross, because the resulting F_1 parents all have the same linkage phase (how the alleles were joined in the parental generation). Thus, after selfing of F_1 plants, all segregating F_2 progeny are informative and linkage disequilibrium is maximized, the linkage phase is known, there are only two QTL alleles, and (except for backcross progeny) the frequency of each QTL allele is 0.5.

[0081] Numerous statistical methods for determining whether markers are genetically linked to a QTL (or to another marker) are known to those of skill in the art and include, for example and without limitation, standard linear models, such as ANOVA or regression mapping (Haley and Knott (1992) Heredity 69:315); and maximum likelihood methods, such as expectation-maximization algorithms (*e.g.*, Lander and Botstein (1989) Genetics 121:185-99; Jansen (1992) Theor. Appl. Genet. 85:252-60; Jansen (1993) Biometrics 49:227-31; Jansen (1994) "Mapping of quantitative trait loci by using genetic markers: an overview of biometrical models," In J. W. van Ooijen and J. Jansen (eds.), Biometrics in Plant breeding: applications of molecular markers, pp. 116-24, CPRO-DLO Metherlands; Jansen (1996) Genetics 142:305-11; and Jansen and Stam (1994) Genetics 136:1447-55).

[0082] Exemplary statistical methods include single point marker analysis; interval mapping (Lander and Botstein (1989) Genetics 121:185); composite interval mapping; penalized regression analysis; complex pedigree analysis; MCMC analysis; MQM analysis (Jansen (1994) Genetics 138:871); HAPLO-IM+ analysis, HAPLO-MQM analysis, and HAPLO-MQM+ analysis; Bayesian MCMC; ridge regression; identity-by-descent analysis; and Haseman-Elston regression, any of which are suitable in the context of particular embodiments of the invention. Alternative statistical methods applicable to complex breeding populations that may be used to identify and localize QTLs in particular examples are described in U.S. Patent 6,399,855 and PCT International Patent Publication No. WO0149104 A2. All of these approaches are computationally intensive and are usually performed with the assistance of a computer based system and specialized software.

Appropriate statistical packages are available from a variety of public and commercial sources, and are known to those of skill in the art.

[0083] Marker: Although specific DNA sequences that encode proteins are generally well-conserved across a species, other regions of DNA (*e.g.*, non-coding DNA and introns) tend to develop and accumulate polymorphism, and therefore, may be variable between individuals of the same species. The genomic variability can be of any origin, for example, the variability may be due to DNA insertions, deletions, duplications, repetitive DNA elements, point mutations, recombination events, and the presence and sequence of transposable elements. Such regions may contain useful molecular genetic markers. In general, any differentially inherited polymorphic trait (including nucleic acid polymorphisms) that segregates among progeny is a potential marker.

[0084] As used herein, the terms "marker" and "molecular marker" refer to a nucleotide sequence or encoded product thereof (*e.g.*, a protein) used as a point of reference when identifying a linked locus. Thus, a marker may refer to a gene or nucleotide sequence that can be used to identify plants having a particular allele. A marker may be described as a variation at a given genomic locus. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, or "SNP"), or a long one, for example, a microsatellite/simple sequence repeat ("SSR"). A "marker allele" or "marker allele form" refers to the version of the marker that is present in a particular individual. The term "marker" as used herein may refer to a cloned segment of chromosomal DNA, and may also or alternatively refer to a DNA molecule that is complementary to a cloned segment of chromosomal DNA. The term also refers to nucleic acid sequences complementary to genomic marker sequences, such as nucleic acid primers and probes.

[0085] A marker may be described, for example, as a specific polymorphic genetic element at a specific location in the genetic map of an organism. A genetic map may be a graphical representation of a genome (or a portion of a genome, such as a single chromosome) where the distances between landmarks on the chromosome are measured by the recombination frequencies between the landmarks. A genetic landmark can be any of a variety of known polymorphic markers, for example and without limitation: simple sequence repeat (SSR) markers; restriction fragment length polymorphism (RFLP) markers; and single nucleotide polymorphism (SNP) markers. As one example, SSR markers can be derived from genomic or expressed nucleic acids (*e.g.*, expressed sequence tags (ESTs)).

[0086] Additional markers include, for example and without limitation, ESTs; amplified fragment length polymorphisms (AFLPs) (Vos *et al.* (1995) Nucl. Acids Res. 23:4407; Becker *et al.* (1995) Mol. Gen. Genet. 249:65; Meksem *et al.* (1995) Mol. Gen. Genet. 249:74); randomly amplified polymorphic DNA (RAPD), and isozyme markers. Isozyme markers may be employed as genetic markers, for example, to track isozyme markers or other types of markers that are linked to a particular first marker. Isozymes are multiple forms of enzymes that differ from one another with respect to amino acid sequence (and therefore with respect to their encoding nucleic acid sequences). Some isozymes are multimeric enzymes containing slightly different subunits. Other isozymes are either multimeric or monomeric, but have been cleaved from a pro-enzyme at different sites in the pro-enzyme amino acid sequence. Isozymes may be characterized and analyzed at the protein level or at the nucleic acid level. Thus, any of the nucleic acid based methods described herein can be used to analyze isozyme markers in particular examples.

[0087] "Genetic markers" include alleles that are polymorphic in a population, where the alleles of may be detected and distinguished by one or more analytic methods (*e.g.*, RFLP analysis, AFLP analysis, isozyme marker analysis, SNP analysis, and SSR analysis). The term "genetic marker" may also refer to a genetic locus (a "marker locus") that may be used as a point of reference when identifying a genetically linked locus (*e.g.*, QTL). Such a marker may also be referred to as a "QTL marker."

[0088] The nature of the foregoing physical landmarks (and the methods used to detect them) vary, but all of these markers are physically distinguishable from each other (as well as from the plurality of alleles of any one particular marker) on the basis of polynucleotide length and/or sequence. Numerous methods for detecting molecular markers and identifying marker alleles are well-established. A wide range of protocols are known to one of skill in the art for detecting this variability, and these protocols are frequently specific for the type of polymorphism they are designed to detect. Such protocols include, for example and without limitation, PCR amplification; detection of single-strand conformation polymorphism (SSCP), *e.g.*, *via* electrophoresis; and self-sustained sequence replication (3SR) (*see* Chan and Fox (1999) Reviews in Medical Microbiology 10:185-96).

[0089] The primary motivation for developing molecular marker technologies from the perspective of plant breeders has been to increase breeding efficiency through MAS. A molecular marker allele that demonstrates linkage disequilibrium with a desired phenotypic trait (*e.g.*, a QTL) provides a useful tool for the selection of the desired trait in a plant population. The key components to the implementation of an MAS approach are the creation of a dense (information rich) genetic map of molecular markers in the plant germplasm; the detection of at least one QTL based on statistical associations between marker and phenotypic variability; the definition

of a set of particular useful marker alleles based on the results of the QTL analysis; and the use and/or extrapolation of this information to the current set of breeding germplasm to enable marker-based selection decisions to be made.

[0090] Genetic variability, for example as determined in a mapping population, may be observed between different populations of the same species (*e.g.*, canola). In spite of the variability in the genetic map that may occur between populations of the same species, genetic map and marker information derived from one population generally remains useful across multiple populations for the purposes of identification and/or selection of plants and/or germplasm comprising traits that are linked to the markers and counter-selection of plants and/or germplasm comprising undesirable traits.

[0091] Two types of markers used in particular MAS protocols described herein are SSR and SNP markers. SSR markers include any type of molecular heterogeneity that results in nucleic acid sequence length variability. Exemplary SSR markers are short (up to several hundred base pairs) segments of DNA that consist of multiple tandem repeats of a short (i.e., between two and ten) base-pair sequence. The repeated sequences, therefore, could be two, three, four, five, six, seven, eight, nine, or ten base pairs long. These repeated sequences result in highly polymorphic DNA regions of variable length due to poor replication fidelity (*e.g.*, by polymerase slippage). SSRs appear to be randomly dispersed through the genome, and are generally flanked by conserved regions. SSR markers may also be derived from genic sequences (in the form of a cDNA, a partial cDNA, or an EST), as well as non-genic material.

[0092] The heterogeneity of SSR markers make them well-suited for use as molecular genetic markers. For example, SSR genomic variability is inherited, and it is multi-allelic, co-dominant, and reproducibly detectable. The proliferation of increasingly sophisticated amplification-based detection techniques (*e.g.*, PCR-based techniques) provides a variety of sensitive methods for the detection of nucleotide sequence heterogeneity between samples. Probes (*e.g.*, nucleic acid primers) may be designed to hybridize to conserved regions that flank the SSR, and the probes may be used to amplify the variable SSR region. The differently sized amplicons generated from an SSR region have characteristic and reproducible sizes. Differently sized SSR amplicons observed from two homologous chromosomes from an individual, or from different individuals, in the plant population define SSR marker alleles. As long as there exist at least two SSR marker alleles that produce PCR products with different sizes, the SSR may be employed as a marker.

[0093] Linkage (dis)equilibrium: As used herein, the term "linkage equilibrium" refers to the situation where two markers independently segregate; *i.e.*, the markers sort randomly among progeny. Markers that show

linkage equilibrium are considered unlinked (whether or not they lie on the same chromosome). As used herein, the term "linkage disequilibrium" refers to the situation where two markers segregate in a non-random manner; *i.e.*, the markers have a recombination frequency of less than 50% (and thus by definition, are separated by less than 50 cM on the same linkage group). In some examples, markers that show linkage disequilibrium are considered linked.

[0094] Linked, tightly linked, and extremely tightly linked: As used herein, linkage between genes or markers may refer to the phenomenon in which genes or markers on a chromosome show a measurable probability of being passed on together to individuals in the next generation. Thus, linkage of one marker to another marker or gene may be measured and/or expressed as a recombination frequency. The closer two genes or markers are to each other, the closer to "1" this probability becomes. Thus, the term "linked" may refer to one or more genes or markers that are passed together with a gene with a probability greater than 0.5 (which is expected from independent assortment where markers/genes are located on different chromosomes). When the presence of a gene contributes to a phenotype in an individual, markers that are linked to the gene may be said to be linked to the phenotype. Thus, the term "linked" may refer to a relationship between a marker and a gene, or between a marker and a phenotype.

[0095] A relative genetic distance (determined by crossing over frequencies and measured in centimorgans (cM)) is generally proportional to the physical distance (measured in base pairs) that two linked markers or genes are separated from each other on a chromosome. One centimorgan is defined as the distance between two genetic markers that show a 1% recombination frequency (*i.e.*, a crossing-over event occurs between the two markers once in every 100 cell divisions). In general, the closer one marker is to another marker or gene (whether the distance between them is measured in terms of genetic distance or physical distance), the more tightly they are linked. Because chromosomal distance is approximately proportional to the frequency of recombination events between traits, there is an approximate physical distance that correlates with recombination frequency. For example, in canola, 1 cM correlates, on average, to about 400 kb.

[0096] Thus, the term "linked" may refer herein to one or more genes or markers that are physically located within about 4.0 Mb of one another on the same canola chromosome (*i.e.*, about 10 cM). Thus, two "linked" genes or markers may be separated by 4.1 Mb; about 4.0 Mb; about 3.0 Mb; about 2.5 Mb; 2.1 Mb; 2.00 Mb; about 1.95 Mb; about 1.90 Mb; about 1.85 Mb; about 1.80 Mb; about 1.75 Mb; about 1.70 Mb; about 1.65 Mb; about 1.60 Mb; about 1.55 Mb; about 1.50 Mb; about 1.45 Mb; about 1.40 Mb; about 1.35 Mb; about 1.30 Mb; about 1.25 Mb; about 1.20 Mb; about 1.15 Mb; about 1.10 Mb; about 1.05 Mb; about 1.00 Mb; about 0.95 Mb; about 0.90 Mb; about 0.85 Mb; about 0.80 Mb; about 0.75 Mb; about 0.70 Mb; about 0.65 Mb; about 0.60 Mb;

about 0.55 Mb; about 0.50 Mb; about 0.45 Mb; about 0.40 Mb; about 0.35 Mb; about 0.30 Mb; about 0.25 Mb; about 0.20 Mb; about 0.15 Mb; about 0.10 Mb; about 0.05 Mb; about 0.025 Mb; and about 0.01 Mb.

[0097] As used herein, the term "tightly-linked" may refer to one or more genes or markers that are located within about 2.0 Mb of one another on the same chromosome. Thus, two "tightly-linked" genes or markers may be separated by 2.1 Mb; about 1.75 Mb; about 1.5 Mb; about 1.0 Mb; about 0.9 Mb; about 0.8 Mb; about 0.7 Mb; about 0.6 Mb; about 0.55 Mb; 0.5 Mb; about 0.45 Mb; about 0.4 Mb; about 0.35 Mb; about 0.3 Mb; about 0.25 Mb; about 0.2 Mb; about 0.15 Mb; about 0.1 Mb; and about 0.05 Mb.

[0098] As used herein, the term "extremely tightly-linked" may refer to one or more genes or markers that are located within about 500 kb of one another on the same chromosome. Thus, two "extremely tightly-linked" genes or markers may be separated by 600 kb; about 450 kb; about 400 kb; about 350 kb; about 300 kb; about 250 kb; about 200 kb; about 175 kb; about 150 kb; about 125 kb; about 120 kb; about 115 kb; about 110 kb; about 105 kb; 100 kb; about 95 kb; about 90 kb; about 85 kb; about 80 kb; about 75 kb; about 70 kb; about 65 kb; about 60 kb; about 55 kb; about 50 kb; about 45 kb; about 40 kb; about 35 kb; about 30 kb; about 25 kb; about 20 kb; about 15 kb; about 10 kb; about 5 kb; and about 1 kb.

[0099] The closer a particular marker is to a gene that encodes a polypeptide that contributes to a particular phenotype (whether measured in terms of genetic or physical distance), the more tightly-linked is the particular marker to the phenotype. In view of the foregoing, it will be appreciated that markers linked to a particular gene or phenotype include those markers that are tightly linked, and those markers that are extremely tightly linked, to the gene or phenotype. In some embodiments, the closer a particular marker is to a gene that encodes a polypeptide that contributes to a blackleg resistance phenotype (whether measured in terms of genetic or physical distance), the more tightly-linked is the particular marker to the blackleg resistance phenotype. Thus, linked, tightly linked, and extremely tightly genetic markers of a blackleg resistance phenotype in canola may be useful in MAS programs to identify canola varieties comprising improved blackleg resistance (when compared to parental varieties and/or at least one particular conventional variety), to identify individual canola plants comprising blackleg resistance (or improved blackleg resistance), and to breed this trait into other canola varieties to introduce or improve blackleg resistance.

[00100] In some embodiments, the linkage relationship between a molecular marker and a phenotype may be expressed as a "probability" or "adjusted probability." Within this context, a probability value is the statistical likelihood that a particular combination of a phenotype and the presence or absence of a particular marker allele form is random. Thus, the lower the probability score, the greater the likelihood that the phenotype and the

particular marker allele form will co-segregate. In some examples, the probability score may be described as "significant" or "non-significant." In particular examples, a probability score of 0.05 (p = 0.05 (a 5% probability)) of random assortment is considered a "significant" indication of co-segregation. However, a significant probability may in other examples be any probability of less than 50% (p = 0.5). For instance, a significant probability may be less than 0.25; less than 0.20; less than 0.15; or less than 0.1.

[00101] In some embodiments, a marker that is linked to a blackleg resistance phenotype may be selected from the QTL markers of canola linkage group N7 that are illustrated in **Figure 1**. In some embodiments, a marker that is linked to a blackleg resistance phenotype may be selected from those markers that are located within about 10 cM of a QTL marker illustrated in **Figure 1**. Thus, marker that is linked to a blackleg resistance phenotype may be, for example, within 10 cM; 9 cM; 8 cM; 7 cM; 6 cM; 5 cM; 4 cM; 3 cM; 2 cM; 1 cM; 0.75 cM; 0.5 cM; 0.25 cM; or less, from a QTL marker illustrated in **Figure 1**.

[00102] A plant breeder can advantageously use molecular markers to identify desired individuals by identifying marker alleles that show a statistically significant probability of co-segregation with a desired phenotype (*e.g.*, blackleg resistance), manifested as linkage disequilibrium. By identifying a molecular marker or clusters of molecular markers that co-segregate with a quantitative trait, the breeder is thus identifying a QTL. By identifying and selecting a marker allele (or desired alleles from multiple markers) that associates with the desired phenotype, the plant breeder is able to rapidly select the phenotype by selecting for the proper molecular marker allele (*i.e.*, MAS). The more molecular markers that are placed on the genetic map, the more potentially useful that map becomes for conducting MAS.

[00103] Marker set: As used herein, a "set" of markers or probes refers to a specific collection of markers or probes (or data derived therefrom) that may be used to identify individuals comprising a trait of interest. In some embodiments, a set of markers linked to the blackleg resistance phenotype may be used to identify canola plants comprising blackleg resistance. Data corresponding to a marker set or probe set (or data derived from the use of such markers or probes) may be stored in an electronic medium. While each marker in a marker set may possess utility with respect to trait identification, individual markers selected from the set and subsets including some, but not all, of the markers may also be effective in identifying individuals comprising the trait of interest.

[00104] Allele: As used herein, the term "allele" refers to one of two or more different nucleotide sequences that occur at a specific locus. For example, a first allele may occur on one chromosome, while a second allele may occur on a second homologous chromosome; *e.g.*, as occurs for different chromosomes of a heterozygous

individual, or between different homozygous or heterozygous individuals in a population. In some embodiments, a particular allele at a particular locus may be linked to an agronomically desirable phenotype (*e.g.*, blackleg resistance). In some embodiments, a particular allele at the locus may allow the identification of plants that do not comprise the agronomically desirable phenotype (*e.g.*, blackleg susceptibility) such that those plants may be removed from a breeding program or planting. A marker allele may segregate with a favorable phenotype, therefore providing the benefit of identifying plants comprising the phenotype. An "allelic form of a chromosome segment" may refer to a chromosome segment that comprises a marker allele nucleotide sequence that contributes to, or is linked to, a particular phenotype at one or more genetic loci physically located on the chromosome segment.

[00105] "Allele frequency" may refer to the frequency (expressed as a proportion or percentage) at which an allele is present at a locus within a plant, within a line, or within a population of lines. Thus, for an allele "A," a diploid individual of genotype "AA," "Aa," or "aa," has an allele frequency of 1.0, 0.5, or 0.0, respectively. The allele frequency within a line may be estimated by averaging the allele frequencies of a sample of individuals from that line. Similarly, the allele frequency within a population of lines may be calculated by averaging the allele frequencies of lines that make up the population. For a population with a finite number of individuals or lines, an allele frequency may be expressed as a count of individuals or lines (or any other specified grouping) containing the allele.

[00106] A marker allele "positively" correlates with a trait when the marker is linked to the trait, and when presence of the marker allele is an indicator that the desired trait or trait form will occur in a plant comprising the allele. A marker allele "negatively" correlates with a trait when the marker is linked to the trait, and when presence of the marker allele is an indicator that the desired trait or trait form will not occur in a plant comprising the allele.

[00107] A "homozygous" individual has only one form of allele at a given locus (*e.g.*, a diploid plant has a copy of the same allele form at a particular locus for each of two homologous chromosomes). An individual is "heterozygous" if more than one allele form is present at the locus (*e.g.*, a diploid individual has one copy of a first allele form and one copy of a second allele form at the locus). The term "homogeneity" refers to members of a group that have the same genotype (*i.e.*, the same allele frequency) at one or more specific loci of interest. In contrast, the term "heterogeneity" refers to individuals within a group that differ in genotype at one or more specific loci of interest.

[00108] Any technique that may be used to characterize the nucleotide sequence at a locus may be used to identify a marker allele. Methods for marker allele detection include, for example and without limitation, molecular identification methods (*e.g.*, amplification and detection of a marker amplicon). For example, an allelic form of an SSR marker, or of a SNP marker, may be detected by an amplification based technology. In a typical amplification-based detection method, a marker locus or a portion of the marker locus is amplified (using, *e.g.*, PCR, LCR, and transcription using a nucleic acid isolated from a canola plant of interest as an amplification template), and the resulting amplified marker amplicon is detected. In some embodiments, plant RNA may be utilized as the template for an amplification reaction. In some embodiments, plant genomic DNA may be utilized as the template for the amplification reaction. In some examples, the QTL marker is an SNP marker, and the detected allele is a SNP marker allele, and the method of detection is allele specific hybridization (ASH). In some examples, the QTL marker is an SSR marker, and the detected allele is an SSR marker allele.

[00109] ASH technology is based on the stable annealing of a short, single-stranded, oligonucleotide probe to a completely complementary single-strand target nucleic acid. Detection may be accomplished *via* detection of an isotopic or non-isotopic label attached to the probe. For each polymorphism, two or more different ASH probes may be designed to have identical DNA sequences, except at site of a polymorphism. Each probe may be perfectly homologous with one allele sequence, so that the range of probes can distinguish all the known alternative allele sequences. When each probe is hybridized to target DNA under appropriate probe design and hybridization conditions, a single-base mismatch between the probe and target DNA prevents hybridization. In this manner, only one of the alternative probes will hybridize to a target sample that is homozygous for an allele. Samples that are heterozygous or heterogeneous for two alleles will hybridize to both of two alternative probes.

[00110] ASH markers may be used as dominant markers, where the presence or absence of only one allele is determined from hybridization or lack of hybridization by only one probe. The alternative allele may be inferred from a lack of hybridization. In examples, ASH probe and target molecules may be RNA or DNA molecules; a target molecule may comprise any length of nucleotides beyond the sequence that is complementary to the probe; the probe may be designed to hybridize with either strand of a DNA target; and the size of the probe may be varied to conform with the requirements of different hybridization conditions.

[00111] Amplified variable sequences refer to amplified sequences of the plant genome that exhibit high nucleic acid residue variability between members of the same species. All organisms have variable genomic sequences, and each organism (with the exception of a clone) has a different set of variable sequences. Once

identified, the presence of specific variable sequence can be used to predict phenotypic traits. DNA from a plant may in some examples be used as a template for amplification with primers that flank a variable sequence of DNA. The variable sequence may be amplified and then sequenced.

[00112] Self-sustained sequence replication may also and alternatively be used to identify genetic markers. Self-sustained sequence replication refers to a method of nucleic acid amplification using target nucleic acid sequences that are replicated exponentially *in vitro* under substantially isothermal conditions, using three enzymatic activities involved in retroviral replication: reverse transcriptase; RNAse H; and a DNA-dependent RNA polymerase. Guatelli *et al.* (1990) Proc. Natl. Acad. Sci. USA 87:1874. By mimicking the retroviral strategy of RNA replication by means of cDNA intermediates, this reaction accumulates cDNA and RNA copies of the original target.

[00113] Data representing detected marker allele(s) may be transmitted (for example, electronically; and *via* infrared, wireless, or optical transmission) to a computer or computer-readable medium for analysis or storage.

[00114] For example, an amplification primer or amplification primer pair may be admixed with a genomic nucleic acid isolated from a first canola plant or germplasm, wherein the primer or primer pair is complementary or partially complementary to at least a portion of a marker locus, and the primer or primer pair is capable of initiating DNA polymerization by a DNA polymerase using the canola genomic nucleic acid as a template. The primer or primer pair (*e.g.*, a primer pair provided in **Table 3** is extended in a DNA polymerization reaction utilizing a DNA polymerase and a template genomic nucleic acid to generate at least one amplicon.

[00115] "Positional cloning" refers to a particular cloning procedure in which a target nucleic acid is identified and isolated by its genomic proximity to a marker. For example, a genomic nucleic acid clone may include all or part of two more chromosomal regions that are proximal to one another. If a marker can be used to identify the genomic nucleic acid clone from a genomic library, standard methods such as sub-cloning and/or sequencing may be used to identify and or isolate sub-sequences of the clone that are located near the marker.

[00116] Locus: As used herein, the term "locus" refers to a position on the genome that corresponds to a measurable characteristic (*e.g.*, a trait) or polymorphism. An SNP locus is defined by a probe that hybridizes to DNA contained within the locus.

[00117] Marker-assisted breeding: As used herein, the term "marker-assisted breeding" may refer to an approach to breeding directly utilizing MAS for one or more traits (*e.g.*, improved blackleg resistance). In current practice, plant breeders attempt to identify easily detectable traits, such as flower color, seed coat appearance, or isozyme variants that are linked to an agronomically desired trait. The plant breeders then follow the agronomic trait in the segregating, breeding populations by following the segregation of the easily detectable trait. However, there are very few of these linkage relationships available for use in plant breeding.

[00118] Marker-assisted breeding provides a time- and cost-efficient process for improvement of plant varieties. Several examples of the application of marker-assisted breeding involve the use of isozyme markers. *See*, *e.g.*, Tanksley and Orton, eds. (1983) Isozymes in Plant Breeding and Genetics, Amsterdam: Elsevier. One example is an isozyme marker associated with a gene for resistance to a nematode pest in tomato. The resistance, controlled by a gene designated *Mi*, is located on chromosome 6 of tomato and is very tightly linked to Aps1, an acid phosphatase isozyme. Use of the *Aps1* isozyme marker to indirectly select for the *Mi* gene provided the advantages that segregation in a population can be determined unequivocally with standard electrophoretic techniques; the isozyme marker can be scored in seedling tissue, obviating the need to maintain plants to maturity; and co-dominance of the isozyme marker alleles allows discrimination between homozygotes and heterozygotes. *See* Rick (1983) in Tanksley and Orton, *supra*.

[00119] Probe: In some embodiments, the presence of a marker in a plant may be detected through the use of a nucleic acid probe. A probe may be a DNA molecule or an RNA molecule. RNA probes can be synthesized by means known in the art, for example, using a DNA molecule template. A probe may contain all or a portion of the nucleotide sequence of the marker and additional, contiguous nucleotide sequence from the plant genome. This is referred to herein as a "contiguous probe." The additional, contiguous nucleotide sequence is referred to as "upstream" or "downstream" of the original marker, depending on whether the contiguous nucleotide sequence from the plant chromosome is on the 5' or the 3' side of the original marker, as conventionally understood. As is recognized by those of ordinary skill in the art, the process of obtaining additional, contiguous nucleotide sequence for inclusion in a marker may be repeated nearly indefinitely (limited only by the length of the chromosome), thereby identifying additional markers along the chromosome.

[00120] An oligonucleotide probe sequence may be prepared synthetically or by cloning. Suitable cloning vectors are well-known to those of skill in the art. An oligonucleotide probe may be labeled or unlabeled. A wide variety of techniques exist for labeling nucleic acid molecules, including, for example and without limitation: radiolabeling by nick translation; random priming; tailing with terminal deoxytransferase; or the like, where the nucleotides employed are labeled, for example, with radioactive ^{32}P. Other labels which may

be used include, for example and without limitation: Fluorophores (*e.g.*, FAM and VIC); enzymes; enzyme substrates; enzyme cofactors; enzyme inhibitors; and the like. Alternatively, the use of a label that provides a detectable signal, by itself or in conjunction with other reactive agents, may be replaced by ligands to which receptors bind, where the receptors are labeled (for example, by the above-indicated labels) to provide detectable signals, either by themselves, or in conjunction with other reagents. *See*, *e.g.*, Leary *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:4045-9.

[00121] A probe may contain a nucleotide sequence that is not contiguous to that of the original marker; this probe is referred to herein as a "noncontiguous probe." The sequence of the noncontiguous probe is located sufficiently close to the sequence of the original marker on the genome so that the noncontiguous probe is genetically linked to the same gene or trait (*e.g.*, blackleg resistance). For example, in some embodiments, a noncontiguous probe is located within about 10 cM; 9 cM; 8 cM; 7 cM; 6 cM; 5 cM; 4 cM; 3 cM; 2 cM; 1 cM; 0.75 cM; 0.5 cM; 0.25 cM; or less, from a QTL marker illustrated in **Figure 1**

[00122] A probe may be an exact copy of a marker to be detected. A probe may also be a nucleic acid molecule comprising, or consisting of, a nucleotide sequence which is substantially identical to a cloned segment of the subject organism's (*e.g.*, canola) chromosomal DNA. As used herein, the term "substantially identical" may refer to nucleotide sequences that are more than 85% identical. For example, a substantially identical nucleotide sequence may be 85.5%; 86%; 87%; 88%; 89%; 90%; 91%; 92%; 93%; 94%; 95%; 96%; 97%; 98%; 99% or 99.5% identical to a reference sequence.

[00123] A probe may also be a nucleic acid molecule that is "specifically hybridizable" or "specifically complementary" to an exact copy of the marker to be detected ("DNA target"). "Specifically hybridizable" and "specifically complementary" are terms that indicate a sufficient degree of complementarity such that stable and specific binding occurs between the nucleic acid molecule and the DNA target. A nucleic acid molecule need not be 100% complementary to its target sequence to be specifically hybridizable. A nucleic acid molecule is specifically hybridizable when there is a sufficient degree of complementarity to avoid non-specific binding of the nucleic acid to non-target sequences under conditions where specific binding is desired, for example, under stringent hybridization conditions.

[00124] Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ and/or Mg^{++} concentration) of the hybridization buffer will determine the stringency of hybridization, though wash times

also influence stringency. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are known to those of ordinary skill in the art, and are discussed, for example, in Sambrook *et al.* (ed.) Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11; and Hames and Higgins (eds.) Nucleic Acid Hybridization, IRL Press, Oxford, 1985. Further detailed instruction and guidance with regard to the hybridization of nucleic acids may be found, for example, in Tijssen, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," in Laboratory Techniques in Biochemistry and Molecular Biology- Hybridization with Nucleic Acid Probes, Part I, Chapter 2, Elsevier, NY, 1993; and Ausubel *et al.*, Eds., Current Protocols in Molecular Biology, Chapter 2, Greene Publishing and Wiley-Interscience, NY, 1995.

[00125] As used herein, "stringent conditions" encompass conditions under which hybridization will only occur if there is less than 50% mismatch between the hybridization molecule and the DNA target. "Stringent conditions" include further particular levels of stringency. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 50% sequence mismatch will not hybridize; conditions of "high stringency" are those under which sequences with more than 20% mismatch will not hybridize; and conditions of "very high stringency" are those under which sequences with more than 10% mismatch will not hybridize.

[00126] The following are representative, non-limiting hybridization conditions.

[00127] Very High Stringency (detects sequences that share at least 90% sequence identity): Hybridization in 5x SSC buffer at 65 °C for 16 hours; wash twice in 2x SSC buffer at room temperature for 15 minutes each; and wash twice in 0.5x SSC buffer at 65 °C for 20 minutes each.

[00128] High Stringency (detects sequences that share at least 80% sequence identity): Hybridization in 5x-6x SSC buffer at 65-70 °C for 16-20 hours; wash twice in 2x SSC buffer at room temperature for 5-20 minutes each; and wash twice in 1x SSC buffer at 55-70 °C for 30 minutes each.

[00129] Moderate Stringency (detects sequences that share at least 50% sequence identity): Hybridization in 6x SSC buffer at room temperature to 55 °C for 16-20 hours; wash at least twice in 2x-3x SSC buffer at room temperature to 55 °C for 20-30 minutes each.

[00130] With respect to all probes discussed, *supra*, the probe may comprise additional nucleic acid sequences, for example, promoters; transcription signals; and/or vector sequences. Any of the probes discussed, *supra*,

may be used to define additional markers that are linked to a gene involved blackleg resistance in canola, and markers thus identified may be equivalent to exemplary markers named in the present disclosure, and thus are within the scope of the invention.

[00131] Sequence identity: The term "sequence identity" or "identity," as used herein in the context of two nucleic acid or polypeptide sequences, may refer to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

[00132] As used herein, the term "percentage of sequence identity" may refer to the value determined by comparing two optimally aligned sequences (*e.g.*, nucleic acid sequences) over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleotide or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window, and multiplying the result by 100 to yield the percentage of sequence identity.

[00133] Methods for aligning sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in, for example: Smith and Waterman (1981) Adv. Appl. Math. 2:482; Needleman and Wunsch (1970) J. Mol. Biol. 48:443; Pearson and Lipman (1988) Proc. Natl. Acad. Sci. U.S.A. 85:2444; Higgins and Sharp (1988) Gene 73:237-44; Higgins and Sharp (1989) CABIOS 5:151-3; Corpet *et al.* (1988) Nucleic Acids Res. 16:10881-90; Huang *et al.* (1992) Comp. Appl. Biosci. 8:155-65; Pearson *et al.* (1994) Methods Mol. Biol. 24:307-31; Tatiana *et al.* (1999) FEMS Microbiol. Lett. 174:247-50. A detailed consideration of sequence alignment methods and homology calculations can be found in, *e.g.*, Altschul *et al.* (1990) J. Mol. Biol. 215:403-10.

[00134] The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST™; Altschul *et al.* (1990)) is available from several sources, including the National Center for Biotechnology Information (Bethesda, MD), and on the internet, for use in connection with several sequence analysis programs. A description of how to determine sequence identity using this program is available on the internet under the "help" section for BLAST™. For comparisons of nucleic acid sequences, the "Blast 2 sequences" function of the BLAST™ (Blastn) program may be employed using the default BLOSUM62 matrix set to default parameters. Nucleic acid sequences with even greater similarity to the reference sequences will show increasing percentage identity when assessed by this method.

[00135] Nucleic acid molecule: As used herein, the term "nucleic acid molecule" may refer to a polymeric form of nucleotides, which may include both sense and anti-sense strands of RNA, cDNA, genomic DNA, and synthetic forms and mixed polymers of the above. A nucleotide may refer to a ribonucleotide, deoxyribonucleotide, or a modified form of either type of nucleotide. A "nucleic acid molecule" as used herein is synonymous with "nucleic acid" and "polynucleotide." The term includes single- and double-stranded forms of DNA. A nucleic acid molecule can include either or both naturally occurring and modified nucleotides linked together by naturally occurring and/or non-naturally occurring nucleotide linkages.

[00136] An "exogenous" molecule is a molecule that is not native to a specified system (*e.g.*, a germplasm, variety, elite variety, and/or plant) with respect to nucleotide sequence and /or genomic location for a polynucleotide, and with respect to amino acid sequence and/or cellular localization for a polypeptide. In embodiments, exogenous or heterologous polynucleotides or polypeptides may be molecules that have been artificially supplied to a biological system (*e.g.*, a plant cell, a plant gene, a particular plant species or variety, and/or a plant chromosome) and are not native to that particular biological system. Thus, the designation of a nucleic acid as "exogenous" may indicate that the nucleic acid originated from a source other than a naturally-occurring source, or it may indicate that the nucleic acid has a non-natural configuration, genetic location, or arrangement of elements.

[00137] In contrast, for example, a "native" or "endogenous" nucleic acid is a nucleic acid (*e.g.*, a gene) that does not contain a nucleic acid element other than those normally present in the chromosome or other genetic material on which the nucleic acid is normally found in nature. An endogenous gene transcript is encoded by a nucleotide sequence at its natural chromosomal locus, and is not artificially supplied to the cell.

[00138] The term "recombinant" refers to a material (*e.g.*, recombinant nucleic acid, recombinant gene, recombinant polynucleotide, and/or recombinant polypeptide) that has been altered by human intervention. For example, the arrangement of the parts or elements of a recombinant molecule may not be a native arrangement, and/or the primary sequence of the recombinant molecule may been changed from its native sequence in some way. A material may be altered to produce a recombinant material within or removed from its natural environment or state. An open reading frame of a nucleic acid is recombinant if the nucleotide sequence of the open reading frame has been removed from it natural context and cloned into any type of artificial nucleic acid (*e.g.*, a vector). Protocols and reagents to produce recombinant molecules, especially recombinant nucleic acids, are common and routine in the art. The term "recombinant" may also herein refer to a cell or organism

that comprises recombinant material (*e.g.*, a plant and/or plant cell that comprises a recombinant nucleic acid). In some examples, a recombinant organism is a transgenic organism.

[00139] As used herein, the term "introduced," when referring to translocation of a heterologous or exogenous nucleic acid into a cell, refers to the incorporation of the nucleic acid into the cell using any methodology available in the art. This term encompasses nucleic acid introduction methods including, for example and without limitation, transfection; transformation; and transduction.

[00140] As used herein, the term "vector" refers to a polynucleotide or other molecules that is capable of transferring at least one nucleic acid segment(s) into a cell. A vector may optionally comprise components/elements that mediate vector maintenance and enable its intended use (*e.g.*, sequences necessary for replication, genes imparting drug or antibiotic resistance, a multiple cloning site, and/or operably-linked promoter/enhancer elements that enable the expression of a cloned gene). Vectors may be derived, for example, from plasmids, bacteriophages, or plant or animal viruses. A "cloning vector," "shuttle vector," or "subcloning vector" generally comprises operably-linked elements to facilitate cloning or subcloning steps (*e.g.*, a multiple cloning site containing multiple restriction endonuclease sites).

[00141] The term "expression vector," as used herein, refers to a vector comprising operably-linked polynucleotide sequences that may facilitate expression of a coding sequence in a particular host organism. For example, a bacterial expression vector may facilitate expression of a coding sequence in a bacterium. A plant expression vector may facilitate expression of a coding sequence in a plant cell. Polynucleotide sequences that facilitate expression in prokaryotes may include, for example and without limitation, a promoter; an operator; and a ribosome binding site. Eukaryotic expression vectors (*e.g.*, a plant expression vector) comprise promoters, enhancers, termination, and polyadenylation signals (and other sequences) that are generally different from those used in prokaryotic expression vectors.

[00142] Single-nucleotide polymorphism: As used herein, the term "single-nucleotide polymorphism" (SNP) may refer to a DNA sequence variation occurring when a single nucleotide in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual. Within a population, SNPs can be assigned a minor allele frequency that is the lowest allele frequency at a locus that is observed in a particular population. This is simply the lesser of the two allele frequencies for single-nucleotide polymorphisms. Different populations are expected to exhibit at least slightly different allele frequencies. Particular populations may exhibit significantly different allele frequencies. In some examples, markers linked to SCN resistance are SNP markers.

[00143] SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. An SNP in which both forms lead to the same polypeptide sequence is termed "synonymous" (sometimes called a silent mutation). If a different polypeptide sequence is produced, they are termed "non-synonymous." A non-synonymous change may either be missense or nonsense, where a missense change results in a different amino acid, and a nonsense change results in a premature stop codon. SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA. SNPs are usually biallelic and thus easily assayed in plants and animals. Sachidanandam (2001) Nature 409:928-33.

[00144] Plant: As used herein, the term "plant" may refer to a whole plant, a cell or tissue culture derived from a plant, and/or any part of any of the foregoing. Thus, the term "plant" encompasses, for example and without limitation, whole plants; plant components and/or organs (*e.g.*, leaves, stems, and roots); plant tissue; seed; and a plant cell. A plant cell may be, for example and without limitation, a cell in and/or of a plant, a cell isolated from a plant, and a cell obtained through culturing of a cell isolated from a plant. Thus, the term "canola plant" may refer to, for example and without limitation, a whole canola plant; multiple canola plants; canola plant cell(s); canola plant protoplast; canola tissue culture (*e.g.*, from which a canola plant can be regenerated); canola plant callus; canola plant parts (*e.g.*, canola seed, canola flower, canola cotyledon, canola leaf, canola stem, canola bud, canola root, and canola root tip); and canola plant cells that are intact in canola plants or in parts of canola plants.

[00145] A "transgenic plant" is a plant comprising within at least one of its cells an exogenous polynucleotide. In examples, the exogenous polynucleotide is stably-integrated within the genome of the cell, such that the polynucleotide may be inherited in successive generations. In some examples, the heterologous polynucleotide may be integrated into the genome as part of a recombinant expression cassette. The term "transgenic" is used herein to refer to any cell, cell line, callus, tissue, plant part, or plant, the genotype of which has been altered by the presence of a exogenous nucleic acid. Thus, this term encompasses transgenic organisms and cells that have been initially altered to comprise the exogenous polynucleotide, and those organisms and cells created by crosses or asexual propagation of the initial transgenic organism or cell. The term "transgenic," as used herein, does not encompass genome (chromosomal or extra-chromosomal) alternations introduced by conventional plant breeding methods (*e.g.*, crosses of only non-transgenic organisms) or by naturally-occurring events (*e.g.*, random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, and spontaneous mutation).

[00146] A plant "line," "variety," or "strain" is a group of individual plants having the same parentage. Plants of a line generally are inbred to some degree, and are generally homozygous and homogeneous at most genetic loci. A "subline" may refer to an inbred subset of descendants from a common progenitor that are genetically distinct from other similarly inbred subsets descended from the same progenitor. In some embodiments, a "subline" may be produced by inbreeding seed from an individual canola plant selected at the F_3 to F_5 generation until the residual segregating loci are homozygous across most or all loci.

[00147] Commercial canola varieties are typically produced by aggregating the self-pollinated progeny ("bulking") of a single F_3 to F_5 plant from a controlled cross between 2 genetically different parents. While such a variety typically appears uniform, a self-pollinating variety derived from the selected plant eventually (for example, by the F_8 generation) becomes a mixture of homozygous plants that may vary in genotype at any locus that was heterozygous in the originally selected F_3 to F_5 plant. In embodiments described herein, marker-based sublines that differ from each other based on qualitative marker polymorphism at the DNA level at one or more specific loci, are produced by genotyping a sample of seed derived from individual self-pollinated progeny derived from a selected F_3 to F_5 plant. Such a seed sample may be genotyped directly as seed, or as plant tissue grown from seed. In some examples, seed sharing a common genotype at one or more specified marker locus are bulked to produce a subline that is genetically homogenous at one or more locus that is linked to a trait of interest (*e.g.*, blackleg resistance).

[00148] An "ancestral line" refers to a parent line that is or has been used as a source of genetic material, for example, for the development of elite lines. An "ancestral population" refers to a group of ancestors that have contributed the bulk of the genetic variation that was used to develop an elite line. "Descendants" are progeny of ancestors, and descendants may be separated from their ancestors by many generations of breeding. For example, elite lines are the descendants of their ancestors. A pedigree may be used to describe the relationship between a descendant and each of its ancestors. A pedigree may span one or more generations, and thus may describe relationships between a descendant and its ancestors removed by 1, 2, 3, 4, etc. generations.

[00149] An "elite line" or "elite strain" refers to an agronomically superior line that has been bred and selected (often through many cycles) for superior agronomic performance. Numerous elite canola lines are available and known to those of skill in the art. An elite population is an assortment of elite lines or individuals from elite lines that may be used to represent the state of the art in terms of the available agronomically superior genotypes of a given crop species (*e.g.*, canola). Similarly, an elite germplasm or elite strain of germplasm is an agronomically superior germplasm. An elite germplasm may be obtained from a plant with superior

agronomic performance, and may capable of being used to generate a plant with superior agronomic performance, such as a canola of an existing or newly-developed elite line.

[00150] In contrast to elite lines, an "exotic line" or "exotic strain" (or an "exotic germplasm") refers to a line or germplasm obtained from a canola not belonging to an available elite canola line or strain of germplasm. In the context of a cross between two canola plants or germplasms, an exotic germplasm is not closely related by descent to the elite germplasm with which it is crossed. Most commonly, exotic germplasm has been selected to introduce a novel genetic element (*e.g.*, an allele form of interest) into a breeding program.

[00151] Trait or phenotype: The terms "trait" and "phenotype" are used interchangeably herein to refer to a measurable or observable heritable characteristic. A phenotype may in some examples be directly controlled by a single gene or genetic locus (*i.e.*, a single gene trait). In other examples, a phenotype may be the result of an interaction between several genes (a complex trait). Thus, a QTL can act through a single gene mechanism or by a polygenic mechanism. In some examples, a trait or phenotype can be assigned a "phenotypic value," which corresponds to a quantitative value measured for the phenotypic trait.

[00152] The term "molecular phenotype" may refer to a phenotype that is detectable at the level of a population of (one or more) molecules. In some examples, the molecular phenotype may only be detectable at the molecular level. The detectable molecules of the phenotype may be nucleic acids (*e.g.*, genomic DNA or RNA); proteins; and/or metabolites. For example, a molecular phenotype may be an expression profile for one or more gene products (*e.g.*, at a specific stage of plant development, or in response to an environmental condition or stress).

[00153] Blackleg resistance: For the purposes of the present disclosure, a trait of particular interest is "blackleg resistance." Those in the art understand that blackleg resistance is predominantly determined by heritable genetic factors. Thus, for example, the selection of a particular canola variety for cultivation may be based at least in part on the characteristic disease (for instance, blackleg) resistance of that particular variety under normal field growing conditions (*e.g.*, conditions without drought, disease, and adequate soil nutrients). In examples, a canola plant having a blackleg susceptibility may comprise a blackleg susceptibility rating of 0, 1, 3, 5, 7 or 9, (See **Table 1**), where a rating of 9 indicates great susceptibility, or limited resistance to, blackleg disease.

[00154] In some embodiments, "blackleg resistance" is determined by comparison with the characteristic blackleg resistance of a wild-type or parental variety. Thus, a first canola comprising a blackleg resistance

phenotype may have "increased" or "greater" levels of blackleg resistance (or "decreased" or "lower" levels of blackleg susceptibility) relative to a wild-type canola, or relative to a parental canola variety from which the first canola was derived. Increased or greater are relative terms, indicating that the plant resists blackleg disease better, or to a greater degree, than a similar wild-type plant. Decreased and lowered are relative terms, indicating that the plant is more susceptible to blackleg disease or is susceptible to a greater degree, than a similar wild-type plant.

Detection of Markers for Blackleg resistance in Canola

[00155] Methods for detecting (identifying) canola plants or germplasm that carry particular alleles of blackleg resistance marker loci are a feature of some embodiments. In some embodiments, any of a variety of marker detection protocols available in the art may be used to detect a marker allele, depending on the type of marker being detected. In examples, suitable methods for marker detection may include amplification and identification of the resulting amplified marker by, for example and without limitation, PCR; LCR; and transcription-based amplification methods (*e.g.*, ASH, SSR detection, RFLP analysis, and many others).

[00156] In general, a genetic marker relies on one or more property of nucleic acids for its detection. For example, some techniques for detecting genetic markers utilize hybridization of a probe nucleic acid to a nucleic acid corresponding to the genetic marker (*e.g.*, an amplified nucleic acid produced using a genomic canola DNA molecule as a template). Hybridization formats including, for example and without limitation, solution phase; solid phase; mixed phase; and *in situ* hybridization assays may be useful for allele detection in particular embodiments. An extensive guide to the hybridization of nucleic acids may be found, for example, in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology- Hybridization with Nucleic Acid Probes Elsevier, NY.

[00157] Markers corresponding to genetic polymorphisms between members of a population may be detected by any of numerous methods including, for example and without limitation, nucleic acid amplification-based methods; and nucleotide sequencing of a polymorphic marker region. Many detection methods (including amplification-based and sequencing-based methods) may be readily adapted to high throughput analysis in some examples, for example, by using available high throughput sequencing methods, such as sequencing by hybridization.

[00158] Amplification primers for amplifying SSR-type marker loci are included in particular examples of some embodiments. Table 6 provides specific primers for amplification of particular markers described herein. However, one of skill will immediately recognize that other sequences on either side of the given primers may

be used in place of the given primers, so long as the primers are capable of amplifying a nucleotide sequence comprising the allele to be detected. Further, the precise probe used for allele detection may vary. For example, any probe capable of identifying the region of a marker amplicon to be detected may be substituted for the exemplary probes listed herein. Further, the configuration of amplification primers and detection probes may also vary. Thus, embodiments are not limited to the primers and probes specifically recited herein. Although many specific examples of primers are provided herein (*see* Table 6), suitable primers to be used with the invention may be designed using any suitable method. For example, equivalent primers may be designed using any suitable software program, such as for example and without limitation, LASERGENE®.

[00159] Molecular markers may be detected by established methods available in the art including, for example and without limitation: ASH, or other methods for detecting SNPs; AFLP detection; amplified variable sequence detection; RAPD detection; RFLP detection; self-sustained sequence replication detection; SSR detection; SSCP detection; and isozyme markers detection. While the exemplary markers provided in FIG. 1 and Table 6 are SSR markers, any of the aforementioned marker types may be employed in particular embodiments to identify chromosome segments encompassing a genetic element that contributes to a blackleg resistance phenotype in canola.

[00160] For example, markers that comprise RFLPs may be detected, for example, by hybridizing a probe (which is typically a sub-fragment or synthetic oligonucleotide corresponding to a sub-fragment) of the nucleic acid to be detected to restriction-digested genomic DNA. The restriction enzyme is selected so as to provide restriction fragments of at least two alternative (or polymorphic) lengths in different individuals or populations. Determining one or more restriction enzyme(s) that produces informative fragments for each cross is a simple procedure that is easily accomplished by those of skill in the art after provision of the target DNA sequence. After separation by length in an appropriate matrix (*e.g.*, agarose or polyacrylamide) and transfer to a membrane (*e.g.*, nitrocellulose or nylon), a labeled probe may be hybridized under conditions that result in equilibrium binding of the probe to the target, followed by removal of excess probe by washing, and detection of the labeled probe.

[00161] In some embodiments, an amplification step is utilized as part of a method to detect/genotype a marker locus. However, an amplification step is not in all cases a requirement for marker detection. For example, an unamplified genomic DNA may be detected simply by performing a Southern blot on a sample of genomic DNA. Separate detection probes may also be omitted in amplification/detection methods, for example and without limitation, by performing a real time amplification reaction that detects product formation by modification of an amplification primer upon incorporation into a product; incorporation of labeled nucleotides

into an amplicon; and by monitoring changes in molecular rotation properties of amplicons as compared to unamplified precursors (*e.g.*, by fluorescence polarization).

[00162] PCR, RT-PCR, real-time PCR, and LCR are in particularly broad use as amplification and amplification-detection methods for amplifying and detecting nucleic acids (*e.g.*, those comprising marker loci). Details regarding the use of these and other amplification methods can be found in any of a variety of standard texts including, for example, Sambrook *et al.*, Molecular Cloning- A Laboratory Manual (2000) 3rd Ed., Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Current Protocols in Molecular Biology, (supplemented through 2002) F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc.; and PCR Protocols A Guide to Methods and Applications (1990) Innis *et al.* eds) Academic Press Inc., San Diego, CA. Additional details regarding detection of nucleic acids in plants can also be found, for example, in Plant Molecular Biology (1993) Croy (ed.) BIOS Scientific Publishers, Inc.

[00163] Additional details regarding techniques sufficient to direct persons of skill through particular *in vitro* amplification and detection methods, including the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Qβ-replicase amplification, and other RNA polymerase-mediated techniques (*e.g.*, NASBA), and examples thereof, may also be found in, for example: U.S. Patent 4,683,202; Arnheim and Levinson (1991) J. NIH Res. 3:81-94; Kwoh *et al.* (1989) Proc. Natl. Acad. Sci. USA 86:1173; Guatelli *et al.* (1990), *supra*; Lomell *et al.* (1989) J. Clin. Chem. 35:1826; Landegren *et al.* (1988) Science 241:1077-80; Van Brunt (1990) Biotechnology 8:291-4; Wu and Wallace (1989) Gene 4:560; Barringer *et al.* (1990) Gene 89:117; and Sooknanan and Malek (1995) Biotechnology 13:563-4. Improved methods of amplifying large nucleic acids by PCR, which may be useful in some applications of positional cloning, are further described in Cheng *et al.* (1994) Nature 369: 684, and the references cited therein, in which PCR amplicons of up to 40 kb are generated.

[00164] Many available biology texts also have extended discussions regarding PCR and related amplification methods. One of skill will appreciate that essentially any RNA can be converted into a double-stranded DNA that is suitable for restriction digestion, PCR amplification, and sequencing using reverse transcriptase and a polymerase (*e.g.*, by RT-PCR).

[00165] In some embodiments, a nucleic acid probe may be used to detect a nucleic acid that comprises a marker allele nucleotide sequence. Such probes can be used, for example, in positional cloning to isolate nucleotide sequences that are linked to a marker allele sequence. Nucleic acid probes that are useful in particular embodiments are not limited by any particular size constraint. In some embodiments, a nucleic acid

probe may be, for example and without limitation, at least 20 nucleotides in length; at least 50 nucleotides in length; at least 100 nucleotides in length; and at least 200 nucleotides in length. Nucleic acid probes to a marker locus may be cloned and/or synthesized.

[00166] Any suitable label may be used with a probe in particular examples. Detectable labels suitable for use with nucleic acid probes include any composition detectable by spectroscopic, radioisotopic, photochemical, biochemical, immunochemical, electrical, optical, or chemical means. Thus, a hybridized probe may be detected using, for example, autoradiography, fluorography, or other similar detection techniques, depending on the particular label to be detected. Useful labels include biotin (for staining with labeled streptavidin conjugate), magnetic beads, fluorescent dyes, radiolabels, enzymes, and colorimetric labels. Other labels include ligands that bind to antibodies or specific binding targets labeled with fluorophores, chemiluminescent agents, and enzymes. A probe may also comprise radiolabelled PCR primers that are used to generate a radiolabelled amplicon. Additional information regarding labeling strategies for labeling nucleic acids, and corresponding detection strategies may be found, for example, in Haugland (1996) Handbook of Fluorescent Probes and Research Chemicals, Sixth Edition, Molecular Probes, Inc., Eugene OR; and Haugland (2001) Handbook of Fluorescent Probes and Research Chemicals, Eighth Edition, Molecular Probes, Inc., Eugene, OR (Available on CD ROM). In particular examples, PCR detection and quantification is carried out using dual-labeled fluorogenic oligonucleotide probes, for example, TaqMan® probes (Applied Biosystems).

[00167] In some embodiments, primers are not labeled, and marker PCR amplicons may be visualized, for example, following their size resolution (*e.g.*, following agarose gel electrophoresis). In particular examples, ethidium bromide staining of PCR amplicons following size resolution allows visualization of differently size amplicons corresponding to different marker alleles.

[00168] Primers for use in embodiments are not limited to those capable of generating an amplicon of any particular size. For example, primers used to amplify particular marker loci and alleles are not limited to those amplifying the entire region of the relevant locus. The primers may generate an amplicon of any suitable length that is longer or shorter than those given in the allele definitions. In examples, marker amplification may produce an amplicon that is, for example and without limitation, at least 20 nucleotides in length; at least 50 nucleotides in length; at least 100 nucleotides in length; and at least 200 nucleotides in length.

[00169] Synthetic methods for making oligonucleotides and useful compositions comprising oligonucleotides (*e.g.*, probes, primers, molecular beacons, PNAs, and LNAs) are generally well-known by those of skill in the art. For example, oligonucleotides may be synthesized chemically according to the solid

phase phosphoramidite triester method described in, for example, Beaucage and Caruthers (1981) Tetrahedron Letts. 22(20):1859-62. Such methods may employ an automated synthesizer, for example and without limitation, as described in Needham-VanDevanter *et al.* (1984) Nucleic Acids Res. 12:6159-68. Oligonucleotides (including modified oligonucleotides) may also be ordered from a variety of commercial sources including, for example and without limitation, The Midland Certified Reagent Company; The Great American Gene Company; ExpressGen Inc.; and Operon Technologies Inc. Similarly, PNAs may be custom ordered from any of a variety of sources including, for example and without limitation, PeptidoGenic; HTI Bio-Products, Inc.; BMA Biomedicals Ltd (U.K.); and Bio.Synthesis, Inc.

[00170] In some embodiments, an *in silico* method may be used to detect a marker allele. For example, the sequence of a nucleic acid comprising a marker sequence may be stored in a computer. The desired marker locus sequence (or its homolog) may be identified using an appropriate nucleic acid search algorithm, as provided by, for example and without limitation, BLAST™, or even simple word processors.

[00171] In some embodiments, a marker allele is detected using a PCR-based detection method, where the size or sequence of a PCR amplicon comprising the marker is indicative of the absence or presence of a particular marker allele. In some examples, PCR primers are hybridized to conserved regions flanking the polymorphic marker region. PCR primers so used to amplify a molecular marker are sometimes referred to in the art as "PCR markers," or simply "markers."

[00172] A primary motivation for development of molecular markers in crop species is the potential for increased efficiency in plant breeding through marker assisted selection (MAS). Genetic markers that are linked to a trait or gene of interest may be used to identify plants that contain a desired marker allele at one or more loci, which plants are thus expected to transfer the desired marker allele, along with the trait or gene of interest, to their progeny. Genetic markers may be used to identify plants that contain a particular genotype at one locus, or at several unlinked or linked loci (*e.g.*, a haplotype). Similarly, marker alleles described herein may be introgressed into any desired canola genetic background, germplasm, plant, line, variety, etc., as part of an overall MAS breeding program designed to enhance canola yield.

[00173] According to some embodiments, markers described herein provide the means to identify canola plants and germplasm that comprise blackleg resistance (or increased blackleg resistance, or decreased blackleg susceptibility) by identifying plants and germplasm comprising a specific allele at a locus such as DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327,

DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359, and/or a marker locus linked to at least one of the foregoing. By identifying plants lacking a marker allele that co-segregates with blackleg resistance, blackleg susceptible plants and germplasm (or plants with a lesser degree of blackleg resistance) may be identified, for example, for elimination from subsequent crosses and breeding.

[00174] According to the foregoing, embodiments of the invention include molecular markers that have a significant probability of co-segregation with a QTL that contributes to or imparts a blackleg resistance phenotype. These QTL markers find use in marker assisted selection for desired traits (blackleg resistance), and also have other uses. Embodiments of the invention are not limited to any particular method for the detection or analysis of these markers.

Introgression of Markers for Blackleg resistance into Canola Lines

[00175] As set forth, *supra*, identification of canola plants or germplasm that includes a marker allele or alleles that is/are linked to blackleg resistant phenotype provides a basis for performing marker assisted selection of canola. In some embodiments, at least one canola plant that comprises at least one marker allele that is positively correlated blackleg resistance is selected, while canola plants that comprise marker alleles that are negatively correlated with blackleg resistance may be selected against.

[00176] Desired marker alleles that are positively correlated blackleg resistance may be introgressed into canola having a particular (*e.g.*, elite or exotic) genetic background, so as to produce an introgressed blackleg resistant canola plant or germplasm. In some embodiments, a plurality of blackleg resistance markers may be sequentially or simultaneous selected and/or introgressed into canola. The particular combinations of blackleg resistance markers that may be selected for in a single plant or germplasm is not limited, and can include a combination of markers such as those set forth in **Table 2**, any markers linked to the markers recited in **Table 2**, or any markers located within the QTL intervals defined herein.

[00177] In embodiments, the ability to identify QTL marker alleles that are positively correlated with blackleg resistance of a canola plant provides a method for selecting plants that have favorable marker loci as well. For example, any plant that is identified as comprising a desired marker allele (*e.g.*, a marker allele that positively correlates with blackleg resistance) may be selected for, while plants that lack the allele (or that comprise an allele that negatively correlates with blackleg resistance) may be selected against. Thus, in particular embodiments, subsequent to identification of a marker allele in a first plant or germplasm, an

introgression method includes selecting the first canola plant or germplasm, or selecting a progeny of the first plant or germplasm. In some examples, the resulting selected canola plant or germplasm may be crossed with a second canola plant or germplasm (*e.g.*, an elite canola or an exotic canola), so as to produce progeny comprising the marker allele and desirable characteristics and/or alleles of the second plant or germplasm.

[00178] In some embodiments, a method of introgressing a blackleg resistance QTL may include, for example, providing at least one marker linked to blackleg resistance (*e.g.*, a marker that co-segregates with blackleg resistance); determining the marker allele in a first plant or germplasm comprising blackleg resistance QTL; and introgressing the marker allele into a second canola plant or germplasm, so as to produce an introgressed canola plant or germplasm. In particular embodiments, the second canola plant or germplasm may comprise improved blackleg resistance as compared to the first canola plant or germplasm, while the introgressed canola plant or germplasm will comprise a blackleg resistance as compared to the second plant or germplasm. As discussed in more detail below, an introgressed canola plant or germplasm produced by these and other embodiments are also included in embodiments of the invention.

[00179] In some embodiments, where an introgressed canola plant or germplasm is produced by any of the methods provided herein, the introgressed canola plant or germplasm may be characterized by the blackleg susceptibility or resistance of the plant. An introgressed plant or germplasm may comprise, for example and without limitation, a blackleg susceptibility rating of 0, 1, 3, or 5. In some examples, such an introgressed canola plant or germplasm comprises a blackleg susceptibility rating of 0, 1, 3, 5, or 7.

[00180] In addition to introgressing selected marker alleles (*e.g.*, through standard breeding methods) into desired genetic backgrounds, so as to introgress a blackleg resistance QTL into the background, transgenic approaches may be used in some embodiments to produce blackleg resistance canola plants and/or germplasm. In some embodiments, an exogenous nucleic acid (*e.g.*, a gene or open reading frame) that is linked to at least one marker described herein in canola may be introduced into a target plant or germplasm. For example, a nucleic acid coding sequence linked to at least one marker described herein may be cloned from canola genomic DNA (*e.g.*, *via* positional cloning) and introduced into a target plant or germplasm.

[00181] Thus, particular embodiments include methods for producing a canola plant or germplasm comprising a blackleg resistance phenotype, wherein the method comprises introducing an exogenous nucleic acid into a target canola plant or progeny thereof, wherein the exogenous nucleic acid is substantially identical to a nucleotide sequence that is linked to at least one positively-correlated marker allele at one or more marker locus that is linked to blackleg resistance. In some examples, the marker locus may be selected from:

DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359. In some embodiments, a plurality of linked markers may be used to construct a transgenic plant. Which of the markers described herein that are used in such a plurality is within the discretion of the practitioner.

[00182] Any of a variety of methods can be used to provide an exogenous nucleic acid to a canola plant or germplasm. In some embodiments, a nucleotide sequence is isolated by positional cloning, and is identified by linkage to a marker allele that is positively correlated with blackleg resistance. For example, the nucleotide sequence may correspond to an open reading frame (ORF) that encodes a polypeptide that, when expressed in a canola plant, results in or contributes to the canola plant having blackleg resistance. The nucleotide sequence may then be incorporated into an exogenous nucleic acid molecule. The precise composition of the exogenous nucleic acid may vary. For example, an exogenous nucleic acid may comprise an expression vector to provide for expression of the nucleotide sequence in the plant wherein the exogenous nucleic acid is introduced.

[00183] Markers linked to blackleg resistance may be introgressed (for example, thereby introgressing a blackleg resistance phenotype) into a canola plant or germplasm utilizing a method comprising marker assisted selection. In embodiments, MAS is performed using polymorphic markers that have been identified as having a significant likelihood of co-segregation with a blackleg resistance trait. Such markers (*e.g.*, those set forth in FIG. 2) are presumed to map within or near a gene or genes that contribute to the blackleg resistance of the plant (compared to a plant comprising the wild-type gene or genes). Such markers may be considered indicators for the trait, and may be referred to as QTL markers. In embodiments, a plant or germplasm is tested for the presence of a positively correlated allele in at least one QTL marker.

[00184] In embodiments, linkage analysis is used to determine which polymorphic marker allele demonstrates a statistical likelihood of co-segregation with a blackleg resistance phenotype. Following identification of such a positively correlated marker allele for the blackleg resistance phenotype, the marker may then be used for rapid, accurate screening of plant lines for the blackleg resistance allele without the need to grow the plants through their life cycle and await phenotypic evaluations. Furthermore, the identification of the marker permits genetic selection for the particular blackleg resistance allele, even when the molecular identity of the actual blackleg resistance QTL is unknown. A small tissue sample (for example, from the first leaf of the plant) may be taken from a progeny canola plant produced by a cross and screened with the

appropriate molecular marker. Thereby, it may be rapidly determined whether the progeny should be advanced for further breeding.

[00185] In some embodiments comprising MAS, a polymorphic QTL marker locus may be used to select a plant that contains a marker allele (or alleles) that is positively correlated with a blackleg resistance phenotype. For example, a nucleic acid corresponding to the marker nucleic acid allele may be detected in a biological sample from the plant to be selected. This detection may take the form of hybridization of a probe nucleic acid to a marker allele or amplicon thereof (*e.g.*, using allele-specific hybridization, Southern analysis, northern analysis, *in situ* hybridization, and hybridization of primers followed by PCR amplification of a region of the marker). After the presence (or absence) of the particular marker allele in the biological sample is verified, the plant is selected, and may in some examples be used to make progeny plants by selective breeding.

[00186] Canola plant breeders desire combinations of blackleg resistance marker loci with markers/genes other desirable traits (*e.g.*, high yield) to develop improved canola varieties. Screening large numbers of samples by non-molecular methods (*e.g.*, trait evaluation in canola plants) is generally expensive, time consuming, and unreliable. Use of the polymorphic markers described herein, which are linked to blackleg resistance QTL, provides an effective method for selecting desirable varieties in breeding programs. Advantages of marker-assisted selection over field evaluations for blackleg resistance include, for example, that MAS can be done at any time of year, regardless of the growing season. Moreover, as set forth, *supra*, environmental effects are largely irrelevant to marker-assisted selection.

[00187] When a population is segregating for multiple marker loci linked to one or more traits (*e.g.*, multiple markers linked to blackleg resistance), the efficiency of MAS compared to phenotypic screening becomes even greater, because all of the marker loci may be evaluated in the lab together from a single sample of DNA. In particular embodiments of the invention, the DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359 markers may be assayed simultaneously or sequentially from a single sample, or from a plurality of parallel samples.

[00188] Another use of MAS in plant breeding is to assist the recovery of the recurrent parent genotype by backcross breeding. Backcrossing is usually performed for the purpose of introgressing one or a few markers or QTL loci from a donor parent (*e.g.*, a parent comprising desirable blackleg resistance marker loci) into an

otherwise desirable genetic background from a recurrent parent (*e.g.*, an otherwise high yielding canola line). The more cycles of backcrossing that are done, the greater the genetic contribution of the recurrent parent to the resulting introgressed variety. In some examples, many cycles of backcrossing may be carried out, for example, because blackleg resistance plants may be otherwise undesirable, *e.g.*, due to low yield, low fecundity, etc. In contrast, strains which are the result of intensive breeding programs may have excellent yield, fecundity, etc., merely being deficient in one desirable respect, such as blackleg susceptibility. In marker assisted backcrossing of specific markers from a donor source, which may or may not constitute an elite genetic background to an elite variety that will serve as the recurrent line, the practitioner may select among backcross progeny for the donor marker, and then use repeated backcrossing to the recurrent line to reconstitute as much of the recurrent line's genome as possible.

[00189] According to the foregoing, markers and methods described herein may be utilized to guide marker assisted selection or breeding of canola varieties with the desired complement (set) of allelic forms of chromosome segments associated with superior agronomic performance (*e.g.*, blackleg resistance, along with any other available markers for yield, disease resistance, etc.). Any of the described marker alleles may be introduced into a canola line *via* introgression (*e.g.*, by traditional breeding, *via* transformation, or both) to yield a canola plant with superior agronomic performance. If nucleic acids from a plant are positive for a desired genetic marker allele, the plant may be self-fertilized in some embodiments to create a true breeding line with the same genotype, or it may be crossed with a plant comprising the same marker allele, or other desired markers and/or characteristics to create a sexually-crossed hybrid generation.

[00190] Often, a method of the present invention is applied to at least one related canola plant such as from progenitor or descendant lines in the subject canola plants pedigree such that inheritance of the desired blackleg resistance allele can be traced. The number of generations separating the canola plants being subject to the methods of the present invention will generally be from 1 to 20, commonly 1 to 5, and typically 1, 2, or 3 generations of separation, and quite often a direct descendant or parent of the canola plant will be subject to the method (i.e., one generation of separation).

[00191] Genetic diversity is important in breeding programs. With limited diversity, the genetic gain achieved in a breeding program will eventually plateau when all of the favorable alleles have been fixed within the elite population. Therefore, one objective of plant breeding is to incorporate diversity into an elite pool without losing the genetic gain that has already been made, and with the minimum possible investment. MAS provide an indication of which genomic regions, and which favorable alleles from the original ancestors, have been selected for and conserved over time, facilitating efforts to incorporate favorable variation from exotic

germplasm sources (parents that are unrelated to the elite gene pool) in the hopes of finding favorable alleles that do not currently exist in the elite gene pool. Thus, in some embodiments, markers described herein may be used for MAS in crosses involving (elite x exotic) canola lines by subjecting segregating progeny to MAS to maintain major yield alleles, along with the blackleg resistance marker alleles herein.

[00192] The molecular marker loci and alleles described herein (*e.g.*, DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359) may be used in some embodiments, as indicated previously, to identify a blackleg resistance QTL, which may then be cloned by familiar procedures. Such increase blackleg resistance clones may be first identified by their genetic linkage to markers described herein. For example, "positional gene cloning" takes advantage of the physical proximity of a blackleg resistance marker to define an isolated chromosomal fragment containing a blackleg resistance QTL gene. The isolated chromosomal fragment may be produced by such well-known methods as, for example and without limitation, digesting chromosomal DNA with one or more restriction enzymes, by amplifying a chromosomal region using PCR, and any suitable alternative amplification reaction. The digested or amplified fragment may subsequently be ligated into a vector suitable for replication and/or expression of the inserted fragment. Markers that are adjacent to an ORF associated with a phenotypic trait may be specifically hybridized to a DNA clone (*e.g.*, a clone from a genomic DNA library), thereby identifying a clone on which the ORF (or a fragment of the ORF) is located. If a marker is more distant from the blackleg resistance QTL gene, a fragment containing the ORF may be identified by successive rounds of screening and isolation of clones, which together comprise a contiguous sequence of DNA. This process is commonly referred to as "chromosome walking," and it may be used to produce a "contig" or "contig map."

[00193] Protocols sufficient to guide one of skill through the isolation of clones associated with linked markers are found in, for example, Sambrook *et al.* (ed.) Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Ausubel *et al.*, Eds., Current Protocols in Molecular Biology, Chapter 2, Greene Publishing and Wiley-Interscience, NY, 1995.

Plants Comprising Markers for Blackleg resistance

[00194] Some embodiments include methods for making a canola plant, and further include these canola plants, *per se*. In particular embodiments, such a method may comprise crossing a first parent canola plant comprising at least one marker allele that is positively correlated with blackleg resistance with a second canola

plant at a marker linked to blackleg resistance described herein, and growing the female canola plant under plant growth conditions to yield canola plant progeny. Such canola plant progeny may be assayed for marker alleles linked to blackleg resistance, and desired progeny may be selected. Such progeny plants, or seed thereof, may be subject to a variety of uses including, for example and without limitation, they may be sold commercially for canola production; used for food; processed to obtain a desired canola product (*e.g.*, canola oil or canola meal); and/or further utilized in subsequent rounds of breeding. Canola plants according to some embodiments include progeny plants that comprise at least one of the allelic forms of the markers described herein, such that further progeny are capable of inheriting the marker allele.

[00195] Some embodiments include methods for producing a canola plant comprising blackleg resistance. In particular embodiments, such methods may include production of such a plant by conventional plant breeding or by introducing an exogenous DNA (*e.g.*, a transgene) into a canola variety or plant.

[00196] Thus, some embodiments include host cells and organisms that are transformed with nucleic acids corresponding to a blackleg resistance QTL identified using at least one marker linked to blackleg resistance described herein. In some examples, such nucleic acids may include chromosome intervals (*e.g.*, genomic fragments), ORFs, and/or cDNAs that encode expression products that contribute to a blackleg resistance phenotype.

[00197] Host cells may be genetically engineered (*e.g.*, transduced, transfected, transformed, etc.) with a vector (*e.g.*, a cloning vector, shuttle vector, or expression vector) that comprises an ORF linked to a marker of blackleg resistance. Vectors include, for example and without limitation, plasmids; phagemids; *Agrobacterium*; viruses; naked polynucleotides (linear or circular); and conjugated polynucleotides. Many vectors may be introduced into bacteria, especially for the purpose of propagation and expansion.

[00198] Vectors may be introduced into plant tissues, cultured plant cells, and plant protoplasts by any of a variety of standard methods known in the art including, for example and without limitation: electroporation (From *et al.* (1985) Proc. Natl. Acad. Sci. USA 82:5824); infection by viral vectors such as cauliflower mosaic virus (CaMV) (*see*, *e.g.*, U.S. Patent 4,407,956); ballistic penetration by small particles comprising the nucleic acid (Klein *et al.* (1987) Nature 327:70); use of pollen as vector (PCT International Patent Publication No. WO 85/01856); and use of *Agrobacterium tumefaciens* or *A. rhizogenes* carrying a T-DNA plasmid in which DNA fragments are cloned (Fraley *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:4803). Any suitable method, including without limitation the specific methods explicitly identified herein, which provides for effective

introduction of a nucleic acid into a cell or protoplast, may be employed in certain embodiments of the invention.

[00199] Engineered host cells can be cultured in conventional nutrient media or media modified for, for example, activating promoters or selecting transformants. In some embodiments, host plant cells may be cultured into transgenic plants. Plant regeneration from cultured protoplasts is described in, for example, Evans *et al.* (1983) "Protoplast Isolation and Culture," In Handbook of Plant Cell Cultures 1, MacMillan Publishing Co., NY, pp. 124-176; Davey (1983) "Recent Developments in the Culture and Regeneration of Plant Protoplasts," In Protoplasts, Birkhauser, Basel, pp. 12-29; Dale (1983) "Protoplast Culture and Plant Regeneration of Cereals and Other Recalcitrant Crops," In Protoplasts, *supra*, pp. 31-41; and Binding (1985) "Regeneration of Plants," In Plant Protoplasts, CRC Press, Boca Raton, FL, pp. 21-73. Additional resources providing useful details regarding plant cell culture and regeneration include Payne *et al.* (1992) Plant Cell and Tissue Culture in Liquid Systems, John Wiley & Sons, Inc., NY; Gamborg and Phillips (eds.) (1995) Plant Cell, Tissue and Organ Culture; Fundamental Methods, Springer Lab Manual, Springer-Verlag (Berlin Heidelberg NY); and R. R. D. Croy (Ed.) Plant Molecular Biology (1993) Bios Scientific Publishers, Oxford, UK (ISBN 0 12 198370 6).

[00200] Transformed plant cells that are produced using any of the above transformation techniques may be cultured to regenerate a whole plant that possesses the transformed genotype and thus the desired phenotype. Such regeneration techniques generally rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker that has been introduced into the cell together with the desired nucleotide sequences. Regeneration and growth processes used to produce a whole plant generally include the steps of selection of transformant cells and shoots; rooting the transformant shoots; and growth of the plantlets in soil.

[00201] Plant transformation with nucleic acids that provide blackleg resistance (*e.g.*, that comprise markers described herein) may be used to transform species other than canola. For example, it is contemplated that expression products from QTLs that contribute to or provide a blackleg resistance phenotype in canola can also confer blackleg resistance when transformed and expressed in other agronomically and horticulturally important plant species. Such species include dicots, for example and without limitation, of the genera: *Sinapis, Raphanus, Descurainia, Sisymbrium, and Thlaspi* in addition to the various members of the Brassicaceae family, including oilseed rape (canola), rutabaga (swede), oilseed turnip rape, turnip, kale, cress, radish, horseradish, stock, bok choy, broccoli, and vegetable and seed crops of cabbage.. Common crop plants which

may be used in particular examples include, for example and without limitation: canola, broccoli, brussel sprouts, cabbage, cauliflower, kale, and kohlrabi,.

Systems for Detecting and/or Correlating Blackleg resistance Markers

[00202] Systems, including automated systems, for identifying plants that comprise at least one marker linked to the blackleg resistance phenotype in canola, and/or for correlating presence of a specific linked marker allele with blackleg resistance, are also included in some embodiments. Exemplary systems may include probes useful for allele detection at a marker locus described herein; a detector for detecting labels on the probes; appropriate fluid handling elements and temperature controllers, for example, that mix probes and templates and/or amplify templates; and/or system instructions that correlate label detection to the presence of a particular marker locus or allele.

[00203] In particular embodiments, a system for identifying a canola plant predicted to have blackleg resistance is provided. Such a system may include, for example and without limitation: a set of marker primers and/or probes configured to detect at least one allele of at least one marker linked to blackleg resistance (*e.g.*, DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359); a detector that is configured to detect one or more signal outputs from the set of marker probes or primers, or amplicon thereof, thereby identifying the presence or absence of the allele; and system instructions that correlate the presence or absence of the allele with blackleg resistance.

[00204] A system that performs marker detection and/or correlation may include a detector that is configured to detect one or more signal outputs from the set of marker probes or primers, or amplicon thereof. The precise configuration of the detector may depend on the type of label used to detect a marker allele. Particular examples may include light detectors and/or radioactivity detectors. For example, detection of light emission or other property of a labeled probe may be indicative of the presence or absence of a marker allele interacting with the probe (*e.g.*, via specific hybridization). The detector(s) optionally monitors one or a plurality of signals from an amplification reaction. For example, a detector may monitor optical signals which correspond to "real time" amplification assay results.

[00205] A wide variety of signal detection devices are available including, for example and without limitation, photo multiplier tubes; spectrophotometers; CCD arrays; arrays and array scanners; scanning

detectors; phototubes and photodiodes; microscope stations; galvo-scanns; and microfluidic nucleic acid amplification detection appliances. In addition to the type of label used to detect a marker allele, the precise configuration of a detector may depend, in part, on the instrumentation that is most conveniently obtained for the user. Detectors that detect fluorescence, phosphorescence, radioactivity, pH, charge, absorbance, luminescence, temperature, or magnetism may be used in some examples.

[00206] The precise form of instructions provided in a system according to some embodiments may similarly vary, depending on the components of the system. For example, instructions may be present as system software in one or more integrated unit(s) of the system, or they may be present in one or more computers or computer readable media operably coupled to a detector. In some examples, system instructions include at least one reference table that includes a correlation between the presence or absence of a particular marker allele in a plant or germplasm and the presence or absence of blackleg resistance. Instructions may also include directions for establishing a user interface with the system; *e.g.*, to permit a user to view results of a sample analysis and to input parameters into the system.

[00207] A system may include in particular embodiments components for storing or transmitting computer readable data representing or designating detected marker alleles, for example, in an automated (*e.g.*, fully automated) system. For example, a computer readable media may be provided that includes cache, main, and storage memory, and/or other electronic data storage components (*e.g.*, hard drives, floppy drives, and storage drives) for storage of computer code. Data representing alleles detected by the method of the present invention can also be electronically, optically, or magnetically transmitted in a computer data signal embodied in a transmission medium over a network, such as an intranet or internet or combinations thereof. A system may also or alternatively transmit data *via* wireless, infrared, or other available transmission alternatives.

[00208] During operation, the system typically comprises a sample that is to be analyzed, such as a plant tissue, or material isolated from the tissue such as genomic DNA, amplified genomic DNA, cDNA, amplified cDNA, RNA, amplified RNA, or the like.

[00209] In some embodiments, a system may be comprised of separate elements, or may alternatively be integrated into a single unit for convenient detection of markers alleles, and optionally for additionally performing marker-phenotype correlations. In particular embodiments, the system may also include a sample, for example and without limitation, genomic DNA; amplified genomic DNA; cDNA; amplified cDNA; RNA; and amplified RNA, from canola or from a selected canola plant tissue.

[00210] Automated systems provided in some embodiments optionally include components for sample manipulation; *e.g.*, robotic devices. For example, an automated system may include a robotic liquid control armature for transferring solutions (*e.g.*, plant cell extracts) from a source to a destination (*e.g.*, from a microtiter plate to an array substrate) that may be operably linked to a digital computer (*e.g.*, in an integrated computer system). An input device for entering data to the digital computer to control high throughput liquid transfer by the robotic liquid control armature (and, optionally, to control transfer by the armature to the solid support) may also be a feature of an automated system. Many automated robotic fluid handling systems are commercially available. For example, a variety of automated systems that utilize various Zymate™ systems, and typically include, robotics and fluid handling modules, are available from Caliper Technologies Corp. (Hopkinton, MA). Similarly, the common ORCA® robot, which is used in a variety of laboratory systems (*e.g.*, for microtiter tray manipulation) is also commercially available from, for example, Beckman Coulter, Inc. (Fullerton, CA). As an alternative to conventional robotics, microfluidic systems for performing fluid handling and detection are now widely available from Caliper Technologies and Agilent technologies (Palo Alto, CA).

[00211] In particular embodiments, a system for molecular marker analysis may include, for example and without limitation, a digital computer comprising high-throughput liquid control software; a digital computer comprising image analysis software for analyzing data from marker labels; a digital computer comprising data interpretation software; a robotic liquid control armature for transferring solutions from a source to a destination; an input device (*e.g.*, a computer keyboard) for entering data into the system (*e.g.*, to control high throughput liquid transfer by the robotic liquid control armature); and an image scanner for digitizing label signals from labeled probes.

[00212] Optical images (*e.g.*, hybridization patterns) viewed and/or recorded by a camera or other device (*e.g.*, a photodiode and data storage device) may be further processed in any of the embodiments herein. For example and without limitation, such images may be processed by digitizing the image and/or storing and analyzing the image on a computer. A variety of commercially available peripheral equipment and software is available for digitizing, storing and analyzing a digitized video or digitized optical image, for example, using various computer and programming platforms.

[00213] Some embodiments also include kits useful for identifying plants that comprise at least one marker linked to blackleg resistance in canola, and/or for correlating presence of a specific linked marker allele with blackleg resistance. In some examples, such a kit may include appropriate primers or probes for detecting at least one marker linked to blackleg resistance and particular marker alleles; and instructions for using the primers or probes to detect the at least one marker and correlate the marker allele with blackleg resistance. A

kit may in some examples include packaging materials for packaging probes, primers, and/or instructions; and controls (*e.g.*, control amplification reactions that include probes, primers or template nucleic acids for amplifications, and molecular size markers).

[00214] In some embodiments, a kit or system for identifying plants that comprise at least one marker linked to blackleg resistance in canola, and/or for correlating presence of a specific linked marker allele with blackleg resistance may include nucleic acids that detect particular SSR QTL markers described herein. For example, a system or kit may comprise an amplification primer pair capable of initiating DNA polymerization by a DNA polymerase on a canola nucleic acid template to generate a canola marker amplicon, where the marker amplicon corresponds to a canola marker selected from DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359. For example, the primer pair that is specific for the marker can be selected from the primer pairs set forth in **Table 3**, or their equivalents.

EXAMPLES

[00215] The following examples are offered to illustrate, but not to limit, certain embodiments of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only, and persons skilled in the art will recognize various reagents, techniques, systems, and parameters that can be altered without departing from the spirit or scope of the invention.

Example 1: Plant material and DNA extraction

[00216] A total of 700 BC1F1 progeny from cross Nex845CL//Nex845CL/NT152343-20 were used for fine mapping of *Rlm1*. NT152343-20 was the *Rlm1* resistance donor. Recurrent parent Nex845CL, a Dow AgroSciences (DAS) cultivar with Omega 9 oil profile and Clearfield® herbicide tolerance, was susceptible to *Rlm1*.

[00217] DNA was extracted using the MagAttract DNA extraction method (Qiagen, Valencia, CA) using the Biocel 1800 (Agilent Technologies, Santa Clara, CA). DNA was quantified using the Nanodrop 8000 Spectrophotometer (Thermo Scientific, Rockford, IL) per manufacturer's instructions.

Example 2: Phenotyping the mapping population

Plant grow out

[00218] A total of 25 BC1F2 families (24 plants/family) derived from 25 BC1F1 progeny, which showed recombination among the markers in the Rlm1 region, were selected for phenotyping. Seeds were sown in soil in a 72-well insert flats in growth chambers. Normal plant growing conditions were maintained (16:8 light/dark photoperiods, 19°C at day time and 16°C at night). Watering and fertilization were applied as normal.

Inoculation

[00219] Inoculation was done at 8 days after planting when cotyledons were fully expanded. Each lobe of the cotyledon was wounded using a tissue teeth forceps. Ten microliters (µl) of *Leptosphaeria maculans (L. maculans)* pycnidiospore suspension in H_2O at a concentration of 2 x 10^7 spores/ml using a repeating pipette was dropped on each lobe. Four droplets on two cotyledons were dropped on a single plant. Inoculated cotyledons were kept at room temperature for 6 - 8 hours before being moved back to growth chambers.

Rating

[00220] Disease symptoms were recorded on 8-9 days post inoculation (dpi). Symptoms started to appear in 6-7 dpi as limited necrotic tissue around the wound in resistant phenotypes and as a faint bleaching around wound in susceptible phenotypes. The resistance was rated into six scales (0, 1, 3, 5, 7 and 9) according to lesion size, amount of dark margin around the wound, and presence of tissue collapse and sporulation (**Table 1**).

[00221] **Table 1.** Disease rating and description for *L. maculans*.

Rating	Description
0	No darkening around wound; the same as water control.
1	Limited blackening around wound, lesion diameter = 0.5-1.5 mm; faint chlorotic halo may be present; sporulation absent.
3	Dark necrotic lesions, 1.5-3.0 mm, chlorotic halo may be present; sporulation absent.
5	3-6 mm lesion, sharply delimited by dark necrotic margin; may show grey-green tissue collapse as in scales 7 and 9 or dark necrosis throughout; sporulation absent.

7	Grey-green tissue collapse 3-5 mm diameter; sharply delimited, no darkened margin; sporulation absent.
9	Rapid tissue collapse, accompanied by profuse sporulation in large, more than 5 mm lesion with diffuse margins.

Disease Index calculation and classification

[00222] A disease index (DI) was calculated based on the formula:

[00223]

$$DI = \frac{\sum_{i=0}^{9} (I \times j)}{n}$$

[00224] Where n = 24 (total inoculated BC1F2 plants/family), i = rating scales (0-9), and j = number of plant/scale. Plants with a DI <= 3 were classified as resistant (R); those with a DI = 3-5 were classified as intermediate resistant; those with a DI > 5 were classified as susceptible (S).

Example 3: The KBioscience Competitive Allele-Specific PCR genotyping system (KASPar™)

[00225] The KASPar™ genotyping system is comprised of two components (1) the SNP-specific assay (a combination of three unlabelled primers), and (2) the universal Reaction Mix, supplied at 2X concentration, and containing Taq polymerase enzyme, the passive reference dye, ROX, 50mM $MgCl_2$, and DMSO. The three primers, allele-specific 1 (A1), allele-specific 2 (A2), and common (C1), or reverse, were designed using the assay design algorithm of the workflow manager, Kraken (KBiosciences, Hoddesdon, Hertfordshire, UK).

[00226] An Assay Mix of the 3 primers was made, consisting of 12 micromolar (μM) each of A1 and A2 and 30 μM of C1. The universal Reaction Mix was diluted to 1x and an additional amount of $MgCl_2$ is added so that the final $MgCl_2$ concentration of Reaction Mix at 1X concentration is 1.8 millimolar (mM). DNA was dispensed into 1536 well PCR plates at a concentration of 2.5 ng/μl per well and was dried down in the plates in a 65° C oven for 1 hour and 15 minutes. The Assay Mix and universal Reaction Mix were combined in a 1:54 ratio and 2.0 μl was dispensed into the DNA plates using a liquid handler robot. A HydroCycler 32 (LGC Genomics, Beverly, MA) was used for thermocycling with the following touchdown

conditions: 94° C for 15 minutes; 10 cycles of 94° C for 20 seconds; 65 to 57° C with -0.8°C/cycle for 60 seconds; 29 cycles of 94°C for 20 seconds, 57°C for 60 seconds; 3-6 cycles of 94°C for 20 seconds, 57°C for 60 seconds. After thermocycling was complete, allele-specific fluorescent intensities were read using a PHERAStar® Spectrofluorometer (BMG LabTech, Cary, NC) at room temperature and data was uploaded to the Kraken system for analysis.

[00227] KASPar™ uses the fluorophores FAM and VIC for distinguishing genotypes. The passive reference dye ROX is also used to allow normalization of variations in signal caused by differences in well-to-well liquid volume. In Kraken, the FAM and VIC data are plotted on the *x*- and *y*- axes, respectively. Genotypes can then be determined according to sample clusters.

[00228] A proprietary high-density consensus map was leveraged and 101 SNP markers mapped in the Rlm1 region of linkage group (LG) N7 were selected for KASPar™ assay development. The two mapping parents Nex845CL and NT152343-20 were screened with the 101 KASPar™ markers and 36 of the markers were polymorphic between the two parents. The 700 BC1F1 progeny were genotyped with 27 of those markers (**Tables 2 and 3**). A total of 25 BC1F2 families (24 plants/family) derived from 25 BC1F1 progeny, which showed recombination among the markers in the Rlm1 gene region, were selected for phenotyping.

[00229] **Table 2.** Sequences and physical locations of 27 SNP markers for fine mapping of Rlm1. GD: Genetic distance in consensus map; Nex845CL: Rlm1 susceptible parent; NT152343-20: Rlm1 resistant donor.

Marker	GD (cM)	SNP	NT152343-20 Allele	Physical location in Darmor reference genome V4.1 (bp)	SEQ ID NO
DBSNP00397	78.9	[A/G]	GG	chrA07:20065374..20066820	1
DBSNP12121	81.9	[A/G]	GG	chrA07:20288346..20289689	2
DBSNP01841	82.5	[C/G]	CC	chrA07:20297357..20298610	3
DBSNP04821	85.9	[A/G]	GG	chrA07:20739631..20740053	4
DBSNP32292	86.2	[T/G]	GG	chrA07:20819679..20819985	5
DBSNP217314	86.2	[A/C]	AA	chrA07_random:1914969..1915264	6
DBSNP62256	86.2	[A/G]	GG	chrA07_random:1912617..1913159	7
DBSNP248920	86.2	[T/C]	TT	chrC06:27737872..27738141	8
DBSNP32465	86.2	[A/C]	AA	chrA07:20896457..20896778	9
DBSNP217319	86.2	[A/G]	GG	chrA07:20909101..20909221	10
DBSNP217320	86.2	[A/G]	GG	chrA07:20916310..20916430	11
DBSNP217322	86.2	[T/C]	CC	chrA07:20917301..20917420	12
DBSNP217327	86.2	[T/C]	CC	chrA07:20923897..20924016	13
DBSNP217328	86.2	[T/C]	CC	chrA07:20935220..20935420	14
DBSNP51173	86.2	[T/C]	CC	chrA07:20935235..20935820	15
DBSNP233856	86.2	[A/C]	AA	chrA07:20942726..20943026	16
DBSNP217329	86.2	[A/C]	CC	chrA07:20943033..20943150	17
DBSNP217331	86.2	[T/C]	TT	chrA07:20946748..20946871	18
DBSNP46180	86.2	[A/G]	GG	chrA07:20959006..20959796	19
DBSNP217333	86.2	[T/C]	CC	chrA07:20959498..20959798	20
DBSNP02628	86.2	[T/G]	GG	chrA07:20960043..20961363	21
DBSNP58735	86.2	[A/G]	GG	chrA07:20974974..20975594	22
DBSNP217338	86.2	[A/G]	GG	chrA07:20978933..20979036	23
DBSNP217339	86.2	[T/C]	CC	chrA07:20983136..20983436	24
DBSNP217340	86.2	[T/C]	CC	chrA07:20985095..20985215	25
DBSNP10650	86.2	[A/G]	GG	chrA07:21120083..21120274	26
DBSNP27359	86.6	[A/G]	GG	chrA07:21351587..21351903	27

[00230] **Table 3.** Primer sequences of 27 KASPar assays for fine mapping of Rlm1.

Marker	Primer	Sequence (5′ → 3′)	SEQ ID NO:
DBSNP00397	A1	GAAGGTGACCAAGTTCATGCTGTTTTGCAGGGGTGCATGACTCA	28
	A2	GAAGGTCGGAGTCAACGGATTTTGCAGGGGTGCATGACTCG	29
	C1	CAACATCACATGCGTGAGTCCAGAA	30
DBSNP12121	A1	GAAGGTGACCAAGTTCATGCTAAAAGGCAGAAAGAGAGACCTTGTTA	31
	A2	GAAGGTCGGAGTCAACGGATTAAAGGCAGAAAGAGAGACCTTGTTG	32
	C1	GCTGGGTTCAAAGGTGGTGTAGATA	33
DBSNP01841	A1	GAAGGTGACCAAGTTCATGCTGTACTACAACAATATTCCAAACGTCTC	34
	A2	GAAGGTCGGAGTCAACGGATTGTACTACAACAATATTCCAAACGTCTG	35
	C1	CTTAGGGTTTGGATCCACACCAAGAA	36
DBSNP04821	A1	GAAGGTGACCAAGTTCATGCTTTTAAAGGAGTTAATATCCTTATGTCGGT	37
	A2	GAAGGTCGGAGTCAACGGATTAAAGGAGTTAATATCCTTATGTCGGC	38
	C1	CTCATCATTGCTTATTGCCTGTCATGAAT	39
DBSNP32292	A1	GAAGGTGACCAAGTTCATGCTTTGAGGATACTAAAGATGAAGTTGAAGTA	40
	A2	GAAGGTCGGAGTCAACGGATTGAGGATACTAAAGATGAAGTTGAAGTC	41
	C1	AAGAACTCTGTTTCTAATTAGCTATGGCAT	42
DBSNP217314	A1	GAAGGTGACCAAGTTCATGCTGCCTCAGCCCAGTAGAGCAGA	43
	A2	GAAGGTCGGAGTCAACGGATTCCTCAGCCCAGTAGAGCAGC	44
	C1	GGTCGAACATGGGTTGGGCCTA	45
DBSNP62256	A1	GAAGGTGACCAAGTTCATGCTTGTAAATTTTAGTTTGGTTTTGCTTTTTTCAA	46
	A2	GAAGGTCGGAGTCAACGGATTGTAAATTTTAGTTTGGTTTTGCTTTTTTCAG	47
	C1	CGAAGATGTGTTTCCCTGGGCATAT	48
DBSNP248920	A1	GAAGGTGACCAAGTTCATGCTCATATGGTCGTATGAAGTAACAAAACTTAA	49
	A2	GAAGGTCGGAGTCAACGGATTATATGGTCGTATGAAGTAACAAAACTTAG	50
	C1	CGGTTTCAAATGGAAATATACTCGGGTTT	51
DBSNP32465	A1	GAAGGTGACCAAGTTCATGCTTTTCATCTTCCTCCTGTCCAGCT	52
	A2	GAAGGTCGGAGTCAACGGATTCATCTTCCTCCTGTCCAGCG	53

	C1	CCTTAGTAACTCATTCTCCTACAGAGAAA	54
DBSNP217319	A1	GAAGGTGACCAAGTTCATGCTCGGCTTCACTGAACCGATTACCA	55
	A2	GAAGGTCGGAGTCAACGGATTGGCTTCACTGAACCGATTACCG	56
	C1	CTTTTCGTGTGGAACCATTGGTATAAGTT	57
DBSNP217320	A1	GAAGGTGACCAAGTTCATGCTACTAAAACTGAAAAAAACTTGATTTTAGAACAT	58
	A2	GAAGGTCGGAGTCAACGGATTACTAAAACTGAAAAAAACTTGATTTTAGAACAC	59
	C1	TATATWCTCCTGGCTGAGAGTGTTGAAA	60
DBSNP217322	A1	GAAGGTGACCAAGTTCATGCTATGGGCATTCTTTTGTTCTACACAACA	61
	A2	GAAGGTCGGAGTCAACGGATTGGGCATTCTTTTGTTCTACACAACG	62
	C1	GGCCTATAATAAGAGTATGGGTAGAAGAA	63
DBSNP217327	A1	GAAGGTGACCAAGTTCATGCTAGTGGATGTAATAAATATCAAAAGCATTGATTT	64
	A2	GAAGGTCGGAGTCAACGGATTGTGGATGTAATAAATATCAAAAGCATTGATTC	65
	C1	GTGTTATTGATAACGACAMTGATCTCTTTA	66
DBSNP217328	A1	GAAGGTGACCAAGTTCATGCTGTGTAGGTTTCCCTTTCCCTGAA	67
	A2	GAAGGTCGGAGTCAACGGATTGTGTAGGTTTCCCTTTCCCTGAG	68
	C1	CTTTATACTTTATCCCCAAGTTTCAGCATA	69
DBSNP51173	A1	GAAGGTGACCAAGTTCATGCTGTGTAGGTTTCCCTTTCCCTGAA	70
	A2	GAAGGTCGGAGTCAACGGATTGTGTAGGTTTCCCTTTCCCTGAG	71
	C1	CTTTATCCCCAAGTTTCAGCATAACACAA	72
DBSNP233856	A1	GAAGGTGACCAAGTTCATGCTCAGCACCAACCATGCTTCCATGT	73
	A2	GAAGGTCGGAGTCAACGGATTAGCACCAACCATGCTTCCATGG	74
	C1	CACCCTTCAAGGATTCTTGCTGCTT	75
DBSNP217329	A1	GAAGGTGACCAAGTTCATGCTAGGAGGTGAGAAAGACAGGAAGA	76
	A2	GAAGGTCGGAGTCAACGGATTGGAGGTGAGAAAGACAGGAAGC	77
	C1	ACTGCTCCTGTWAGCTGCGGTATT	78
DBSNP217331	A1	GAAGGTGACCAAGTTCATGCTGTCATCAAATTTACTACACCTAAATATGGT	79
	A2	GAAGGTCGGAGTCAACGGATTCATCAAATTTACTACACCTAAATATGGC	80
	C1	AGAAGCTTTATTCCTCGTAATCTCCTYTTT	81
DBSNP46180	A1	GAAGGTGACCAAGTTCATGCTAATAAGCAGAATCAGTATTTGGCCAGA	82
	A2	GAAGGTCGGAGTCAACGGATTAAGCAGAATCAGTATTTGGCCAGG	83

	C1	TTACATGGCAATCCTGTTTTGTCCACATT	84
DBSNP217333	A1	GAAGGTGACCAAGTTCATGCTGAACCAGTAATTGCCATGTATGGTATT	85
	A2	GAAGGTCGGAGTCAACGGATTAACCAGTAATTGCCATGTATGGTATC	86
	C1	GAAACATTTCGAGCCAATAAGAACAGCAT	87
DBSNP02628	A1	GAAGGTGACCAAGTTCATGCTCCACGCAGATCATCGATGGTAATA	88
	A2	GAAGGTCGGAGTCAACGGATTCACGCAGATCATCGATGGTAATC	89
	C1	GGAGTCAAGACCAATCCTCGTTGAA	90
DBSNP58735	A1	GAAGGTGACCAAGTTCATGCTCCGTGGCTCGAGCCAATTACTTA	91
	A2	GAAGGTCGGAGTCAACGGATTCGTGGCTCGAGCCAATTACTTG	92
	C1	TAGGACGGAAGTTTAAGTCTATGCCATAA	93
DBSNP217338	A1	GAAGGTGACCAAGTTCATGCTTAACCTCATCACTCTCACATTCT	94
	A2	GAAGGTCGGAGTCAACGGATTCTTAACCTCATCACTCTCACATTCC	95
	C1	CATTGGAGGGACTTCAGCAAGAACTA	96
DBSNP217339	A1	GAAGGTGACCAAGTTCATGCTGAGTAACCAAGTGAAAACAGCTGCA	97
	A2	GAAGGTCGGAGTCAACGGATTAGTAACCAAGTGAAAACAGCTGCG	98
	C1	GAAGCAAAAAATGTCCTTATTATTGTGGAT	99
DBSNP217340	A1	GAAGGTGACCAAGTTCATGCTTAAACTTTCGACTCAAAACTTTCATGAGA	100
	A2	GAAGGTCGGAGTCAACGGATTAACTTTCGACTCAAAACTTTCATGAGG	101
	C1	CCTCATAGAGCAAAAACGTRGGTTCTA	102
DBSNP10650	A1	GAAGGTGACCAAGTTCATGCTAGGAGCAACTTGGTTGAACAGATCT	103
	A2	GAAGGTCGGAGTCAACGGATTGAGCAACTTGGTTGAACAGATCC	104
	C1	CCAAAGTGTCAATGAGGCACATCTGTT	105
DBSNP27359	A1	GAAGGTGACCAAGTTCATGCTGCCACGCACCATTGTCT	106
	A2	GAAGGTCGGAGTCAACGGATTGCTGCCACGCACCATTGTCC	107
	C1	TGAACTCGATGGTTATGTCGGAATTTGTA	108

Example 4: Linkage analysis and map construction

[00231] MAPMAKER/EXP 3.0 (Lander *et al.* 1987) was used to construct a linkage group (LG) at LOD score 10.0 to confirm that the markers were mapped with their phenotypic trait together on LG N7. MAPMAKER/EXP 3.0 requires only one input file from each population, referred to as a locus genotype file. In the locus genotype file of the population, elite parent alleles were called "A", donor parent alleles were called "B", while heterozygous alleles were called "H". Missing data were represented with a dash (-) in the locus genotype file.

[00232] A high-resolution map of 7.0 cM and 36 SNP markers was constructed (**Figure 1**). The Rlm1 locus was fine mapped to a 177 Kb region (chrA07:209430266..21120083) (**Figure 1; Table 2**) in the publicly available reference genome of *B. napus* genotype Darmor between SNP markers DBSNP233856 (chrA07:20942726..20943026) and DBSNP10650 (chrA07:21120083..21120274) (**Figure 1; Table 2**). Twenty four SNP markers within 1.0 cM to the Rlm1 locus were identified for MAS (**Figure 1; Table 2**). The Rlm1 locus was mapped to a 6.6 cM region and co-segregated with SNP markers DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339 and DBSNP217340. The high-throughput SNP markers developed will greatly facilitate the stacking of multiple traits, and increase breeding accuracy and efficacy in breeding of canola cultivars with blackleg resistance.

What may be claimed is:

1. A method for identifying a first canola plant or germplasm that comprises blackleg resistance, the method comprising:

detecting in the first canola plant or germplasm at least one marker linked to blackleg resistance, wherein the at least one marker is selected from the group consisting of DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359.

2. The method according to claim 1, wherein the method comprises detecting in the first canola plant or germplasm a marker linked to at least one of DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359.

3. The method according to claim 2, wherein the detected marker displays a genetic recombination frequency of less than about 10% with the at least one marker of DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359.

4. The method according to claim 1, wherein the detected marker is selected from the group consisting of DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359.

5. The method according to claim 1, wherein the germplasm is a canola line or canola variety.

6. The method according to claim 1, wherein the detecting comprises detecting at least one allelic form of a single nucleotide polymorphism (SNP).

7. The method according to claim 1, wherein the detecting comprises amplifying the marker or a portion of the marker to yield an amplified marker amplicon, and detecting the resulting amplified marker amplicon.

8. The method according to claim 7, wherein the amplifying comprises:

admixing an amplification primer or amplification primer pair with a nucleic acid isolated from the first canola plant or germplasm, wherein the primer or primer pair is complementary or partially complementary to at least a portion of the marker, and is capable of initiating DNA polymerization by a DNA polymerase using the canola nucleic acid as a template; and

extending the primer or primer pair in a DNA polymerization reaction comprising a DNA polymerase and a template nucleic acid to generate at least one amplicon.

9. The method according to claim 8, wherein the nucleic acid is a DNA molecule or RNA molecule.

10. The method according to claim 7, wherein the amplifying comprises utilizing a polymerase chain reaction (PCR) or ligase chain reaction (LCR) using a nucleic acid isolated from the first canola plant or germplasm as a template in the PCR or LCR.

11. A method for producing an introgressed canola plant or germplasm, the method comprising:

introgressing at least one marker allele that is positively correlated with blackleg resistance from a first canola plant or germplasm into a second canola plant or germplasm, to produce an introgressed canola plant or germplasm, wherein the at least one marker is selected from the group consisting of DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359.

12. The method according to claim 11, wherein the second canola plant or germplasm displays lower blackleg resistance as compared to the first canola plant or germplasm, and wherein the introgressed canola plant or germplasm displays an increased resistance to blackleg disease as compared to the second plant or germplasm.

13. The introgressed canola plant or germplasm produced by the method according to claim 11.

14. The method according to claim 11, wherein the second canola plant or germplasm is a plant or germplasm from an elite canola variety or an exotic canola variety.

15. The method according to claim 2, wherein the detected marker is determined using the mapping population Nex845CL//Nex845CL/NT152343-20.

16. The method according to claim 1, wherein the detected marker is determined using software selected from TASSEL™, GeneFlow™, MapManager-QTX™, MAPMAKER/EXP 3.0 and QTL Cartographer

17. The method according to claim 1, wherein the method comprises electronically transmitting or electronically storing data representing the detected marker in a computer readable medium.

18. The method according to claim 1, wherein the method comprises selecting the first canola plant or germplasm.

19. The method according to claim 1, wherein the method comprises selecting a progeny of the first canola plant or germplasm.

20. The method according to claim 1, comprising crossing the first canola plant or germplasm with a second canola plant or germplasm.

21. The method according to claim 20, wherein the second canola plant or germplasm is a plant or germplasm from an elite canola variety or an exotic canola variety.

22. A method for producing a transgenic plant having a blackleg resistance phenotype, the method comprising:

introducing one or more exogenous nucleic acids into a target plant to produce a transgenic plant, wherein at least one of the one or more exogenous nucleic acids comprises an endogenous nucleotide sequence

from a canola plant that is linked in the canola plant to at least one marker selected from the group consisting of DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359, wherein the resulting transgenic plant displays blackleg resistance.

23. The method according to claim 22, wherein the target plant is a canola plant.

24. The method according to claim 22, wherein the endogenous nucleotide sequence from the canola plant is linked in the canola plant to the at least one marker such that the endogenous nucleotide sequence displays a genetic recombination frequency of not more than about 10% with the at least one marker.

25. The method according to claim 22, wherein the nucleotide sequence is isolated by positional cloning, and is identified by linkage to the at least one marker.

26. The method according to claim 22, wherein the exogenous nucleic acid corresponds to an open reading frame (ORF) that encodes a polypeptide that, when expressed in a canola plant, results in the canola plant having blackleg resistance.

27. The method according to claim 22, wherein the exogenous nucleic acid comprises an expression vector.

28. A system for identifying a canola plant predicted to have a blackleg resistance phenotype, the system comprising:

a set of marker probes or primers configured to detect at least one marker linked to blackleg resistance, wherein the marker is selected from the group consisting of DBSNP00397, DBSNP12121, DBSNP01841, DBSNP04821, DBSNP32292, DBSNP217314, DBSNP62256, DBSNP248920, DBSNP32465, DBSNP217319, DBSNP217320, DBSNP217322, DBSNP217327, DBSNP217328, DBSNP51173, DBSNP233856, DBSNP217329, DBSNP217331, DBSNP46180, DBSNP217333, DBSNP02628, DBSNP58735, DBSNP217338, DBSNP217339, DBSNP217340, DBSNP10650 and DBSNP27359;

a detector that is configured to detect one or more signal outputs from the set of marker probes or primers, or an amplicon thereof, thereby identifying the presence or absence of the marker; and

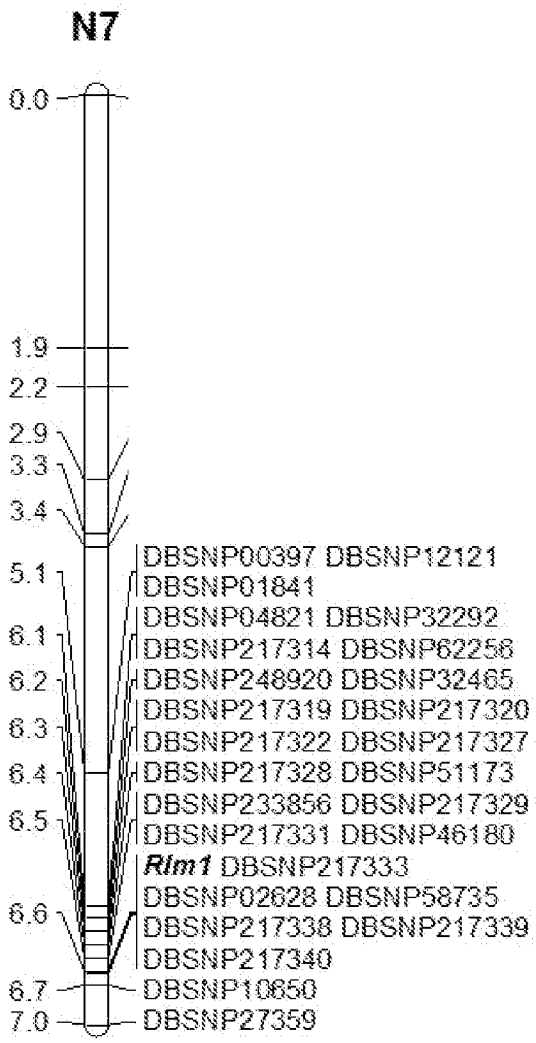
system instructions that correlate the presence or absence of the marker with the blackleg resistance phenotype.

29. The system of claim 28, wherein the detector detects one or more light emission, wherein the light emission is indicative of the presence or absence of a marker allele.

30. The system of claim 28, wherein the instructions comprise at least one reference table that includes a correlation between the presence or absence of a marker allele at the at least one detected marker and the blackleg resistance phenotype.

31. The system of claim 28, wherein the system comprises a nucleic acid sample from a plant or germplasm.

Figure 1


N7
0.0
1.9
2.2
2.9
3.3
3.4
5.1
6.1
6.2
6.3
6.4
6.5
6.6
6.7
7.0
DBSNP00397 DBSNP12121
DBSNP01841
DBSNP04821 DBSNP32292
DBSNP217314 DBSNP62256
DBSNP248920 DBSNP32465
DBSNP217319 DBSNP217320
DBSNP217322 DBSNP217327
DBSNP217328 DBSNP51173
DBSNP233856 DBSNP217329
DBSNP217331 DBSNP46180
Rim1 DBSNP217333
DBSNP02628 DBSNP58735
DBSNP217338 DBSNP217339
DBSNP217340
DBSNP10650
DBSNP27359

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/46336

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 15/29, C12N 15/54, C12N 15/82 (2019.01)

CPC - A01H 1/04, A01H 5/10, C12N 15/8282

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2015/0074850 A1 (DOW AGROSCIENCES LLC) 12 March 2015 (12.03.2015) claim 1; para [0032]	1-10, 15-21, 28-31
A	Genbank Accession No. FX692374 "TSA: Brassica rapa subsp. pekinensis RNA, contig: CF3_12005, transcribed RNA sequence" 17 December 2014 [online]. [Retrieved on 03 December 2019]. Retrieved from the internet: <URL: https://www.uniprot.org/uniprot/D8SB13> Entire document	1-10, 15-21, 28-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 December 2019	27 DEC 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/46336

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+, claims 1-10, 15-21 and 28-31, directed to a method or system for identifying a canola plant predicted to have a blackleg resistance phenotype. The method will be searched to the extent that the marker encompasses DBSNP00397. It is believed that claims 1-10, 15-21 and 28-31 encompass this first named invention, and thus these claims will be searched without fee to the extent that the marker encompasses DBSNP00397. Additional marker(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected marker(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be marker DBSNP12121, (claims 1-10, 15-21 and 28-31).

--continued on first extra sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10, 15-21, 28-31, limited to DBSNP00397

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 19/46336

--continued from Box III: Observations where unity of invention is lacking--

Group II+, claims 11-14 and 22-27, directed to a method for producing a plant having a blackleg resistance phenotype. Group II+ will be searched upon payment of additional fees. The method may be searched, for example, to encompass marker DBSNP00397 for an additional fee and election as such. It is believed that claims 11-14 and 22-27 read on this exemplary invention. Additional marker(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected marker(s). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be marker DBSNP12121 (claims 11-14 and 22-27).

The inventions listed as Groups I+ and II+ do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features

Group I+ has the special technical feature of identifying a canola plant predicted to have a blackleg resistance phenotype, that is not required by Group II+.

Group II+ has the special technical feature of producing a plant having a blackleg resistance phenotype, that is not required by Group I+.

The inventions of Group I+ and II+ each include the special technical feature of a unique marker, and is considered a distinct technical feature.

Common technical features

The inventions of Group I+ and Group II+ share the common technical feature of a canola plant having a marker that confers a blackleg resistance phenotype.

No technical features are shared between the markers of Group I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Group I+ were considered to share the technical features of including:

- a method for identifying a first canola plant or germplasm that comprises blackleg resistance, the method comprising: detecting in the first canola plant or germplasm at least one marker linked to blackleg resistance, or

- a system for identifying a canola plant predicted to have a blackleg resistance phenotype, the system comprising: a set of marker probes or primers configured to detect at least one marker linked to blackleg resistance, a detector that is configured to detect one or more signal outputs from the set of marker probes or primers, or an amplicon thereof, thereby identifying the presence or absence of the marker; and system instructions that correlate the presence or absence of the marker with the blackleg resistance phenotype,

these shared technical features are previously taught by US 2015/0074850 A1 to Dow Agrosciences LLC (hereinafter 'Dow').

No technical features are shared between the markers of Group II+ and, accordingly, these groups lack unity a priori.

Additionally, even if Group II+ were considered to share the technical features of including:

- a method for producing an introgressed canola plant or germplasm, the method comprising: introgressing at least one marker allele that is positively correlated with blackleg resistance from a first canola plant or germplasm into a second canola plant or germplasm, to produce an introgressed canola plant or germplasm, or

- a method for producing a transgenic plant having a blackleg resistance phenotype, the method comprising: introducing one or more exogenous nucleic acids into a target plant to produce a transgenic plant, wherein at least one of the one or more exogenous nucleic acids comprises an endogenous nucleotide sequence from a canola plant that is linked in the canola plant to at least one marker,

these shared technical features are previously taught by Dow.

Dow teaches a method for identifying a first canola plant or germplasm that comprises blackleg resistance, the method comprising: detecting in the first canola plant or germplasm at least one marker linked to blackleg resistance (claim 1 "method for identifying a first canola plant or germplasm that comprises blackleg resistance, the method comprising: detecting in the first canola plant or germplasm at least one marker linked to blackleg resistance").

Dow teaches a system for identifying a canola plant predicted to have a blackleg resistance phenotype, the system comprising: a set of marker probes or primers configured to detect at least one marker linked to blackleg resistance, a detector that is configured to detect one or more signal outputs from the set of marker probes or primers, or an amplicon thereof, thereby identifying the presence or absence of the marker; and system instructions that correlate the presence or absence of the marker with the blackleg resistance phenotype (para [0032] "systems and kits for identifying a canola plant that is likely to comprise blackleg resistance. Particular examples of such systems and kits may comprise a set of nucleic acid probes, each comprising a nucleotide sequence that is specifically hybridizable to a nucleotide sequence that is linked in canola to at least one marker that is linked to blackleg resistance...a detector that is configured to detect one or more signal outputs from the set of nucleic acid probes, or an amplicon thereof, thereby identifying the presence or absence of the at least one marker...").

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Dow teaches a method for producing an introgressed canola plant or germplasm, the method comprising: introgressing at least one marker allele that is positively correlated with blackleg resistance from a first canola plant or germplasm into a second canola plant or germplasm, to produce an introgressed canola plant or germplasm (claim 10 "method for producing an introgressed canola plant or germplasm, the method comprising: introgressing at least one marker allele that is positively correlated with blackleg resistance from a first canola plant or germplasm into a second canola plant or germplasm").

Dow teaches a method for producing a transgenic plant having a blackleg resistance phenotype, the method comprising: introducing one or more exogenous nucleic acids into a target plant to produce a transgenic plant, wherein at least one of the one or more exogenous nucleic acids comprises an endogenous nucleotide sequence from a canola plant that is linked in the canola plant to at least one marker (claim 17 "method for producing a transgenic plant having a blackleg resistance phenotype, the method comprising: introducing one or more exogenous nucleic acid into a target plant to produce a transgenic plant, wherein at least one of the one or more exogenous nucleic acid comprises an endogenous nucleotide sequence from a canola plant that is linked in the canola plant to at least one marker").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+ and II+ inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.