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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 February 2022 (24.02.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/040134 A1

(51) International Patent Classification:
C12N 15/82 (2006.01)

(21) International Application Number:
PCT/US2021/046227

(22) International Filing Date:
17 August 2021 (17.08.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/067,090 18 August 2020 (18.08.2020) US
63/154,960 01 March 2021 (01.03.2021) US

(71) Applicant: **PIONEER HI-BRED INTERNATIONAL, INC.** [US/US]; 7100 NW 62nd Avenue, PO Box 1014, Johnston, Iowa 50131-1014 (US).

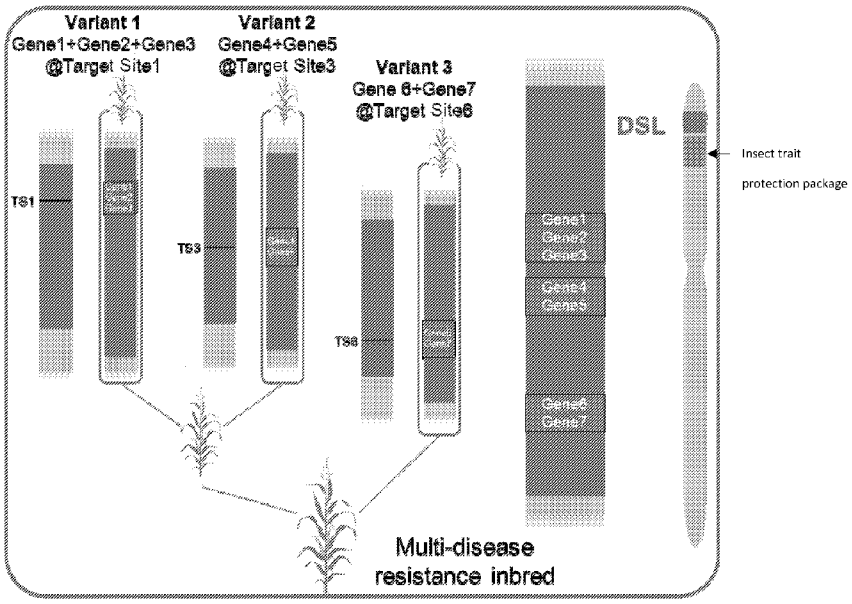
(72) Inventors: **FRANK, Mary J.**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US). **GAO, Huirong**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US). **HABBEN, Jeffrey**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US). **HUMBERT, Sabrina**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US). **KRISHNAMURTHY, Nandini**; 2412 NE 10TH CT, Grimes, Iowa 50111 (US). **LASSNER, Michael**; 4262 SE Belmont, Apartment 301, Portland, Oregon 97215 (US). **LI, Bailin**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US). **MEELEY, Robert B.**; 4511 Beaver Avenue, Des Moines, Iowa 50310 (US). **PERUGINI, Leandro Daniel**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US). **TABOR, Girma M.**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US). **WOLTERS, Petra J.**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US).

(74) Agent: **SHRESTHA, Jay**; 7250 NW 62nd Avenue, PO Box 552, Johnston, Iowa 50131-0552 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD,

(54) Title: MULTIPLE DISEASE RESISTANCE GENES AND GENOMIC STACKS THEREOF

FIG. 1



(57) Abstract: The field is molecular biology, and more specifically, methods for chromosomal engineering of multiple native genes, such as disease resistance genes in a genomic locus using site-specific editing to produce plants. Also described herein are methods of generating heterologous genomic locus in a plant that comprises a plurality of intraspecies polynucleotide sequences.

WO 2022/040134 A1

[Continued on next page]

ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

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

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

TITLE

MULTIPLE DISEASE RESISTANCE GENES AND GENOMIC STACKS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This Application claims the benefit of U.S. Provisional Application No. 63/154,960, filed on March 1, 2021, and 63/067,090, filed on August 18, 2020, each of which is incorporated herein by reference in its entirety.

FIELD

The field is molecular biology, and more specifically, methods for chromosomal engineering of multiple native genes, such as disease resistance genes in a genomic locus using site-specific editing to produce plants.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The official copy of the sequence listing is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file named 7823WO_ST25.txt created on August 10, 2021 and having a size 249 kilobytes and is filed concurrently with the specification. The sequence listing contained in this ASCII formatted document is part of the specification and is herein incorporated by reference in its entirety.

BACKGROUND

Plants contain a variety of genes and allelic variations thereof in their chromosomes. But those genes and alleles are often not linked in a manner to facilate faster breeding in combination with other traits such as insect resistance and herbicide tolerance. For example, resistance against multiple diseases is an essential component of crop improvement especially as disease pressure and patterns are quickly evolving under a changing climate. Resistance against a specific disease is typically achieved by introgressing a genomic region from a resistant source to an elite line. This process is time consuming and often leads to yield drag and other deleterious effects. In addition, introgressing loci conferring resistance against multiple diseases becomes impractical (in the context of time and resources) because of the number of loci involved and difficult in the case of genetically linked loci. This disclosure provides various methods and compositions to overcome some of these difficulties in breeding with multiplie loci and provides a platform for chromosomal engineering of gene stacks, such

as for example, disese resistant genes.

SUMMARY

Limitations of conventional breeding for introgressing a genomic region from a source to an elite line can be overcome by the compositions and methods described herein.

Presented herein are embodiments that describe a method for defining a region of the crop genome specifically engineered to confer disease resistance against multiple diseases, pathogen races, and combinations thereof. Further, disclosed herein is a method for inserting multiple disease resistance genes by gene editing and combining them within the defined region. Furthermore, disclosed herein is a method for deploying the engineered region in combination with other traits in a product context.

Provided are methods for generating a non-native, heterologous genomic locus in a crop plant cell that comprises a plurality of intraspecies polynucleotide sequences are provided herein. The methods include introducing two or more intraspecies polynucleotide sequences to a predetermined genomic locus in the plant cell, wherein the introducing step does not result in integration of a transgene or a foreign polynucleotide that is not native to the plant; the intraspecies polynucleotides confer one or more agronomic characteristics to the plant; at least one or more of the intraspecies polynucleotides are from different chromosome or the intraspecies polyucleotides are not located in the same chromosome in their native configuration compared to the heterologous genomic locus, prior to their integration into the heterologous genomic locus; and the introducing step comprises at least one site-directed genome modification that is not traditional breeding. In one embodiment, the genomic locus is adjacent to a genomic locus that comprises one or more transgenic traits, the transgenic traits comprising a plurality of polynucleotides that are not from the same plant species. In another embodiment, the transgenic traits comprise one or more traits conferring resistance to one or more insects. In yet another embodiment, the transgenic trait comprises a herbicide tolerance trait.

In one embodiment, the genomic locus is defined by a chromosomal region that is about 1 to about 5 cM or an equivalent physical chromosomal map distance for the crop plant species. In another embodiment, the chromosomal region is about 10Kb to about 50Mb. In some aspects, the plant is a corn, soy, canola, or cotton plant.

Also provided are methods of generating a disease super locus in an elite crop plant genome to increase trait introgression efficiency in the elite crop plant, the method comprising

introducing a plurality of disease resistance traits at a predetermined genomic locus of the crop plant chromosome by engineering insertion of one or more disease resistant genes, genomic translocation of one or more disease resistant genes through targeted chromosomal engineering, engineering duplication of one or more disease resistant genes at the genomic locus by targeted genome modification, modifying the genomic locus by introducing one or more insertions, deletions or substitions of nucleotides in the genome, or a combination of the foregoing. In one embodiment, the disease super locus is present in linkage disequilbrium with a transgenic trait. In another embodiment, the transgenic trait is selected from the group consisting of insect resistance, herbicide tolerance, and an agronomic trait. In yet another embodiment, the transgenic trait is a pre-existing commercial trait. In another embodiment, the trait introgression efficiency is increased by reducing the backcrosses by at least 50% or by reducing the backcrosses by three generations. In another embodiment, the trait introgression efficiency is increased by reducing the backcrosses by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%. In yet another embodiment, the trait introgression efficiency is increased by reducing the backcrosses by at least one, two, three, or four generations.

Also provided are methods for obtaining a plant cell with a modified genomic locus comprising at least two heterologous polynucleotide sequences that confer enhanced disease resistance to at least one plant disease, or at least two traits resulting in resistance to at least one disease through two different modes of action, wherein said at least two polynucleotide sequences are heterologous to the corresponding genomic locus and are from the same plant species. The methods include introducing a site-specific modification at at least one target site in a genomic locus in a plant cell; introducing at least two polynucleotide sequences that confer enhanced disease resistance to the target site; and obtaining the plant cell having a genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance. In one embodiment, the at least one target site comprises a target site selected from Table 2. In another embodiment, at least one of the two heterologous polynucleotides further comprise a site-specific modification. In yet another embodiment, the site-specific modification is genetic or epigenetic modification. In one embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein the polypeptide sequence has at least 90% identity to a polypeptide sequence selected from the group consisting of RppK (SEQ ID NO: 11), Ht1 (SEQ ID NO: 8), NLB18 (SEQ ID NOs: 3 or 5), NLR01 (SEQ ID No: 29), NLR02 (SEQ ID No: 26), RCG1 (SEQ ID Nos: 31), and RCG1b (SEQ ID Nos: 33).In another embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein

polypeptide sequence has at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a polypeptide sequence selected from the group consisting of RppK (SEQ ID NO: 11), Ht1 (SEQ ID NO: 8), NLB18 (SEQ ID NOs: 3 or 5), NLR01 (SEQ ID No: 29), NLR02 (SEQ ID No: 26), RCG1 (SEQ ID Nos: 31), and RCG1b (SEQ ID Nos: 33). In yet another embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein the polypeptide sequence has at least 90% identity to a polypeptide sequence selected from the group consisting of PRR03 (SEQ ID No: 36), PRR01 (SEQ ID No: 38), NLR01 (SEQ ID No: 41), and NLR04 (SEQ ID No: 44). In another embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein polypeptide sequence has at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a polypeptide sequence selected from the group consisting of PRR03 (SEQ ID No: 36), PRR01 (SEQ ID No: 38), NLR01 (SEQ ID No: 41), and NLR04 (SEQ ID No: 44).

Further provided are methods for obtaining a plant cell with a modified genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance to at least one plant disease, or at least two traits resulting in resistance to at least one disease through two different modes of action, wherein said at least two polynucleotide sequences are heterologous to the corresponding genomic locus. In one embodiment, the method comprises introducing a double-strand break or site-specific modification at one or more target sites in a genomic locus in a plant cell; introducing at least two polynucleotide sequences that confer enhanced disease resistance; and obtaining a plant cell having a genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance. In one embodiment, the at least one target site comprises a target site selected from Table 2. In another embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein the polypeptide sequence has at least 90% identity to a polypeptide sequence selected from the group consisting of RppK (SEQ ID NO: 11), Ht1 (SEQ ID NO: 8), NLB18 (SEQ ID NOs: 3 or 5), NLR01 (SEQ ID No: 29), NLR02 (SEQ ID No: 26), RCG1 (SEQ ID Nos: 31), and RCG1b (SEQ ID Nos: 33).In another embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein polypeptide sequence has at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a polypeptide sequence selected from the group consisting of RppK (SEQ ID NO: 11), Ht1 (SEQ ID NO: 8), NLB18 (SEQ ID NOs: 3 or 5), NLR01 (SEQ ID No: 29), NLR02 (SEQ ID No: 26), RCG1 (SEQ ID Nos: 31), and RCG1b (SEQ ID Nos: 33). In yet another embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein the polypeptide sequence has at least 90% identity to a

polypeptide sequence selected from the group consisting of PRR03 (SEQ ID No: 36), PRR01 (SEQ ID No: 38), NLR01 (SEQ ID No: 41), and NLR04 (SEQ ID No: 44). In another embodiment, the polynucleotide sequence encodes a polypeptide sequence wherein polypeptide sequence has at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to a polypeptide sequence selected from the group consisting of PRR03 (SEQ ID No: 36), PRR01 (SEQ ID No: 38), NLR01 (SEQ ID No: 41), and NLR04 (SEQ ID No: 44).

Further provided are plants comprising a modified genomic locus, the locus comprising at least a first modified target site and second modified target site, wherein the first modified target site comprises a first polynucleotide sequence that confers enhanced disease resistance to a first plant disease, and wherein the second modified target site comprises a second polynucleotide sequence that confers enhanced disease resistance to the first plant disease or to a second plant disease, wherein the first and the second polynucleotide sequences are heterologous to the modified genomic locus and are present within a genomic window of less than about 1 cM.

Also provided are methods for obtaining a plant cell with an modified genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance to at least one plant disease, or at least two traits resulting in resistance to at least one disease through two different modes of action, wherein said at least two polynucleotide sequences are heterologous to the corresponding genomic locus, wherein the genomic locus is located in the distal region of chromosome 1. In one embodiment, the genomic locus is located in the telomeric region.

Further provided are methods of breeding transgenic and native disease traits at a single locus in a plant comprising inserting at a single locus in a plant a first heterologous polynucleotide sequence that confers enhanced disease resistance to a first plant disease, and second heterologous polynucleotide sequence that confers enhanced disease resistance to the first plant disease or to a second plant disease; inserting at least one heterologous polynucleotide sequence encoding an insecticidal polypeptide, agronomic trait polypeptide, or a herbicide resistance polypeptide at the single locus; crossing the plant with the single locus with a different plant; and obtaining a progeny plant comprising the single locus; and wherein the single locus allows for fewer backcrosses compared to a plant with traits at more than one locus.

Also provided are methods of introgressing or forward breeding multiple disease resistance loci into an elite germplasm, wherein the timeframe for inserting two or more

heterologous polynucltotides from different donor plants into the elite line and developing the homozygous resistant lines is shorter. In one embodiment, the methods comprise improving agronomic traits with multiple disease resistance with reduced yield drag from breeding.

Further provided are methods of stacking genetically linked resistance genes from multiple sources. In one aspect, provide are modified modified crop plants comprising at least two, at least three, or at least four trait genes stacked in a single genomic locus, wherein the trait stack in a single locus allows for increased breeding efficiency and wherein the trait stack comprises at least two or more non-transgenic native traits introduced through genome modification, the native traits comprising polynucleotides from the same crop plant. In one embodiment, the trait genes are native traits. In another embodiment, the trait genes are selceted from the group consisting of herbicide tolerance, insect resistance, output traits, or disease resistance.

Further embodiments increase breeding efficiency for stacked traits, wherein the stacked traits are at a single locus and the stacked traits comprise at least two traits resulting in resistance to two different diseases, or at least two traits resulting in resistance to at least one disease through two different modes of action. In some embodiments, the stacked traits further comprise an insect control trait and/or a herbicide resistance trait at the single locus.

Further provided are modified plants comprising at least three disease resistance genes selected from the group consisting of NLB18, Ht1, and RppK, wherein the at least three disease resistence genes are located in the same genomic locus. In one embodiment, the modified plant is a maize plant. In one embodiment, the modified plant further comprises PRR03. In another embodiment, the modified plant further comprises at least one gene selected from NLR01, NLR02, RCG1, RCG1b, PRR03, PRR01, NLR01, and NLR04.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTINGS

FIG. 1 shows an example of a breeding stack approach. Variants 1, 2 and 3 are created independently by inserting respectively 3, 2, and 2 genes of interest at target sites 1, 3 and 6 at the super locus. Variant 1 and variant 2 are combined by crossing using standard breeding methods. Recombinants containing both the insertion at target site 1 and the insertion at target site 3 are selected. The new material is further combined with variant 3 by crossing using standard breeding methods. Recombinants containing the insertions at target sites 1 and 3 and the insertion at target site 6 are selected. The new material is comprised of multiple insertions of one or several genes of interest at several target sites at the super locus.

FIG. 2 is an illustration of possible scenarios to create a multi disease resistance stack. **A.** In a molecular stacking approach, one construct containing one or more genes of interest is used as the repair template to create an insertion of those genes at a target site at the super locus. **B.** In a breeding stack approach, genes of interest are inserted independently at several target sites and later assembled by breeding crosses to obtain the desired set of genes at the super locus. **C.** In a successive transformation approach, one construct containing one or more genes of interest is used as the repair template to create an insertion of those genes at a single target site. The material comprising this first insertion is then used as the transformation background for the next insertion, where another set of one or more genes of interest is inserted at the same or another target site at the super locus. This iterative process may be repeated to obtain the desired combination of genes of interest at the super locus. The three scenarios presented here can be used in combination to assemble the desired set of genes of interest at the super locus.

Description of the Sequence Listing

SEQ ID NO	**Sequence Description**
1	NLB18 (PH26N) genomic fragment
2	NLB18 (PH26N) cDNA 1
3	NLB18 (PH26N) Protein 1
4	NLB18 (PH26N) cDNA 2
5	NLB18 (PH26N) Protein 2
6	PH4GP Ht1 Genomic Sequence with Native Promoter and Terminator
7	PH4GP Ht1 Longer Model CDS Sequence
8	Translation of PH4GP Ht1 Longer Model CDS Sequence
9	Rppk Genomic Fragment
10	Rppk cDNA
11	Rppk Protein
12	DSL1-CR1 Guide with PAM
13	DSL1-CR3 Guide with PAM
14	DSL1-CR4 Guide with PAM
15	DSL1-CR5 Guide with PAM
16	DSL1-CR6 Guide with PAM
17	DSL1-CR7 Guide with PAM
18	DSL1-CR9 Guide with PAM

19	DSL1-CR14 Guide with PAM
20	DSL1-CR17 Guide with PAM
21	DSL1-CR18 Guide with PAM
22	pze-101020971
23	pze-101022341
24	NLR02 genomic frag
25	NLR02 CDS
26	NLR02 Protein
27	NLR01 genomic frag
28	NLR01 CDS
29	NLR01 Protein
30	Rcg1 CDS
31	Rcg1 Protein
32	Rcg1b CDS
33	Rcg1b Protein
34	GLS PRR 03 genomic frag
35	GLS PRR 03 (VAR1) CDS
36	CHR4 GLS PRR 03 (VAR1) AA
37	PRR01 (DRL-019.CDS)
38	PRR01 AA
39	NLR01_GENOMIC
40	NLR01_CDS
41	NLR01_PROTEIN
42	NLR04_GENOMIC
43	NLR04_CDS
44	NLR04_PROTEIN

DETAILED DESCRIPTION

It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended 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, reference to “plant”, “the plant” or “a plant” also includes a plurality of plants; also, depending on the context, use of the term “plant” can also include genetically similar or identical progeny of that plant; use of the term “a nucleic acid” optionally includes, as a practical matter, many copies of that nucleic acid molecule; similarly, the term “probe” optionally (and typically) encompasses many similar or identical probe molecules. 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 to which this disclosure belongs unless clearly indicated otherwise.

Compositions and methods are presented herein to modify the maize genome to produce maize plants that have enhanced resistance diseases including, but not limited to, northern leaf blight, anthracnose stalk rot, grey leaf spot, southern rust, tar spot, Stewart's Bacterial Wilt, Goss's Bacterial Wilt and Blight, Holcus Spot, Bacterial Leaf Blight, Bacterial Stalk Rot, Bacterial Leaf Streak, Bacterial Stripe and Leaf Spot, Chocolate Spot, Kernel Crown Spot, Corn Stunt, Maize Bushy Stunt, Seed Rot, Seedling Blight, and Damping-off, Pythium Root Rot (and Feeder Root Necrosis), Rhizoctonia Crown and Brace Root Rot, Fusarium Root Rot Diseases, Red Root Rot, Southern Corn Leaf Blight, Northern Corn Leaf Blight, Northern Corn Leaf Spot, Rostratum Leaf Spot, Physoderma Brown Spot, Eyespot, Anthracnose Leaf Blight, Gray Leaf Spot, Sorghum Downy Mildew, Java Downy Mildew, Philippine Downy Mildew, Sugarcane Downy Mildew, Rajasthan Downy Mildew, Spontaneum Downy Mildew, Leaf Splitting Downy Mildew, Graminicola Downy Mildew, Crazy Top, Brown Stripe Downy Mildew, Ergot, Common Smut, Head Smut, False Smut, Common Rust, Southern Rust, Tropical Rust, Gibberella Stalk Rot, Diplodia (Stenocarpella) Stalk Rot, Anthracnose Stalk Rot, Charcoal Rot, Fusarium Stalk Rot, Pythium Stalk Rot, Late Wilt, Aspergillus Ear Rot, Diplodia Ear Rot, Fusarium Kernel or Ear Rot, Gibberella Ear Rot or Red Rot, Nigrospora Ear or Cob Rot, Penicillium Ear Rot and Blue Eye, Mycotoxins and Mycotoxicoses, Maize Dwarf Mosaic, Maize Chlorotic Dwarf, Maize Streak, Maize Rough Dwarf, Root-Knot Nematodes, Lesion Nematodes, Sting Nematodes, Needle Nematodes, Stubby-Root Nematodes, Awl Nematodes, Corn Cyst Nematode, Dagger Nematodes, Lance Nematodes, Ring Nematodes, Spiral Nematodes, Stunt Nematodes, disease caused by a parasitic seed plant such as Witchweed, for example.

The term "allele" refers to one of two or more different nucleotide sequences that occur at a specific locus. Allele can include single nucleotide polymorphism (SNP) as well as larger insertions and deletions ("Indel").

The term "intraspecies" refers to organisms within the same species. The term "intraspecies polynucleotide sequence" refers to polynucleotide sequence from the same species such as maize DNA for maize crop, soy DNA for soybean crop, for example.

"Backcrossing" refers to the process whereby hybrid progeny are repeatedly crossed back to one of the parents. In a backcrossing scheme, the "donor" parent refers to the parental plant with the desired gene/genes, locus/loci, or specific phenotype to be introgressed. The

"recipient" parent (used one or more times) or "recurrent" parent (used two or more times) refers to the parental plant into which the gene or locus is being introgressed. For example, see Ragot, M. et al. (1995) Marker-assisted backcrossing: a practical example, in *Techniques et Utilisations des Marqueurs Moleculaires Les Colloques*, Vol. 72, pp. 45-56, and Openshaw et al., (1994) Marker-assisted Selection in *Backcross Breeding, Analysis of Molecular Marker Data*, pp. 41-43. The initial cross gives rise to the F_1 generation; the term "BC_1" then refers to the second use of the recurrent parent, "BC_2" refers to the third use of the recurrent parent, and so on.

A centimorgan ("cM") is a unit of measure of recombination frequency. One cM is equal to a 1% chance that a marker at one genetic locus will be separated from a marker at a second locus due to crossing over in a single generation.

As used herein, the term "chromosomal interval" designates a contiguous linear span of genomic DNA that resides *in planta* on a single chromosome. The genetic elements or genes located on a single chromosomal interval are physically linked. The size of a chromosomal interval is not particularly limited. In some aspects, the genetic elements located within a single chromosomal interval are genetically linked, typically with a genetic recombination distance of, for example, less than or equal to 20 cM, or alternatively, less than or equal to 10 cM. That is, two genetic elements within a single chromosomal interval undergo recombination at a frequency of less than or equal to 20% or 10%.

The phrase "closely linked", in the present application, means that recombination between two linked loci occurs with a frequency of equal to or less than about 10% (i.e., are separated on a genetic map by not more than 10 cM). Put another way, the closely linked loci co-segregate at least 90% of the time. Marker loci are especially useful with respect to the subject matter of the current disclosure when they demonstrate a significant probability of co-segregation (linkage) with a desired trait (e.g., resistance to gray leaf spot). Closely linked loci such as a marker locus and a second locus can display an inter-locus recombination frequency of 10% or less, preferably about 9% or less, still more preferably about 8% or less, yet more preferably about 7% or less, still more preferably about 6% or less, yet more preferably about 5% or less, still more preferably about 4% or less, yet more preferably about 3% or less, and still more preferably about 2% or less. In highly preferred embodiments, the relevant loci display a recombination a frequency of about 1% or less, e.g., about 0.75% or less, more preferably about 0.5% or less, or yet more preferably about 0.25% or less. Two loci that are localized to the same chromosome, and at such a distance that recombination between the two

loci occurs at a frequency of less than 10% (e.g., about 9 %, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.75%, 0.5%, 0.25%, or less) are also said to be "proximal to" each other. In some cases, two different markers can have the same genetic map coordinates. In that case, the two markers are in such close proximity to each other that recombination occurs between them with such low frequency that it is undetectable.

When a gene is introgressed, it is not only the gene that is introduced but also the flanking regions (Gepts. (2002). *Crop Sci;* 42: 1780-1790). This is referred to as "linkage drag." In the case where the donor plant is highly unrelated to the recipient plant, these flanking regions carry additional genes that may code for agronomically undesirable traits. This "linkage drag" may also result in reduced yield or other negative agronomic characteristics even after multiple cycles of backcrossing into the elite line. This is also sometimes referred to as "yield drag."

The term "crossed" or "cross" refers to a sexual cross and involved the fusion of two haploid gametes via pollination to produce diploid progeny (e.g., cells, seeds, or plants). The term encompasses both the pollination of one plant by another and selfing (or self-pollination, e.g., when the pollen and ovule are from the same plant).

The term "Disease Super Locus" or "DSL" as used herein generally refers to a genomic locus comprising at least two different disease resistant genes targeting at leat two different plant diseases, or comprising at least two different disease resistant genes targeting at least one disease through two different modes of action. In one embodiment, the disease resistance genes are within about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cM away from each other. In another embodiment, disease resistance genes are within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000, 100000, or about 1000000 bases away from each other. This DSL may be engineered in a manner that facilitates enhanced breeding with co-located transgenic herbicide and/or insect or other agronomic traits.

A "genetic map" is a description of genetic linkage relationships among loci on one or more chromosomes (or linkage groups) within a given species, generally depicted in a diagrammatic or tabular form. For each genetic map, distances between loci are measured by how frequently their alleles appear together in a population (their recombination frequencies). Alleles can be detected using DNA or protein markers, or observable phenotypes. A genetic map is a product of the mapping population, types of markers used, and the polymorphic

potential of each marker between different populations. Genetic distances between loci can differ from one genetic map to another. However, information can be correlated from one map to another using common markers. One of ordinary skill in the art can use common marker positions to identify positions of markers and other loci of interest on each individual genetic map. The order of loci should not change between maps, although frequently there are small changes in marker orders due to e.g. markers detecting alternate duplicate loci in different populations, differences in statistical approaches used to order the markers, novel mutation or laboratory error.

A “genetic map location” is a location on a genetic map relative to surrounding genetic markers on the same linkage group where a specified marker can be found within a given species.

“Genetic mapping” is the process of defining the linkage relationships of loci through the use of genetic markers, populations segregating for the markers, and standard genetic principles of recombination frequency.

“Genetic markers” are nucleic acids that are polymorphic in a population and where the alleles of which can be detected and distinguished by one or more analytic methods, e.g., RFLP, AFLP, isozyme, SNP, SSR, and the like. The term also refers to nucleic acid sequences complementary to the genomic sequences, such as nucleic acids used as probes. Markers corresponding to genetic polymorphisms between members of a population can be detected by methods well-established in the art. These include, e.g., PCR-based sequence specific amplification methods, detection of restriction fragment length polymorphisms (RFLP), detection of isozyme markers, detection of polynucleotide polymorphisms by allele specific hybridization (ASH), detection of amplified variable sequences of the plant genome, detection of self-sustained sequence replication, detection of simple sequence repeats (SSRs), detection of single nucleotide polymorphisms (SNPs), or detection of amplified fragment length polymorphisms (AFLPs). Well established methods are also known for the detection of expressed sequence tags (ESTs) and SSR markers derived from EST sequences and randomly amplified polymorphic DNA (RAPD).

“Genetic recombination frequency” is the frequency of a crossing over(recombination) between two genetic loci. Recombination frequency can be observed by following the segregation of markers and/or traits following meiosis.

As used herein, the term "haplotype" generally refers to a chromosomal region defined by a genetic characteristic that includes for example, one or more polymorphic molecular

markers. In other words, a haplotype is a set of DNA variations, or polymorphisms, that tend to be inherited together. A haplotype can refer to a combination of alleles or to a set of single nucleotide polymorphisms (SNPs) found on the same chromosome or a chromosomal region. A "haplotype window" generally refers to a chromosomal region that is delineated by statistical analyses and often in linkage disequilibrium. The spatial delineation of a haplotype window may change with available marker density and/or other genotyped information density that can differentiate multiple haplotypes.

The term "heterogeneity" is used to indicate that individuals within the group differ in genotype at one or more specific loci.

An "IBM genetic map" can refer to any of following maps: IBM, IBM2, IBM2 neighbors, IBM2 FPC0507, IBM2 2004 neighbors, IBM2 2005 neighbors, IBM2 2005 neighbors frame, IBM2 2008 neighbors, IBM2 2008 neighbors frame, or the latest version on the maizeGDB website. IBM genetic maps are based on a B73 x Mo17 population in which the progeny from the initial cross were random-mated for multiple generations prior to constructing recombinant inbred lines for mapping. Newer versions reflect the addition of genetic and BAC mapped loci as well as enhanced map refinement due to the incorporation of information obtained from other genetic maps or physical maps, cleaned date, or the use of new algorithms.

The term "inbred" refers to a line that has been bred for genetic homogeneity.

As used herein, the term "elite germplasm" or "elite plant" refers to any germplasm or plant, respectively, that has resulted from breeding and selection for superior agronomic performance.

The term "indel" refers to an insertion or deletion, wherein one line may be referred to as having an inserted nucleotide or piece of DNA relative to a second line, or the second line may be referred to as having a deleted nucleotide or piece of DNA relative to the first line.

The term "introgression" refers to the transmission of a desired allele of a genetic locus from one genetic background to another. For example, introgression of a desired allele at a specified locus can be transmitted 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 desired allele in its genome. Alternatively, for example, transmission of an allele can occur by recombination between two donor genomes, e.g., in a fused protoplast, where at least one of the donor protoplasts has the desired allele in its genome. The desired allele can be, e.g., detected by a marker that is associated with a phenotype, at a QTL, a transgene, or the like. In any case, offspring

comprising the desired allele can be repeatedly backcrossed to a line having a desired genetic background and selected for the desired allele, to result in the allele becoming fixed in a selected genetic background.

The process of "introgressing" is often referred to as "backcrossing" when the process is repeated two or more times.

A "line" or "strain" is a group of individuals of identical parentage that are generally inbred to some degree and that are generally homozygous and homogeneous at most loci (isogenic or near isogenic). A "subline" refers to an inbred subset of descendants that are genetically distinct from other similarly inbred subsets descended from the same progenitor.

As used herein, the term "linkage" is used to describe the degree with which one marker locus is associated with another marker locus or some other locus. The linkage relationship between a molecular marker and a locus affecting a phenotype is given as a "probability" or "adjusted probability". Linkage can be expressed as a desired limit or range. For example, in some embodiments, any marker is linked (genetically and physically) to any other marker when the markers are separated by less than 50, 40, 30, 25, 20, or 15 map units (or cM) of a single meiosis map (a genetic map based on a population that has undergone one round of meiosis, such as e.g. an F_2; the IBM2 maps consist of multiple meioses). In some aspects, it is advantageous to define a bracketed range of linkage, for example, between 10 and 20 cM, between 10 and 30 cM, or between 10 and 40 cM. The more closely a marker is linked to a second locus, the better an indicator for the second locus that marker becomes. Thus, "closely linked loci" such as a marker locus and a second locus display an inter-locus recombination frequency of 10% or less, preferably about 9% or less, still more preferably about 8% or less, yet more preferably about 7% or less, still more preferably about 6% or less, yet more preferably about 5% or less, still more preferably about 4% or less, yet more preferably about 3% or less, and still more preferably about 2% or less. In highly preferred embodiments, the relevant loci display a recombination frequency of about 1% or less, e.g., about 0.75% or less, more preferably about 0.5% or less, or yet more preferably about 0.25% or less. Two loci that are localized to the same chromosome, and at such a distance that recombination between the two loci occurs at a frequency of less than 10% (e.g., about 9 %, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.75%, 0.5%, 0.25%, or less) are also said to be "in proximity to" each other. Since one cM is the distance between two markers that show a 1% recombination frequency, any marker is closely linked (genetically and physically) to any other marker that is in close proximity, e.g., at or less than 10 cM distant. Two closely linked markers on the same

chromosome can be positioned 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.75, 0.5 or 0.25 cM or less from each other.

The term "linkage disequilibrium" refers to a non-random segregation of genetic loci or traits (or both). In either case, linkage disequilibrium implies that the relevant loci are within sufficient physical proximity along a length of a chromosome so that they segregate together with greater than random (i.e., non-random) frequency. Markers that show linkage disequilibrium are considered linked. Linked loci co-segregate more than 50% of the time, e.g., from about 51% to about 100% of the time. In other words, two markers that co-segregate have a recombination frequency of less than 50% (and by definition, are separated by less than 50 cM on the same linkage group.) As used herein, linkage can be between two markers, or alternatively between a marker and a locus affecting a phenotype. A marker locus can be "associated with" (linked to) a trait. The degree of linkage of a marker locus and a locus affecting a phenotypic trait is measured, e.g., as a statistical probability of co-segregation of that molecular marker with the phenotype (e.g., an F statistic or LOD score).

Linkage disequilibrium is most commonly assessed using the measure r^2, which is calculated using the formula described by Hill, W.G. and Robertson, A, Theor. Appl. Genet. 38:226-231(1968). When $r^2 = 1$, complete LD exists between the two marker loci, meaning that the markers have not been separated by recombination and have the same allele frequency. The r^2 value will be dependent on the population used. Values for r^2 above 1/3 indicate sufficiently strong LD to be useful for mapping (Ardlie et al., Nature Reviews Genetics 3:299-309 (2002)). Hence, alleles are in linkage disequilibrium when r^2 values between pairwise marker loci are greater than or equal to 0.33, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0.

As used herein, "linkage equilibrium" describes a situation where two markers independently segregate, i.e., sort among progeny randomly. Markers that show linkage equilibrium are considered unlinked (whether or not they lie on the same chromosome).

A "marker" is a means of finding a position on a genetic or physical map, or else linkages among markers and trait loci (loci affecting traits). The position that the marker detects may be known via detection of polymorphic alleles and their genetic mapping, or else by hybridization, sequence match or amplification of a sequence that has been physically mapped. A marker can be a DNA marker (detects DNA polymorphisms), a protein (detects variation at an encoded polypeptide), or a simply inherited phenotype (such as the 'waxy' phenotype). A DNA marker can be developed from genomic nucleotide sequence or from expressed nucleotide sequences (e.g., from a spliced RNA or a cDNA). Depending on the DNA marker

technology, the marker will consist of complementary primers flanking the locus and/or complementary probes that hybridize to polymorphic alleles at the locus. A DNA marker, or a genetic marker, can also be used to describe the gene, DNA sequence or nucleotide on the chromosome itself (rather than the components used to detect the gene or DNA sequence) and is often used when that DNA marker is associated with a particular trait in human genetics (e.g. a marker for breast cancer). The term marker locus is the locus (gene, sequence or nucleotide) that the marker detects.

Markers that detect genetic polymorphisms between members of a population are well-established in the art. Markers can be defined by the type of polymorphism that they detect and also the marker technology used to detect the polymorphism. Marker types include but are not limited to, e.g., detection of restriction fragment length polymorphisms (RFLP), detection of isozyme markers, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), detection of simple sequence repeats (SSRs), detection of amplified variable sequences of the plant genome, detection of self-sustained sequence replication, or detection of single nucleotide polymorphisms (SNPs). SNPs can be detected e.g. via DNA sequencing, PCR-based sequence specific amplification methods, detection of polynucleotide polymorphisms by allele specific hybridization (ASH), dynamic allele-specific hybridization (DASH), molecular beacons, microarray hybridization, oligonucleotide ligase assays, Flap endonucleases, 5' endonucleases, primer extension, single strand conformation polymorphism (SSCP) or temperature gradient gel electrophoresis (TGGE). DNA sequencing, such as the pyrosequencing technology has the advantage of being able to detect a series of linked SNP alleles that constitute a haplotype. Haplotypes tend to be more informative (detect a higher level of polymorphism) than SNPs.

A "marker allele", alternatively an "allele of a marker locus", can refer to one of a plurality of polymorphic nucleotide sequences found at a marker locus in a population.

"Marker assisted selection" (of MAS) is a process by which individual plants are selected based on marker genotypes.

"Marker assisted counter-selection" is a process by which marker genotypes are used to identify plants that will not be selected, allowing them to be removed from a breeding program or planting.

A "marker haplotype" refers to a combination of alleles or haplotypes at a marker locus.

A "marker locus" is a specific chromosome location in the genome of a species where a specific marker can be found. A marker locus can be used to track the presence of a second

linked locus, e.g., one that affects the expression of a phenotypic trait. For example, a marker locus can be used to monitor segregation of alleles at a genetically or physically linked locus.

A “marker probe” is a nucleic acid sequence or molecule that can be used to identify the presence of a marker locus, e.g., a nucleic acid probe that is complementary to a marker locus sequence, through nucleic acid hybridization. Marker probes comprising 30 or more contiguous nucleotides of the marker locus (“all or a portion” of the marker locus sequence) may be used for nucleic acid hybridization. Alternatively, in some aspects, a marker probe refers to a probe of any type that is able to distinguish (i.e., genotype) the particular allele that is present at a marker locus.

The term "molecular marker" may be used to refer to a genetic marker, as defined above, or an encoded product thereof (e.g., a protein) used as a point of reference when identifying a linked locus. A marker can be derived from genomic nucleotide sequences or from expressed nucleotide sequences (e.g., from a spliced RNA, a cDNA, etc.), or from an encoded polypeptide. The term also refers to nucleic acid sequences complementary to or flanking the marker sequences, such as nucleic acids used as probes or primer pairs capable of amplifying the marker sequence. A "molecular marker probe" is a nucleic acid sequence or molecule that can be used to identify the presence of a marker locus, e.g., a nucleic acid probe that is complementary to a marker locus sequence. Alternatively, in some aspects, a marker probe refers to a probe of any type that is able to distinguish (i.e., genotype) the particular allele that is present at a marker locus. Nucleic acids are "complementary" when they specifically hybridize in solution, e.g., according to Watson-Crick base pairing rules. Some of the markers described herein are also referred to as hybridization markers when located on an indel region, such as the non-collinear region described herein. This is because the insertion region is, by definition, a polymorphism *vis a vis* a plant without the insertion. Thus, the marker need only indicate whether the indel region is present or absent. Any suitable marker detection technology may be used to identify such a hybridization marker, e.g. SNP technology is used in the examples provided herein.

“*Exserohilum turcicum*”, previously referred to as *Helminthosporium turcicum*, is the fungal pathogen that induces northern leaf blight infection. The fungal pathogen is also referred to herein as *Exserohilum* or *Et.*

The phrase “Gray Leaf Spot” or “GLS” refers to a cereal disease caused by the fungal pathogen *Cercospora zeae-maydis*, which characteristically produces long, rectangular, grayish-tan leaf lesions which run parallel to the leaf vein.

"Disease resistance" (such as, for example, northern leaf blight resistance) is a characteristic of a plant, wherein the plant avoids, miminimzes, or reduces the disease symptoms that are the outcome of plant-pathogen interactions, such as maize-*Exserohilum turcicum* interactions. That is, pathogens are prevented from causing plant diseases and the associated disease symptoms, or alternatively, the disease symptoms caused by the pathogen are minimized or lessened.

A "locus" is a position on a chromosome where a gene or marker is located.

"Resistance" is a relative term, indicating that the infected plant produces better plant health or yield of maize than another, similarly treated, more susceptible plant. That is, the conditions cause a reduced decrease in maize survival, growth, and/or yield in a tolerant maize plant, as compared to a susceptible maize plant. One of skill will appreciate that maize plant resistance to northern leaf blight, or the pathogen causing such, can represent a spectrum of more resistant or less resistant phenotypes, and can vary depending on the severity of the infection. However, by simple observation, one of skill can determine the relative resistance or susceptibility of different plants, plant lines or plant families to northern leaf blight, and furthermore, will also recognize the phenotypic gradations of "resistant". For example, a 1 to 9 visual rating indicating the level of resistance to northern leaf blight can be used. A higher score indicates a higher resistance. The terms "tolerance" and "resistance" are used interchangeably herein.

The resistance may be "newly conferred" or "enhanced". "Newly conferred" or "enhanced" resistance refers to an increased level of resistance against a particular pathogen, a wide spectrum of pathogens, or an infection caused by the pathogen(s). An increased level of resistance against a particular fungal pathogen, such as *Et*, for example, constitutes "enhanced" or improved fungal resistance. The embodiments may enhance or improve fungal plant pathogen resistance.

In some embodiments, gene editing may be facilitated through the induction of a double-stranded break (a "DSB") in a defined position in the genome near the desired alteration. DSBs can be induced using any DSB-inducing agent available, including, but not limited to, TALENs, meganucleases, zinc finger nucleases, Cas9-gRNA systems (based on bacterial CRISPR-Cas systems), and the like. In some embodiments, the introduction of a DSB can be combined with the introduction of a polynucleotide modification template.

A polynucleotide modification template may be introduced into a cell by any method known in the art, such as, but not limited to, transient introduction methods, transfection, electroporation, microinjection, particle mediated delivery, topical application, whiskers mediated delivery, delivery via cell-penetrating peptides, or mesoporous silica nanoparticle (MSN)-mediated direct delivery.

The polynucleotide modification template may be introduced into a cell as a single stranded polynucleotide molecule, a double stranded polynucleotide molecule, or as part of a circular DNA (vector DNA). The polynucleotide modification template may also be tethered to the guide RNA and/or the Cas endonuclease. Tethered DNAs can allow for co-localizing target and template DNA, useful in genome editing and targeted genome regulation, and can also be useful in targeting post-mitotic cells where function of endogenous homologous recombination HR machinery is expected to be highly diminished (Mali et al. 2013 *Nature Methods* Vol. 10 : 957-963.) The polynucleotide modification template may be present transiently in the cell or it can be introduced via a viral replicon.

A "modified nucleotide" or "edited nucleotide" refers to a nucleotide sequence of interest that comprises at least one alteration when compared to its non-modified nucleotide sequence, and the alteration is by deliberate human intervention. Such "alterations" include, for example: (i) replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, or (iv) any combination of (i) – (iii). An "edited cell" or an "edited plant cell" refers to a cell containing at least one alteration in the genomic sequence when compared to a control cell or plant cell that does not include such alteration in the genomic sequence.

The term "polynucleotide modification template" or "modification template" as used herein refers to a polynucleotide that comprises at least one nucleotide modification when compared to the target nucleotide sequence to be edited. A nucleotide modification can be at least one nucleotide substitution, addition or deletion. Optionally, the polynucleotide modification template can further comprise homologous nucleotide sequences flanking the at least one nucleotide modification, wherein the flanking homologous nucleotide sequences provide sufficient homology to the desired nucleotide sequence to be edited.

The process for editing a genomic sequence combining DSBs and modification templates generally comprises: providing to a host cell a DSB-inducing agent, or a nucleic acid encoding a DSB-inducing agent, that recognizes a target sequence in the chromosomal sequence, and wherein the DSB-inducing agent is able to induce a DSB in the genomic

sequence; and providing at least one polynucleotide modification template comprising at least one nucleotide alteration when compared to the nucleotide sequence to be edited. The endonuclease may be provided to a cell by any method known in the art, for example, but not limited to transient introduction methods, transfection, microinjection, and/or topical application or indirectly via recombination constructs. The endonuclease may be provided as a protein or as a guided polynucleotide complex directly to a cell or indirectly via recombination constructs. The endonuclease may be introduced into a cell transiently or can be incorporated into the genome of the host cell using any method known in the art. In the case of a CRISPR-Cas system, uptake of the endonuclease and/or the guided polynucleotide into the cell can be facilitated with a Cell Penetrating Peptide (CPP) as described in WO2016073433.

As used herein, a "genomic region" refers to a segment of a chromosome in the genome of a cell. In one embodiment, a genomic region includes a segment of a chromosome in the genome of a cell that is present on either side of the target site or, alternatively, also comprises a portion of the target site. The genomic region may comprise at least 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, 5- 50, 5-55, 5-60, 5-65, 5- 70, 5-75, 5-80, 5-85, 5-90, 5-95, 5-100, 5-200, 5-300, 5-400, 5-500, 5-600, 5-700, 5-800, 5-900, 5-1000, 5-1100, 5-1200, 5-1300, 5-1400, 5-1500, 5-1600, 5-1700, 5-1800, 5-1900, 5-2000, 5-2100, 5-2200, 5-2300, 5-2400, 5-2500, 5-2600, 5-2700, 5-2800. 5-2900, 5-3000, 5-3100 or more bases such that the genomic region has sufficient homology to undergo homologous recombination with the corresponding region of homology.

A "modified plant" refers to any plant that has a heterologous polynucleotide purposefully inserted into its genome, wherein the inserted polynucleotide is heterologous to the plant, heterologous to the position in the genome, or has an altered sequence compared to an unmodified plant from the same genetic background. A modified plant may be created through transgenic applications, genomic modifications including CRISPR or Talens, traditional breeding, or any combination thereof.

The term "site of action" generally refers to a specific physical location or biochemical site within the organism where a specific ligand or polypeptide acts or directly interacts. For example, an effector polypeptide may interact with a disease resistance polypeptide.

The term "mode of action" generally describes a functional or anatomical change resulting from the exposure of an organism to a substance such as polypeptide or regulatory RNA. The term "mode of action" may also refer to a specific mechanism of recognition or action at the cellular or molecular level.

In some embodiments, a modified plant comprises a heterologous polynucleotide, the transcript of which is alternatively spliced into two messenger RNAs encoding two polypeptides, wherein the two polypeptides have a different site of action or mode of action. In some embodiments, the modified plant has increased resistance durability to a plant pathogen when expressing said transcript, which is alternatively spliced into two messenger RNAs encoding two polypeptides, wherein the two polypeptides have a different site of action or mode of action. In other embodiments, the modified plant has increased resistance to more than one plant pathogen when expressing said transcript, which is alternatively spliced into two messenger RNAs encoding two polypeptides, wherein the two polypeptides have a different site of action or mode of action.

In another embodiment, a modified plant comprises at least two heterologous polynucleotides wherein the polynucleotides produce one or more non-coding transcripts or encode one or more polypeptides. In another embodiment, said one or more non-coding transcripts or one or more polypeptides target the same plant pathogen. In another embodiment, said one or more non-coding transcripts or one or more polypeptides target the same plant pathogen via different modes of action.

In one embodiment, a modified plant comprises at least two heterologous polynucleotides wherein the polynucleotides produce one or more non-coding transcripts or encode one or more polypeptides. In another embodiment, said least two heterologous polynucleotides are derived from the same species. In yet another embodiment, said least two heterologous polynucleotides are derived from different species.

TAL effector nucleases (TALEN) are a class of sequence-specific nucleases that can be used to make double-strand breaks at specific target sequences in the genome of a plant or other organism. (See Miller et al. (2011) *Nature Biotechnology* 29:143–148).

Endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain. Endonucleases include restriction endonucleases, which cleave DNA at specific sites without damaging the bases, and meganucleases, also known as homing endonucleases (HEases), which like restriction endonucleases, bind and cut at a specific recognition site, however the recognition sites for meganucleases are typically longer, about 18 bp or more (patent application PCT/US12/30061, filed on March 22, 2012). Meganucleases have been classified into four families based on conserved sequence motifs, the families are the LAGLIDADG, GIY-YIG, H-N-H, and His-Cys box families. These motifs participate in the coordination of metal ions and hydrolysis of phosphodiester bonds. HEases are notable for

their long recognition sites, and for tolerating some sequence polymorphisms in their DNA substrates. The naming convention for meganuclease is similar to the convention for other restriction endonuclease. Meganucleases are also characterized by prefix F-, I-, or PI- for enzymes encoded by free-standing ORFs, introns, and inteins, respectively. One step in the recombination process involves polynucleotide cleavage at or near the recognition site. The cleaving activity can be used to produce a double-strand break. For reviews of site-specific recombinases and their recognition sites, see, Sauer (1994) Curr Op Biotechnol 5:521-7; and Sadowski (1993) *FASEB* 7:760-7. In some examples the recombinase is from the Integrase or Resolvase families.

Zinc finger nucleases (ZFNs) are engineered double-strand break inducing agents comprised of a zinc finger DNA binding domain and a double-strand-break-inducing agent domain. Recognition site specificity is conferred by the zinc finger domain, which typically comprising two, three, or four zinc fingers, for example having a C2H2 structure, however other zinc finger structures are known and have been engineered. Zinc finger domains are amenable for designing polypeptides which specifically bind a selected polynucleotide recognition sequence. ZFNs include an engineered DNA-binding zinc finger domain linked to a non-specific endonuclease domain, for example nuclease domain from a Type IIs endonuclease such as FokI. Additional functionalities can be fused to the zinc-finger binding domain, including transcriptional activator domains, transcription repressor domains, and methylases. In some examples, dimerization of nuclease domain is required for cleavage activity. Each zinc finger recognizes three consecutive base pairs in the target DNA. For example, a 3 finger domain recognized a sequence of 9 contiguous nucleotides, with a dimerization requirement of the nuclease, two sets of zinc finger triplets are used to bind an 18 nucleotide recognition sequence.

Genome editing using DSB-inducing agents, such as Cas9-gRNA complexes, has been described, for example in U.S. Patent Application US 2015-0082478 A1, WO2015/026886 A1, WO2016007347, and WO201625131, all of which are incorporated by reference herein.

The term “Cas gene” herein refers to a gene that is generally coupled, associated or close to, or in the vicinity of flanking CRISPR loci in bacterial systems. The terms “Cas gene”, “CRISPR-associated (Cas) gene” are used interchangeably herein. The term “Cas endonuclease” herein refers to a protein, or complex of proteins, encoded by a Cas gene. A Cas endonuclease as disclosed herein, when in complex with a suitable polynucleotide component, is capable of recognizing, binding to, and optionally nicking or cleaving all or part of a specific

DNA target sequence. A Cas endonuclease as described herein comprises one or more nuclease domains. Cas endonucleases of the disclosure includes those having a HNH or HNH-like nuclease domain and / or a RuvC or RuvC-like nuclease domain. A Cas endonuclease of the disclosure may include a Cas9 protein, a Cpf1 protein, a C2c1 protein, a C2c2 protein, a C2c3 protein, Cas3, Cas 5, Cas7, Cas8, Cas10, or complexes of these.

As used herein, the terms "guide polynucleotide/Cas endonuclease complex", "guide polynucleotide/Cas endonuclease system", " guide polynucleotide/Cas complex", "guide polynucleotide/Cas system", "guided Cas system" are used interchangeably herein and refer to at least one guide polynucleotide and at least one Cas endonuclease that are capable of forming a complex, wherein said guide polynucleotide/Cas endonuclease complex can direct the Cas endonuclease to a DNA target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break) the DNA target site. A guide polynucleotide/Cas endonuclease complex herein can comprise Cas protein(s) and suitable polynucleotide component(s) of any of the four known CRISPR systems (Horvath and Barrangou, 2010, *Science* 327:167-170) such as a type I, II, or III CRISPR system. A Cas endonuclease unwinds the DNA duplex at the target sequence and optionally cleaves at least one DNA strand, as mediated by recognition of the target sequence by a polynucleotide (such as, but not limited to, a crRNA or guide RNA) that is in complex with the Cas protein. Such recognition and cutting of a target sequence by a Cas endonuclease typically occurs if the correct protospacer-adjacent motif (PAM) is located at or adjacent to the 3' end of the DNA target sequence. Alternatively, a Cas protein herein may lack DNA cleavage or nicking activity, but can still specifically bind to a DNA target sequence when complexed with a suitable RNA component. (See also U.S. Patent Application US 2015-0082478 A1, and US 2015-0059010 A1, both hereby incorporated in its entirety by reference).

A guide polynucleotide/Cas endonuclease complex can cleave one or both strands of a DNA target sequence. A guide polynucleotide/Cas endonuclease complex that can cleave both strands of a DNA target sequence typically comprises a Cas protein that has all of its endonuclease domains in a functional state (e.g., wild type endonuclease domains or variants thereof retaining some or all activity in each endonuclease domain). Thus, a wild type Cas protein, or a variant thereof, retaining some or all activity in each endonuclease domain of the Cas protein, is a suitable example of a Cas endonuclease that can cleave both strands of a DNA target sequence. A Cas9 protein comprising functional RuvC and HNH nuclease domains is an example of a Cas protein that can cleave both strands of a DNA target sequence. A guide

polynucleotide/Cas endonuclease complex that can cleave one strand of a DNA target sequence can be characterized herein as having nickase activity (e.g., partial cleaving capability). A Cas nickase typically comprises one functional endonuclease domain that allows the Cas to cleave only one strand (i.e., make a nick) of a DNA target sequence. For example, a Cas9 nickase may comprise (i) a mutant, dysfunctional RuvC domain and (ii) a functional HNH domain (e.g., wild type HNH domain). As another example, a Cas9 nickase may comprise (i) a functional RuvC domain (e.g., wild type RuvC domain) and (ii) a mutant, dysfunctional HNH domain. Non-limiting examples of Cas9 nickases suitable for use herein are disclosed in U.S. Patent Appl. Publ. No. 2014/0189896, which is incorporated herein by reference.

A pair of Cas9 nickases may be used to increase the specificity of DNA targeting. In general, this can be done by providing two Cas9 nickases that, by virtue of being associated with RNA components with different guide sequences, target and nick nearby DNA sequences on opposite strands in the region for desired targeting. Such nearby cleavage of each DNA strand creates a double strand break (i.e., a DSB with single-stranded overhangs), which is then recognized as a substrate for non-homologous-end-joining, NHEJ (prone to imperfect repair leading to mutations) or homologous recombination, HR. Each nick in these embodiments can be at least about 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 (or any integer between 5 and 100) bases apart from each other, for example. One or two Cas9 nickase proteins herein can be used in a Cas9 nickase pair. For example, a Cas9 nickase with a mutant RuvC domain, but functioning HNH domain (i.e., Cas9 HNH+/RuvC-), could be used (e.g., Streptococcus pyogenes Cas9 HNH+/RuvC-). Each Cas9 nickase (e.g., Cas9 HNH+/RuvC-) would be directed to specific DNA sites nearby each other (up to 100 base pairs apart) by using suitable RNA components herein with guide RNA sequences targeting each nickase to each specific DNA site.

A Cas protein may be part of a fusion protein comprising one or more heterologous protein domains (e.g., 1, 2, 3, or more domains in addition to the Cas protein). Such a fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains, such as between Cas and a first heterologous domain. Examples of protein domains that may be fused to a Cas protein herein include, without limitation, epitope tags (e.g., histidine [His], V5, FLAG, influenza hemagglutinin [HA], myc, VSV-G, thioredoxin [Trx]), reporters (e.g., glutathione-5-transferase [GST], horseradish peroxidase [HRP], chloramphenicol acetyltransferase [CAT], beta-galactosidase, beta-glucuronidase [GUS], luciferase, green fluorescent protein [GFP], HcRed, DsRed, cyan fluorescent protein [CFP],

yellow fluorescent protein [YFP], blue fluorescent protein [BFP]), and domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity (e.g., VP16 or VP64), transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. A Cas protein can also be in fusion with a protein that binds DNA molecules or other molecules, such as maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD), GAL4A DNA binding domain, and herpes simplex virus (HSV) VP16. See PCT patent applications PCT/US16/32073, filed May 12, 2016 and PCT/US16/32028 filed May 12, 2016 (both applications incorporated herein by reference) for more examples of Cas proteins.

A guide polynucleotide/Cas endonuclease complex in certain embodiments may bind to a DNA target site sequence, but does not cleave any strand at the target site sequence. Such a complex may comprise a Cas protein in which all of its nuclease domains are mutant, dysfunctional. For example, a Cas9 protein herein that can bind to a DNA target site sequence, but does not cleave any strand at the target site sequence, may comprise both a mutant, dysfunctional RuvC domain and a mutant, dysfunctional HNH domain. A Cas protein herein that binds, but does not cleave, a target DNA sequence can be used to modulate gene expression, for example, in which case the Cas protein could be fused with a transcription factor (or portion thereof) (e.g., a repressor or activator, such as any of those disclosed herein). In other aspects, an inactivated Cas protein may be fused with another protein having endonuclease activity, such as a Fok I endonuclease.

"Cas9" (formerly referred to as Cas5, Csn1, or Csx12) herein refers to a Cas endonuclease of a type II CRISPR system that forms a complex with a crNucleotide and a tracrNucleotide, or with a single guide polynucleotide, for specifically recognizing and cleaving all or part of a DNA target sequence. Cas9 protein comprises a RuvC nuclease domain and an HNH (H-N-H) nuclease domain, each of which can cleave a single DNA strand at a target sequence (the concerted action of both domains leads to DNA double-strand cleavage, whereas activity of one domain leads to a nick). In general, the RuvC domain comprises subdomains I, II and III, where domain I is located near the N-terminus of Cas9 and subdomains II and III are located in the middle of the protein, flanking the HNH domain (Hsu et al, Cell 157:1262-1278). A type II CRISPR system includes a DNA cleavage system utilizing a Cas9 endonuclease in complex with at least one polynucleotide component. For example, a Cas9 can

be in complex with a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA). In another example, a Cas9 can be in complex with a single guide RNA.

The Cas endonuclease can comprise a modified form of the Cas9 polypeptide. The modified form of the Cas9 polypeptide can include an amino acid change (e.g., deletion, insertion, or substitution) that reduces the naturally-occurring nuclease activity of the Cas9 protein. For example, in some instances, the modified form of the Cas9 protein has less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, less than 5%, or less than 1% of the nuclease activity of the corresponding wild-type Cas9 polypeptide (US patent application US20140068797 A1). In some cases, the modified form of the Cas9 polypeptide has no substantial nuclease activity and is referred to as catalytically "inactivated Cas9" or "deactivated cas9 (dCas9)." Catalytically inactivated Cas9 variants include Cas9 variants that contain mutations in the HNH and RuvC nuclease domains. These catalytically inactivated Cas9 variants are capable of interacting with sgRNA and binding to the target site in vivo but cannot cleave either strand of the target DNA.

A catalytically inactive Cas9 can be fused to a heterologous sequence (US patent application US20140068797 A1). Suitable fusion partners include, but are not limited to, a polypeptide that provides an activity that indirectly increases transcription by acting directly on the target DNA or on a polypeptide (e.g., a histone or other DNA-binding protein) associated with the target DNA. Additional suitable fusion partners include, but are not limited to, a polypeptide that provides for methyltransferase activity, demethylase activity, acetyltransferase activity, deacetylase activity, kinase activity, phosphatase activity, ubiquitin ligase activity, deubiquitinating activity, adenylation activity, deadenylation activity, SUMOylating activity, deSUMOylating activity, ribosylation activity, deribosylation activity, myristoylation activity, or demyristoylation activity. Further suitable fusion partners include, but are not limited to, a polypeptide that directly provides for increased transcription of the target nucleic acid (e.g., a transcription activator or a fragment thereof, a protein or fragment thereof that recruits a transcription activator, a small molecule/drug-responsive transcription regulator, etc.). A catalytically inactive Cas9 can also be fused to a FokI nuclease to generate double strand breaks (Guilinger et al. *Nature Biotechnology*, volume 32, number 6, June 2014).

The terms "functional fragment ", "fragment that is functionally equivalent" and "functionally equivalent fragment" of a Cas endonuclease are used interchangeably herein, and refer to a portion or subsequence of the Cas endonuclease sequence of the present disclosure

in which the ability to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break in) the target site is retained.

The terms "functional variant ", "Variant that is functionally equivalent" and "functionally equivalent variant" of a Cas endonuclease are used interchangeably herein, and refer to a variant of the Cas endonuclease of the present disclosure in which the ability to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break in) the target site is retained. Fragments and variants can be obtained via methods such as site-directed mutagenesis and synthetic construction.

Any guided endonuclease (e.g., guided CRISPR-Cas systems) can be used in the methods disclosed herein. Such endonucleases include, but are not limited to Cas9, Cas12f and their variants (see SEQ ID NO: 37 of U.S. Pat. No. 10,934,536, incorporated herein by reference in its entirety) and Cpf1 endonucleases. Many endonucleases have been described to date that can recognize specific PAM sequences (see for example –Jinek et al. (2012) *Science* 337 p 816-821, PCT patent applications PCT/US16/32073, and PCT/US16/32028and Zetsche B et al. 2015. *Cell* 163, 1013) and cleave the target DNA at a specific positions. It is understood that based on the methods and embodiments described herein utilizing a guided Cas system one can now tailor these methods such that they can utilize any guided endonuclease system. Various chromosomal engineering tools and methods are illustrated in PCT/US2021/034704, filed May 28, 2021 and the contents thereof are incorporated herein by reference to the extent they relate to certain targeted chromosome engineering applications.

As used herein, the term "guide polynucleotide", relates to a polynucleotide sequence that can form a complex with a Cas endonuclease and enables the Cas endonuclease to recognize, bind to, and optionally cleave a DNA target site. The guide polynucleotide can be a single molecule or a double molecule. The guide polynucleotide sequence can be a RNA sequence, a DNA sequence, or a combination thereof (a RNA-DNA combination sequence). Optionally, the guide polynucleotide can comprise at least one nucleotide, phosphodiester bond or linkage modification such as, but not limited, to Locked Nucleic Acid (LNA), 5-methyl dC, 2,6-Diaminopurine, 2'-Fluoro A, 2'-Fluoro U, 2'-O-Methyl RNA, phosphorothioate bond, linkage to a cholesterol molecule, linkage to a polyethylene glycol molecule, linkage to a spacer 18 (hexaethylene glycol chain) molecule, or 5' to 3' covalent linkage resulting in circularization. A guide polynucleotide that solely comprises ribonucleic acids is also referred to as a "guide RNA" or "gRNA" (See also U.S. Patent Application US 2015-0082478 A1, and US 2015-0059010 A1, both hereby incorporated in its entirety by reference).

The guide polynucleotide can be a double molecule (also referred to as duplex guide polynucleotide) comprising a crNucleotide sequence and a tracrNucleotide sequence. The crNucleotide includes a first nucleotide sequence domain (referred to as Variable Targeting domain or VT domain) that can hybridize to a nucleotide sequence in a target DNA and a second nucleotide sequence (also referred to as a tracr mate sequence) that is part of a Cas endonuclease recognition (CER) domain. The tracr mate sequence can hybridized to a tracrNucleotide along a region of complementarity and together form the Cas endonuclease recognition domain or CER domain. The CER domain is capable of interacting with a Cas endonuclease polypeptide. The crNucleotide and the tracrNucleotide of the duplex guide polynucleotide can be RNA, DNA, and/or RNA-DNA- combination sequences. In some embodiments, the crNucleotide molecule of the duplex guide polynucleotide is referred to as "crDNA" (when composed of a contiguous stretch of DNA nucleotides) or "crRNA" (when composed of a contiguous stretch of RNA nucleotides), or "crDNA-RNA" (when composed of a combination of DNA and RNA nucleotides). The crNucleotide can comprise a fragment of the cRNA naturally occurring in Bacteria and Archaea. The size of the fragment of the cRNA naturally occurring in Bacteria and Archaea that can be present in a crNucleotide disclosed herein can range from, but is not limited to, 2, 3, 4, 5, 6, 7, 8, 9,10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides. In some embodiments the tracrNucleotide is referred to as "tracrRNA" (when composed of a contiguous stretch of RNA nucleotides) or "tracrDNA" (when composed of a contiguous stretch of DNA nucleotides) or "tracrDNA-RNA" (when composed of a combination of DNA and RNA nucleotides. In one embodiment, the RNA that guides the RNA/ Cas9 endonuclease complex is a duplexed RNA comprising a duplex crRNA-tracrRNA.

The tracrRNA (trans-activating CRISPR RNA) contains, in the 5'-to-3' direction, (i) a sequence that anneals with the repeat region of CRISPR type II crRNA and (ii) a stem loop-containing portion (Deltcheva et al., Nature 471:602-607). The duplex guide polynucleotide can form a complex with a Cas endonuclease, wherein said guide polynucleotide/Cas endonuclease complex (also referred to as a guide polynucleotide/Cas endonuclease system) can direct the Cas endonuclease to a genomic target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break) into the target site. (See also U.S. Patent Application US 2015-0082478 A1, published on March 19, 2015 and US 2015-0059010 A1, both hereby incorporated in its entirety by reference.)

The single guide polynucleotide can form a complex with a Cas endonuclease, wherein said guide polynucleotide/Cas endonuclease complex (also referred to as a guide polynucleotide/Cas endonuclease system) can direct the Cas endonuclease to a genomic target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break) the target site. (See also U.S. Patent Application US 2015-0082478 A1, and US 2015-0059010 A1, both hereby incorporated in its entirety by reference.)

The term "variable targeting domain" or "VT domain" is used interchangeably herein and includes a nucleotide sequence that can hybridize (is complementary) to one strand (nucleotide sequence) of a double strand DNA target site. The percent complementation between the first nucleotide sequence domain (VT domain) and the target sequence can be at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 63%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. The variable targeting domain can be at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length. In some embodiments, the variable targeting domain comprises a contiguous stretch of 12 to 30 nucleotides. The variable targeting domain can be composed of a DNA sequence, a RNA sequence, a modified DNA sequence, a modified RNA sequence, or any combination thereof.

The term "Cas endonuclease recognition domain" or "CER domain" (of a guide polynucleotide) is used interchangeably herein and includes a nucleotide sequence that interacts with a Cas endonuclease polypeptide. A CER domain comprises a tracrNucleotide mate sequence followed by a tracrNucleotide sequence. The CER domain can be composed of a DNA sequence, a RNA sequence, a modified DNA sequence, a modified RNA sequence (see for example US 2015-0059010 A1, incorporated in its entirety by reference herein), or any combination thereof.

The terms "functional fragment ", "fragment that is functionally equivalent" and "functionally equivalent fragment" of a guide RNA, crRNA or tracrRNA are used interchangeably herein, and refer to a portion or subsequence of the guide RNA, crRNA or tracrRNA , respectively, of the present disclosure in which the ability to function as a guide RNA, crRNA or tracrRNA, respectively, is retained.

The terms "functional variant ", "Variant that is functionally equivalent" and "functionally equivalent variant" of a guide RNA, crRNA or tracrRNA (respectively) are used

interchangeably herein, and refer to a variant of the guide RNA, crRNA or tracrRNA, respectively, of the present disclosure in which the ability to function as a guide RNA, crRNA or tracrRNA, respectively, is retained.

The terms "single guide RNA" and "sgRNA" are used interchangeably herein and relate to a synthetic fusion of two RNA molecules, a crRNA (CRISPR RNA) comprising a variable targeting domain (linked to a tracr mate sequence that hybridizes to a tracrRNA), fused to a tracrRNA (trans-activating CRISPR RNA). The single guide RNA can comprise a crRNA or crRNA fragment and a tracrRNA or tracrRNA fragment of the type II CRISPR/Cas system that can form a complex with a type II Cas endonuclease, wherein said guide RNA/Cas endonuclease complex can direct the Cas endonuclease to a DNA target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break) the DNA target site.

The terms "guide RNA/Cas endonuclease complex", "guide RNA/Cas endonuclease system", " guide RNA/Cas complex", "guide RNA/Cas system", "gRNA/Cas complex", "gRNA/Cas system", "RNA-guided endonuclease" , "RGEN" are used interchangeably herein and refer to at least one RNA component and at least one Cas endonuclease that are capable of forming a complex , wherein said guide RNA/Cas endonuclease complex can direct the Cas endonuclease to a DNA target site, enabling the Cas endonuclease to recognize, bind to, and optionally nick or cleave (introduce a single or double strand break) the DNA target site. A guide RNA/Cas endonuclease complex herein can comprise Cas protein(s) and suitable RNA component(s) of any of the four known CRISPR systems (Horvath and Barrangou, 2010, *Science* 327:167-170) such as a type I, II, or III CRISPR system. A guide RNA/Cas endonuclease complex can comprise a Type II Cas9 endonuclease and at least one RNA component (e.g., a crRNA and tracrRNA, or a gRNA). (See also U.S. Patent Application US 2015-0082478 A1, and US 2015-0059010 A1, both hereby incorporated in its entirety by reference).

The guide polynucleotide can be introduced into a cell transiently, as single stranded polynucleotide or a double stranded polynucleotide, using any method known in the art such as, but not limited to, particle bombardment, *Agrobacterium transformation* or topical applications. The guide polynucleotide can also be introduced indirectly into a cell by introducing a recombinant DNA molecule (via methods such as, but not limited to, particle bombardment or Agrobacterium transformation) comprising a heterologous nucleic acid fragment encoding a guide polynucleotide, operably linked to a specific promoter that is

capable of transcribing the guide RNA in said cell. The specific promoter can be, but is not limited to, a RNA polymerase III promoter, which allow for transcription of RNA with precisely defined, unmodified, 5'- and 3'-ends (DiCarlo et al., *Nucleic Acids Res.* 41: 4336-4343; Ma et al., *Mol. Ther. Nucleic Acids* 3:e161) as described in WO2016025131, incorporated herein in its entirety by reference.

The terms "target site", "target sequence", "target site sequence, "target DNA", "target locus", "genomic target site", "genomic target sequence", "genomic target locus" and "protospacer", are used interchangeably herein and refer to a polynucleotide sequence including, but not limited to, a nucleotide sequence within a chromosome, an episome, or any other DNA molecule in the genome (including chromosomal, choloroplastic, mitochondrial DNA, plasmid DNA) of a cell, at which a guide polynucleotide/Cas endonuclease complex can recognize, bind to, and optionally nick or cleave . The target site can be an endogenous site in the genome of a cell, or alternatively, the target site can be heterologous to the cell and thereby not be naturally occurring in the genome of the cell, or the target site can be found in a heterologous genomic location compared to where it occurs in nature. As used herein, terms "endogenous target sequence" and "native target sequence" are used interchangeable herein to refer to a target sequence that is endogenous or native to the genome of a cell. Cells include, but are not limited to, human, non-human, animal, bacterial, fungal, insect, yeast, non-conventional yeast, and plant cells as well as plants and seeds produced by the methods described herein. An "artificial target site" or "artificial target sequence" are used interchangeably herein and refer to a target sequence that has been introduced into the genome of a cell. Such an artificial target sequence can be identical in sequence to an endogenous or native target sequence in the genome of a cell but be located in a different position (i.e., a non-endogenous or non-native position) in the genome of a cell.

An "altered target site", "altered target sequence", "modified target site", "modified target sequence" are used interchangeably herein and refer to a target sequence as disclosed herein that comprises at least one alteration when compared to non-altered target sequence. Such "alterations" include, for example: (i) replacement of at least one nucleotide, (ii) a deletion of at least one nucleotide, (iii) an insertion of at least one nucleotide, or (iv) any combination of (i) – (iii).

The length of the target DNA sequence (target site) can vary, and includes, for example, target sites that are at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides in length. It is further possible that the target site can be palindromic,

that is, the sequence on one strand reads the same in the opposite direction on the complementary strand. The nick/cleavage site can be within the target sequence or the nick/cleavage site could be outside of the target sequence. In another variation, the cleavage could occur at nucleotide positions immediately opposite each other to produce a blunt end cut or, in other Cases, the incisions could be staggered to produce single-stranded overhangs, also called "sticky ends", which can be either 5' overhangs, or 3' overhangs. Active variants of genomic target sites can also be used. Such active variants can comprise at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the given target site, wherein the active variants retain biological activity and hence are capable of being recognized and cleaved by an Cas endonuclease. Assays to measure the single or double-strand break of a target site by an endonuclease are known in the art and generally measure the overall activity and specificity of the agent on DNA substrates containing recognition sites.

A "protospacer adjacent motif" (PAM) herein refers to a short nucleotide sequence adjacent to a target sequence (protospacer) that is recognized (targeted) by a guide polynucleotide/Cas endonuclease system described herein. The Cas endonuclease may not successfully recognize a target DNA sequence if the target DNA sequence is not followed by a PAM sequence. The sequence and length of a PAM herein can differ depending on the Cas protein or Cas protein complex used. The PAM sequence can be of any length but is typically 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides long.

The terms "targeting", "gene targeting" and "DNA targeting" are used interchangeably herein. DNA targeting herein may be the specific introduction of a knock-out, edit, or knock-in at a particular DNA sequence, such as in a chromosome or plasmid of a cell. In general, DNA targeting may be performed herein by cleaving one or both strands at a specific DNA sequence in a cell with an endonuclease associated with a suitable polynucleotide component. Such DNA cleavage, if a double-strand break (DSB), can prompt NHEJ or HDR processes which can lead to modifications at the target site.

A targeting method herein may be performed in such a way that two or more DNA target sites are targeted in the method, for example. Such a method can optionally be characterized as a multiplex method. Two, three, four, five, six, seven, eight, nine, ten, or more target sites may be targeted at the same time in certain embodiments. A multiplex method is typically performed by a targeting method herein in which multiple different RNA components

are provided, each designed to guide an guidepolynucleotide/Cas endonuclease complex to a unique DNA target site.

The terms "knock-out", "gene knock-out" and "genetic knock-out" are used interchangeably herein. A knock-out as used herein represents a DNA sequence of a cell that has been rendered partially or completely inoperative by targeting with a Cas protein; such a DNA sequence prior to knock-out could have encoded an amino acid sequence, or could have had a regulatory function (e.g., promoter), for example. A knock-out may be produced by an indel (insertion or deletion of nucleotide bases in a target DNA sequence through NHEJ), or by specific removal of sequence that reduces or completely destroys the function of sequence at or near the targeting site. In a separate embodiment, a "knock out" may be the result of downregulation of a gene through RNA interference. In some aspects, a double stranded RNA (dsRNA) molecule(s) may be employed in the disclosed methods and compositions to mediate the reduction of expression of a target sequence, for example, by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner. In some embodiments, a native susceptible copy allele of a gene that has a resistant gene counterpart in the DSL is knocked out by RNA interference or gene editing.

The guide polynucleotide/Cas endonuclease system can be used in combination with a co-delivered polynucleotide modification template to allow for editing (modification) of a genomic nucleotide sequence of interest. (See also U.S. Patent Application US 2015-0082478 A1, and WO2015/026886 A1, both hereby incorporated in its entirety by reference.)

The terms "knock-in", "gene knock-in , "gene insertion" and "genetic knock-in" are used interchangeably herein. A knock-in represents the replacement or insertion of a DNA sequence at a specific DNA sequence in cell by targeting with a Cas protein (by HR, wherein a suitable donor DNA polynucleotide is also used). Examples of knock-ins include, but are not limited to, a specific insertion of a heterologous amino acid coding sequence in a coding region of a gene, or a specific insertion of a transcriptional regulatory element in a genetic locus.

Various methods and compositions can be employed to obtain a cell or organism having a polynucleotide of interest inserted in a target site for a Cas endonuclease. Such methods can employ homologous recombination to provide integration of the polynucleotide of Interest at the target site. In one method provided, a polynucleotide of interest is provided to the organism cell in a donor DNA construct. As used herein, "donor DNA" is a DNA construct that comprises a polynucleotide of Interest to be inserted into the target site of a Cas endonuclease. The donor DNA construct may further comprise a first and a second region of homology that

flank the polynucleotide of Interest. The first and second regions of homology of the donor DNA share homology to a first and a second genomic region, respectively, present in or flanking the target site of the cell or organism genome. By "homology" is meant DNA sequences that are similar. For example, a "region of homology to a genomic region" that is found on the donor DNA is a region of DNA that has a similar sequence to a given "genomic region" in the cell or organism genome. A region of homology can be of any length that is sufficient to promote homologous recombination at the cleaved target site. For example, the region of homology can comprise at least 5-10, 5-15, 5-20, 5-25, 5-30, 5-35, 5-40, 5-45, 5- 50, 5-55, 5-60, 5-65, 5- 70, 5-75, 5-80, 5-85, 5-90, 5-95, 5-100, 5-200, 5-300, 5-400, 5-500, 5-600, 5-700, 5-800, 5-900, 5-1000, 5-1100, 5-1200, 5-1300, 5-1400, 5-1500, 5-1600, 5-1700, 5-1800, 5-1900, 5-2000, 5-2100, 5-2200, 5-2300, 5-2400, 5-2500, 5-2600, 5-2700, 5-2800, 5-2900, 5-3000, 5-3100 or more bases in length such that the region of homology has sufficient homology to undergo homologous recombination with the corresponding genomic region. "Sufficient homology" indicates that two polynucleotide sequences have sufficient structural similarity to act as substrates for a homologous recombination reaction. The structural similarity includes overall length of each polynucleotide fragment, as well as the sequence similarity of the polynucleotides. Sequence similarity can be described by the percent sequence identity over the whole length of the sequences, and/or by conserved regions comprising localized similarities such as contiguous nucleotides having 100% sequence identity, and percent sequence identity over a portion of the length of the sequences.

"Percent (%) sequence identity" with respect to a reference sequence (subject) is determined as the percentage of amino acid residues or nucleotides in a candidate sequence (query) that are identical with the respective amino acid residues or nucleotides in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any amino acid conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2 . Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes. The percent identity between the two sequences is a function of the number of identical positions shared by

the sequences (e.g., percent identity of query sequence = number of identical positions between query and subject sequences/total number of positions of query sequence (e.g., overlapping positions)×100).

The amount of homology or sequence identity shared by a target and a donor polynucleotide can vary and includes total lengths and/or regions having unit integral values in the ranges of about 1-20 bp, 20-50 bp, 50-100 bp, 75-150 bp, 100-250 bp, 150-300 bp, 200-400 bp, 250-500 bp, 300-600 bp, 350-750 bp, 400-800 bp, 450-900 bp, 500-1000 bp, 600-1250 bp, 700-1500 bp, 800-1750 bp, 900-2000 bp, 1-2.5 kb, 1.5–3 kb, 2-4 kb, 2.5-5 kb, 3-6 kb, 3.5-7 kb, 4-8 kb, 5-10 kb, or up to and including the total length of the target site. These ranges include every integer within the range, for example, the range of 1-20 bp includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 bps. The amount of homology can also be described by percent sequence identity over the full aligned length of the two polynucleotides which includes percent sequence identity of about at least 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. Sufficient homology includes any combination of polynucleotide length, global percent sequence identity, and optionally conserved regions of contiguous nucleotides or local percent sequence identity, for example sufficient homology can be described as a region of 75-150 bp having at least 80% sequence identity to a region of the target locus. Sufficient homology can also be described by the predicted ability of two polynucleotides to specifically hybridize under high stringency conditions, see, for example, Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, NY); Current Protocols in Molecular Biology, Ausubel et al., Eds (1994) Current Protocols, (Greene Publishing Associates, Inc. and John Wiley & Sons, Inc.); and, Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, (Elsevier, New York).

The structural similarity between a given genomic region and the corresponding region of homology found on the donor DNA can be any degree of sequence identity that allows for homologous recombination to occur. For example, the amount of homology or sequence identity shared by the "region of homology" of the donor DNA and the "genomic region" of the organism genome can be at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity, such that the sequences undergo homologous recombination

The region of homology on the donor DNA can have homology to any sequence flanking the target site. While in some embodiments the regions of homology share significant sequence homology to the genomic sequence immediately flanking the target site, it is recognized that the regions of homology can be designed to have sufficient homology to regions that may be further 5' or 3' to the target site. In still other embodiments, the regions of homology can also have homology with a fragment of the target site along with downstream genomic regions. In one embodiment, the first region of homology further comprises a first fragment of the target site and the second region of homology comprises a second fragment of the target site, wherein the first and second fragments are dissimilar.

As used herein, "homologous recombination" includes the exchange of DNA fragments between two DNA molecules at the sites of homology. The frequency of homologous recombination is influenced by a number of factors. Different organisms vary with respect to the amount of homologous recombination and the relative proportion of homologous to non-homologous recombination. Generally, the length of the region of homology affects the frequency of homologous recombinations: the longer the region of homology, the greater the frequency. The length of the homology region needed to observe homologous recombination is also species-variable. In many cases, at least 5 kb of homology has been utilized, but homologous recombination has been observed with as little as 25-50 bp of homology. See, for example, Singer et al., (1982) *Cell* 31:25-33; Shen and Huang, (1986) *Genetics* 112:441-57; Watt et al., (1985) *Proc. Natl. Acad. Sci. USA* 82:4768-72, Sugawara and Haber, (1992) *Mol Cell Biol* 12:563-75, Rubnitz and Subramani, (1984) *Mol Cell Biol* 4:2253-8; Ayares et al., (1986) *Proc. Natl. Acad. Sci.* USA 83:5199-203; Liskay et al., (1987) *Genetics* 115:161-7.

Homology-directed repair (HDR) is a mechanism in cells to repair double-stranded and single stranded DNA breaks. Homology-directed repair includes homologous recombination (HR) and single-strand annealing (SSA) (Lieber. 2010 *Annu. Rev. Biochem.* 79:181-211). The most common form of HDR is called homologous recombination (HR), which has the longest sequence homology requirements between the donor and acceptor DNA. Other forms of HDR include single-stranded annealing (SSA) and breakage-induced replication, and these require shorter sequence homology relative to HR. Homology-directed repair at nicks (single-stranded breaks) can occur via a mechanism distinct from HDR at double-strand breaks (Davis and Maizels. (2014) PNAS (0027-8424), 111 (10), p. E924-E932).

Alteration of the genome of a plant cell, for example, through homologous recombination (HR), is a powerful tool for genetic engineering. Homologous recombination

has been demonstrated in plants (Halfter et al., (1992) *Mol Gen Genet* 231:186-93) and insects (Dray and Gloor, 1997, *Genetics* 147:689-99). Homologous recombination has also been accomplished in other organisms. For example, at least 150-200 bp of homology was required for homologous recombination in the parasitic protozoan Leishmania (Papadopoulou and Dumas, (1997) *Nucleic Acids Res* 25:4278-86). In the filamentous fungus Aspergillus nidulans, gene replacement has been accomplished with as little as 50 bp flanking homology (Chaveroche et al., (2000) *Nucleic Acids Res* 28:e97). Targeted gene replacement has also been demonstrated in the ciliate Tetrahymena thermophila (Gaertig et al., (1994) *Nucleic Acids Res* 22:5391-8). In mammals, homologous recombination has been most successful in the mouse using pluripotent embryonic stem cell lines (ES) that can be grown in culture, transformed, selected and introduced into a mouse embryo (Watson et al., 1992, Recombinant DNA, 2nd Ed., (Scientific American Books distributed by WH Freeman & Co.).

In some embodiments, methods and compositions are provided for inverting large segments of a chromosome, deleting segments of chromosomes, and relocating segments or genes using CRISPR-Cas technology (US Patent Application 63/301822 filed 29 May 2020). In some aspects, a DSL chromosomal segment may be moved or otherwise altered using chromosomal rearrangement.

In another embodiment, a chromosomal segment may be rearranged into a DSL. In some aspects, a chromosomal segment is at least about 1 kb, between 1 kb and 10 kb, at least about 10 kb, between 10 kb and 20 kb, at least about 20 kb, between 20 kb and 30 kb, at least about 30 kb, between 30 kb and 40 kb, at least about 40 kb, between 40 kb and 50 kb, at least about 50 kb, between 50 kb and 60 kb, at least about 60 kb, between 60 kb and 70 kb, at least about 70 kb, between 70 kb and 80 kb, at least about 80 kb, between 80 kb and 90 kb, at least about 90 kb, between 90 kb and 100 kb, or greater than 100 kb. In some aspects, the segment is at least about 100 kb, between 100 kb and 150 kb, at least about 150 kb, between 150 kb and 200 kb, at least about 200 kb, between 200 kb and 250 kb, at least about 250 kb, between 250 kb and 300 kb, at least about 300 kb, between 300 kb and 350 kb, at least about 350 kb, between 350 kb and 400 kb, at least about 400 kb, between 400 kb and 450 kb, at least about 450 kb, between 450 kb and 500 kb, at least about 500 kb, between 500 kb and 550 kb, at least about 550 kb, between 550 kb and 600 kb, at least about 600 kb, between 600 kb and 650 kb, at least about 650 kb, between 650 kb and 700 kb, at least about 700 kb, between 700 kb and 750 kb, at least about 750 kb, between 750 kb and 800 kb, at least about 800 kb, between 800 kb and 850 kb, at least about 850 kb, between 850 kb and 900 kb, at least about 900 kb, between 900

kb and 950 kb, at least about 950 kb, between 950 kb and 1000 kb, at least about 1000 kb, between 1000 kb and 1050 kb, at least about 1050 kb, between 1050 kb and 1100 kb, or greater than 1100 kb. In some aspects, the segment is at least about 1 Mb, between 1 Mb and 10 Mb, at least about 10 Mb, between 10 Mb and 20 Mb, at least about 20 Mb, between 20 Mb and 30 Mb, at least about 30 Mb, between 30 Mb and 40 Mb, at least about 40 Mb, between 40 Mb and 50 Mb, at least about 50 Mb, between 50 Mb and 60 Mb, at least about 60 Mb, between 60 Mb and 70 Mb, at least about 70 Mb, between 70 Mb and 80 Mb, at least about 80 Mb, between 80 Mb and 90 Mb, at least about 90 Mb, between 90 Mb and 100 Mb, or greater than 100 Mb.

Error-prone DNA repair mechanisms can produce mutations at double-strand break sites. The Non-Homologous-End-Joining (NHEJ) pathways are the most common repair mechanism to bring the broken ends together (Bleuyard et al., (2006) *DNA Repair* 5:1-12). The structural integrity of chromosomes is typically preserved by the repair, but deletions, insertions, or other rearrangements are possible. The two ends of one double-strand break are the most prevalent substrates of NHEJ (Kirik et al., (2000) *EMBO J* 19:5562-6), however if two different double-strand breaks occur, the free ends from different breaks can be ligated and result in chromosomal deletions (Siebert and Puchta, (2002) *Plant Cell* 14:1121-31), or chromosomal translocations between different chromosomes (Pacher et al., (2007) Genetics 175:21-9).

The donor DNA may be introduced by any means known in the art. The donor DNA may be provided by any transformation method known in the art including, for example, Agrobacterium-mediated transformation or biolistic particle bombardment. The donor DNA may be present transiently in the cell or it could be introduced via a viral replicon. In the presence of the Cas endonuclease and the target site, the donor DNA is inserted into the transformed plant's genome.

Further uses for guide RNA/Cas endonuclease systems have been described (See U.S. Patent Application US 2015-0082478 A1, WO2015/026886 A1, US 2015-0059010 A1, US application US 2017/0306349 A1, and US application 62/036,652, all of which are incorporated by reference herein) and include but are not limited to modifying or replacing nucleotide sequences of interest (such as a regulatory elements), insertion of polynucleotides of interest, gene knock-out, gene-knock in, modification of splicing sites and/or introducing alternate splicing sites, modifications of nucleotide sequences encoding a protein of interest,

amino acid and/or protein fusions, and gene silencing by expressing an inverted repeat into a gene of interest.

Polynucleotides of interest and/or traits can be stacked together in a complex trait locus as described in US 2013/0263324-A1 and in PCT/US13/22891, both applications hereby incorporated by reference.

In some embodiments, a maize plant cell comprises a genomic locus with at least one nucleotide sequence that confers enhanced resistance to northern leaf blight and a at least one different plant disease are provided herein. Further plant disesases may include, but are not limited to, grey leaf spot, southern corn rust, and anthracnose stalk rot. The disclosed methods include introducing a double-strand break at one or more target sites in a genomic locus in a maize plant cell; introducing one or more nucleotide sequences that confer enhanced resistance to more than one plant disease, wherein each is flanked by 300-500bp of nucleotide sequences 5′ or 3′ of the corresponding target sites; and obtaining a maize plant cell having a genomic locus comprising one or more nucleotide sequences that confer enhanced resistance to more than one plant disease. The double-strand break may be induced by a nuclease such as but not limited to a TALEN, a meganuclease, a zinc finger nuclease, or a CRISPR-associated nuclease. The method may further comprise growing a maize plant from the maize plant cell having the genomic locus comprising the at least one nucleotide sequence that confers enhanced resistance to northern leaf blight, and the maize plant may exhibit enhanced resistance to northern leaf blight.

A maize plants exhibits enhanced resistance when compared to equivalent plants lacking the nucleotide sequences conferring enhanced resistance at the genomic locus of interest. “Equivalent” means that the plants are genetically similar with the exception of the genomic locus of interest.

In some aspects, the one or more nucleotide sequences that confers enhanced disease resistance include any of the following: RppK (Genomic DNA SEQ ID NO: 9; cDNA SEQ ID NO: 10; Protein SEQ ID NO: 11), *Ht1* (Genomic DNA SEQ ID NO: 6; cDNA SEQ ID NO: 7; Protein SEQ ID NO: 8), NLB18 (Genomic DNA SEQ ID NO: 1; cDNA SEQ ID NO: 2 or 4; Protein SEQ ID NO: 3 or 5), NLR01 (Genomic DNA SEQ ID No: 27; cDNA SEQ ID NO: 28; Protein SEQ ID No: 29), NLR02 (Genomic DNA SEQ ID Nos: 24; cDNA SEQ ID NO: 25; Protein SEQ ID No: 26), RCG1 (cDNA SEQ ID Nos: 30; Protein SEQ ID No: 31), RCG1b (cDNA SEQ ID Nos: 32; Protein SEQ ID No: 33), PRR03 (Genomic DNA SEQ ID Nos: 34; cDNA SEQ ID NO: 35; Protein SEQ ID No: 36), PRR01 (cDNA SEQ ID NO: 37; Protein SEQ

ID No: 38), NLR01 (Genomic DNA SEQ ID Nos: 39; cDNA SEQ ID NO: 40; Protein SEQ ID No: 41), or NLR04 (Genomic DNA SEQ ID Nos: 42; cDNA SEQ ID NO: 43; Protein SEQ ID No: 44), for example.

As used herein a "complex transgenic trait locus" (plural: "complex transgenic trait loci") is a chromosomal segment within a genomic region of interest that comprises at least two altered target sequences that are genetically linked to each other and can also comprise one or more polynucleotides of interest as described hereinbelow. Each of the altered target sequences in the complex transgenic trait locus originates from a corresponding target sequence that was altered, for example, by a mechanism involving a double-strand break within the target sequence that was induced by a double-strand break-inducing agent of the invention. In certain embodiments of the invention, the altered target sequences comprise a transgene.

CTL1 exists on Maize Chromosome 1 in a window of approximately 5 cM (US Patent No. 10,030,245, US Patent Publication No. 2018/0258438A1, US Patent Publication No. 2018/0230476A1). The first maize genomic window that was identified for development of a Complex Trait Locus (CTL) spans from ZM01: 12987435 (flanked by public SNP marker SYN12545) to Zm01:15512479 (flanked by public SNP marker SYN20196) on chromosome 1. Table 1 shows the physical and genetic map position (if available) for a multitude of maize SNP markers (Ganal, M. et al, A Large Maize (*Zea mays L.*) SNP Genotyping Array: Development and Germplasm Genotyping, and Genetic Mapping to Compare with the B73 Reference Genome. PloS one, December 08, 2011DOI: 10.1371) and Cas endonuclease target sites (31 sites) within the genomic window of interest on the maize chromosome 1.

Table 1. Genomic Window comprising a Complex Trait Locus (CTL1) on Chromosome 1 of maize

Name of public SNP markers (*) or Cas endonuclease target site	Cas endonuclease target or SNP marker sequence (SEQ ID NO:)	Physical position (PUB B73v3)	Genetic Position (PUB B73v3)
SYN12545*	1	12987435	36.9
SYN12536*	2	12988556	36.9
49-CR2	3	13488227	
50-CR1	4	13554078	
51-CR1	5	13676343	
SYN14645*	6	13685871	37.4
41-CR2	7	13830316	

72-CR1	8	13841735	
71-CR1	9	13846794	
81-CR1	10	13967499	
73-CR1	11	13986903	
PZE-101023852*	12	14030843	37.6
14-CR4	13	14038610	
74-CR1	14	14089937	
75-CR1	15	14226763	
84-CR1	16	14233410	
76-CR1	17	14245535	
77-CR1	18	14344614	
78-CR1	19	14380330	
PZE-101024424*	20	14506833	37.8
79-CR1	21	14577827	
85-CR1	22	14811592	
19-CR1	23	14816379	
SYN25022*	24	14851517	37.8
86-CR1	25	14951113	
08-CR1	26	14955364	
43-CR1	27	15006039	
11-CR1	28	15066942	
SYN31156*	29	15070918	39.9
47-CR2	30	15081190	
80-CR1	31	15084949	
52-CR2	32	15088711	
87-CR1	33	15158706	
88-CR1	34	15162366	
SYN31166*	35	15169575	40.9
45-CR1	36	15177228	
10-CR3	37	15274433	
44-CR2	38	15317833	
46-CR2	39	15345674	
SYN22238*	40	15491134	41.7
SYN20196*	41	15512479	41.9

In one embodiment, the genomic locus comprises Disease Super Locus 1 (DSL1). In another embodiment, Disease Super Locus 1 (DSL1) is located in the distal region of chromosome 1 approximately 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cM away from Complex Trait Locus 1 (CTL1). In one embodiment, a Disease Super Locus (DSL) is located approximately 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cM away from at least one

different trait locus. In another embodiment, a DSL is located in the telomeric region. In a preferred embodiment, DSL1 is distal to CTL1 within about 0.5 cM to about 5 cM. In yet another embodiment. DSL1 is flanked by pze-101020971 (SEQ ID NO: 22) and pze-101022341 (SEQ ID NO: 23). In some embodiments, CTL1 comprises an insect control trait and a herbacide tolerance trait.

In one aspect, the genomic locus that confers enhanced resistance to northern leaf blight comprises DSL1..

The guide polynucleotide/Cas9 endonuclease system as described herein provides for an efficient system to generate double strand breaks and allows for traits to be stacked in a complex trait locus. Thus, in one aspect, Cas9 endonuclease is used as the DSB-inducing agent, and one or more guide RNAs are used to target the Cas9 to sites in the DSL1 locus.

The maize plants generated by the methods described herein may provide durable and broad spectrum disease resistance and may assist in breeding of disease resistant maize plants. For instance, because the nucleotide sequences that confer enhanced disease resistance in tight linkage with one another (at one locus), this reduces the number of specific loci that require trait introgression through backcrossing and minimizes linkage drag from non-elite resistant donors. In one embodiment, a DSL is located within at least 1 cM, 2 cM, 3 cM, 4 cM, 5 cM, 6 cM, 7 cM, 8 cM, 9 cM, 10 cM, 15 cM, or 20 cM from a QTL for yield stability or disease resistance.

In some embodiments, the maize plants that comprise DSL may be treated with insecticide, fungicide, or biologicals. In one embodiment, the maize plants generated by the methods described herein may require lower levels or fewer number of treatments of fungicide, or biologicals compared to the levels of fungicide, or biologicals required in maize plants that do not comprise DSL. In a further embodiment, the lower levels or fewer number of treatments of fungicide, or biologicals compared to the levels of fungicide, or biologicals required in maize plants that do not comprise DSL may increase the durability of the fungicide, or biologicals.

In one embodiment, the fungicide comprises a fungicide composition selected from the group consisting of azoxystrobin, thiabendazole, fludioxonil, metalaxyl, tebuconazole, prothioconazole, ipconazole, penflufen, and sedaxane. Compositions disclosed herein may comprise fungicides which may include, but are not limited to, the respiration inhibitors, such as azoxystrobin, which target complex III of mitochondrial electron transport; tubulin inhibitors, such as thiabendazole, which bind to beta-tubulin; the osmotic stress related-kinase

inhibitor fludioxonil; an RNA polymerase inhibitor of Oomycetes, a group of fungal-like organisms, such as metalaxyl; inhibitors of sterol biosynthesis, which include inhibitors of the C-14 demethylase of the sterol biosynthesis pathway (commonly referred to as demethylase inhibitors or DMIs), such as tebuconazole, prothioconazole, and ipconazole; a respiration inhibitor which targets complex II mitochondrial electron transport, such as a penflufen; a respiration inhibitor which targets complex II mitochondrial electron transport, such as sedaxane. Other classes of fungicides with different or similar modes of action can be found at frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2016.pdf?sfvrsn=2 (which can be accessed on the world-wide web using the "www" prefix; See Hirooka and Ishii (2013), *Journal of General Plant Pathology*). A fungicide may comprise all or any combination of different classes of fungicides as described herein. In certain embodiments, a composition disclosed herein comprises azoxystrobin, thiabendazole, fludioxonil, and metalaxyl. In another embodiment, a composition disclosed herein comprises a tebuconazole. In another embodiment, a composition disclosed herein comprises prothioconazole, metalaxyl, and penflufen. In another embodiment, a composition disclosed herein comprises ipconazole and metalaxyl. In another embodiment, a composition disclosed herein comprises sedaxane. As used herein, a composition may be a liquid, a heterogeneous mixture, a homogeneous mixture, a powder, a solution, a dispersion or any combination thereof. In another embodiment, a biocontrol agent may be used in combination with a DSL.

Another strategy to reduce the need for refuge is the pyramiding of traits with different modes of action against a target pest. For example, *Bt* toxins that have different modes of action pyramided in one transgenic plant are able to have reduced refuge requirements due to reduced resistance risk. The same may be done for disease resistance and trait durability. In some aspects, two genes targeting the same disease can increase each trait's durability. For example, the combination of NLB18 and Ht1 (SEQ ID NOs: 3 and 8 respectively) expressed in a plant increase the durability of each trait to increase resistance to northern leaf blight. Different modes of action in a pyramid combination also extends the durability of each trait, as resistance is slower to develop to each trait.

In one embodiment, a first Disease Super Locus is stacked with a second Disease Super Locus. In another embodiment, a breeding stack approach is used to obtain a maize plant comprising a first Disease Super Locus stacked with a second Disease Super Locus. In some

embodiments, the second Disease Super Locus has at least one different disease resistance gene from the first Disease Super Locus.

In one embodiment, the polynucleotide sequence encoding a disease resistance gene comprises a heterologous promoter. In another embodiment, the polynucleotide sequence encoding a disease resistance gene comprises a cDNA sequence. In yet another embodiment, polynucleotide sequence encoding a disease resistance gene comprises an endogenous disease resistance locus and further comprises a heterologous expression modulating element (EME).

In one embodiment, DSL comprises a polynucleotide that produces a non-coding transcript or non-coding RNA. In another embodiment, the source of non-coding transcripts could be from non-coding genes, or it could be from repetitive sequences like transposons or retrotransposons. In another embodiment, the non-coding transcripts could be produced by RNAi constructs with a hairpin design. In another embodiemnt, a DSL may comprise one or more polynucleotide sequence that don't encode a polypeptide, but comprise a transposon or repetitive sequence, or a sequence that is transcribed into non-coding transcripts of various sizes such as long non-coding RNAs (lncRNAs), for example. In one embodiment, a non-coding transcript may be processed into small RNAs such as microRNA (miRNA), short-interfering RNA (siRNA), trans-acting siRNA (tasiRNA), and phased siRNA (phasiRNA). In one embodiment, the non-coding genes and sequences in a DSL may share nucleotide sequence homology to specific sequences in plant pathogens or pests, such as viruses, bacteria, oomycetes, fungus, insects, and parasitic plants. A non-coding transcript or processed products such as small RNAs may regulate or modulate the expression of specific genes or sequences in plant pathogens or pests, resulting in reduce pathogen pathogenicity and providing improved resistance in host plant.

In a further embodiment, a plant comprising a Disease Super Locus (DSL) may be stacked with one or more additional Bt insecticidal toxins, including, but not limited to, a Cry3B toxin, a mCry3B toxin, a mCry3A toxin, or a Cry34/35 toxin. In a further embodiment, a plant comprising a DSL may be stacked with one or more additional transgenes containing these Bt insecticidal toxins and other Coleopteran active Bt insecticidal traits for example, event MON863, event MIR604, event 5307, event DAS-59122, event DP-4114, event MON 87411, and event MON88017. In some embodiments, a plant comprising a DSL may be stacked with MON-87429-9 (MON87429 Event); MON87403; MON95379; MON87427; MON87419; MON-00603-6 (NK603); MON-87460-4; LY038; DAS-06275-8; BT176; BT11; MIR162; GA21; MZDT09Y; SYN-05307-1; DP-23211, DP-915635, and DAS-40278-9.

As used herein, "heterologous" in reference to a sequence is a sequence that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous polynucleotide is from a species different from the species from which the polynucleotide was derived, or, if from the same/analogous species, one or both are substantially modified from their original form and/or genomic locus, or the promoter is not the native promoter for the operably linked polynucleotide. In some embodiments a heterologous sequence comprises a polynucleotide encoding a polypeptide that is from the same species in a different location, a "native gene." In some embodiments, a heterologous sequence comprises a native gene and a sequence from a different species. In some embodiments, a DSL comprises at least two heterologous native gene and no polynucleotides a different species.

IV. Maize plant cells, plants, and seeds

"Maize" refers to a plant of the Zea mays L. ssp. mays and is also known as "corn". The use of "ZM" preceding an object described herein refers to the fact that the object is from *Zea mays*.

Maize plants, maize plant cells, maize plant parts and seeds, and maize grain having the modified RppK (Genomic DNA SEQ ID NO: 9; cDNA SEQ ID NO: 10; Protein SEQ ID NO: 11), *Ht1* (Genomic DNA SEQ ID NO: 6; cDNA SEQ ID NO: 7; Protein SEQ ID NO: 8), NLB18 (Genomic DNA SEQ ID NO: 1; cDNA SEQ ID NO: 2 or 4; Protein SEQ ID NO: 3 or 5), NLR01 (Genomic DNA SEQ ID No: 27; cDNA SEQ ID NO: 28; Protein SEQ ID No: 29), NLR02 (Genomic DNA SEQ ID Nos: 24; cDNA SEQ ID NO: 25; Protein SEQ ID No: 26), RCG1 (cDNA SEQ ID Nos: 30; Protein SEQ ID No: 31), RCG1b (cDNA SEQ ID Nos: 32; Protein SEQ ID No: 33), PRR03 (Genomic DNA SEQ ID Nos: 34; cDNA SEQ ID NO: 35; Protein SEQ ID No: 36), PRR01 (cDNA SEQ ID NO: 37; Protein SEQ ID No: 38), NLR01 (Genomic DNA SEQ ID Nos: 39; cDNA SEQ ID NO: 40; Protein SEQ ID No: 41), or NLR04 (Genomic DNA SEQ ID Nos: 42; cDNA SEQ ID NO: 43; Protein SEQ ID No: 44), for example sequences disclosed herein are also provided.

As used herein, the term plant includes plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like. Grain is intended to

mean the mature seed produced by commercial growers for purposes other than growing or reproducing the species.

EXAMPLES

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

Example 1

Designing a suitable locus for genetic engineering of disease resistance traits in maize

Several considerations were taken into account for defining and selecting a region of the maize genome suitable for the development of a disease super locus: ease of product assembly, molecular characteristics and regulatory and stewardship aspects.

One selected locus, Disease Super Locus 1 (DSL1), is located in the distal region of chromosome 1 approximately 0.5 cM away from complex trait locus 1 (CTL1). This distance is specifically chosen and engineered to facilitate breeding stacks with inserted traits, such as insect control traits and/or herbicide tolerance traits inserted at CTL1 landing pads (FIG. 1) and expedite final steps of product assembly. DSL1 spans approximately 3.2 cM or 515 Kbp in a region that does not display major structural variation across a range of germplasm, including a set of representative North American inbreds and a collection of tropical lines. At a more local level, pangenome alignment reveals that most of the region is structurally conserved in non-stiff stalk inbreds.

Identification of target sites for seamless insertion of traits

The DSL1 region was scanned for target sites using a bioinformatics tool searching for protospacer adjacent motifs (PAM) and retrieving the upstream 20-base sequences. The following filters were then applied to select the appropriate target sites and their corresponding guide RNAs.

Target sites were deemed unsuitable if less than 2.5kb away from any native gene annotation. Gene annotations in the target inbred were based on a bioinformatic pipeline combining in silico predictions and *in vivo* evidence. For downstream analytical reasons, target

sites located within 2kb of repetitive regions larger than 200bp were also deemed unsuitable.

Candidate guide RNAs targeting suitable sites were finally inspected in silico for their potential off-target activity using a bioinformatic tool run against the genome assembly. For each candidate guide RNA, a list of potential off-target sites was generated based on bioinformatics analysis, potential off-target hits were dismissed if they presented 3 or more mismatches with the guide including at least one mismatch in the PAM proximal seed sequence (Young, Zastrow-Hayes *et al.* 2019, Sci Rep Apr 30;9(1):672).

A list of potentially acceptable sites in DSL1 is provided in Table 2. FIG. 2 shows a schematic drawing of the locations of target sites.

TABLE 2. Acceptable sites in DSL1

SEQ ID NO:	**Name**	**GUIDE_RNA_WITH_PAM**	**Best B73_v2 hit_Chr01**	**Estimated B73 genetic coordinate**
12	DSL1-CR1	GCACGCTCCAGGTTAATGGCTGG	ZmChr1v2:12883117	46.37
13	DSL1-CR3	GCAGCTGAAATTGAGCCTCCCGG	ZmChr1v2:12917624	46.51
14	DSL1-CR4	GATTAGTCTCGGCATACGTACGG	ZmChr1v2:12918033	46.51
15	DSL1-CR5	GGATAATGGCGTACGTATTGCGG	ZmChr1v2:12921435	46.53
16	DSL1-CR6	GTTTCGAACAGAACGTACGCAGG		
17	DSL1-CR7	GGCTAGGCGTGTCACCATAATGG	ZmChr1v2:12972339	46.74
18	DSL1-CR9	GAATACGAAACTATACCGCGGGG		
19	DSL1-CR14	GACTACCTCTGGGGGTACGTAGG	ZmChr1v2:13502712	49.15
20	DSL1-CR17	GACGGGGACTTAATTATGCGTGG	ZmChr1v2:13527536	49.28

21	DSL1-CR18	GCGATCCGTCACTTGTATATCGG	ZmChr1v2:13550737	49.4

TABLE 3 Markers Flanking DSL1

Probe/Marker	PHI_v2 cM	AlleleA Probe SEQ ID NO:
pze-101020971	45.75	22
pze-101022341	49.45	23

Vector construction of guides and template

To improve their co-expression and presence, the Cas endonuclease and guide RNA expression cassettes were linked into a single DNA construct. A 480-490 bp sequence containing the guide RNA coding sequence, the 12-30 bp variable targeting domain from the chosen maize genomic target site, and part of the U6 promoter were synthesized. The sequence was then cloned to the backbone already have the cas cassette and the rest of the gRNA expression cassette.

Homology-directed repair (HDR) templates were designed to enable the insertion of disease resistance genes at the desired target sites. To optimize delivery, template sequences were synthesized and cloned on the vector backbone containing Cas endonuclease and guide RNA. In this setting, release of the template from the vector is achieved by inserting the target site sequence corresponding to the guide RNA encoded on the vector on each side of the HDR template FIGURE 3). Template sequences included the full genomic region(s) of the disease resistance gene(s) of interest, flanked by homologous arms corresponding to the 100-1000bp region directly adjacent to the cut site.

The plasmids comprising the Cas endonuclease expression cassette, guide RNA expression cassette and HDR template were delivered to maize embryos by Agrobacterium mediated transformation. Upon DNA cleavage at the designated site by Cas endonuclease, templates will be integrated by homology directed repair, resulting in seamless insertion at the cut site of the genomic regions conferring resistance to one or multiple diseases.

Insertion of maize genomic fragments conferring resistance against Northern Leaf Blight and Southern Rust

One genomic fragment may contain a single source of resistance or multiple sources molecularly stacked to create genomic insertions at DSL1. In certain aspects, the coding sequences present within this genomic fragment are driven by their native regulatory sequences, such as native promoter and/or enhancer sequences compared to a transgenic cassette driven by a non-native or heterologous promoter. Single and stacked insertions at different target sites within DSL1 may then be used individually or later combined by breeding. As an example, genomic fragments of NLB18 (Genomic DNA SEQ ID NO: 1; cDNA SEQ ID NO: 2 or 4; Protein SEQ ID NO: 3 or 5) or HT1 (Genomic DNA SEQ ID NO: 6; cDNA SEQ ID NO: 7; Protein SEQ ID NO: 8), conferring resistance against Northern Leaf Blight (U.S. Patent Application No. 16/341,531), and genomic fragment of RppK gene from inbred line K22 (WO2019/236257 (Genomic DNA SEQ ID NO: 9; cDNA SEQ ID NO: 10; Protein SEQ ID NO: 11), conferring resistance against Southern Rust, may be inserted at DSL1 individually or in combination as illustrated in FIG. 4.

Example 2

Introgressing or forward breeding multiple disease resistance loci into elite germplasm

A Disease Super Locus (DSL) where multiple genes are combined within about a 5 cM region to confer resistance to multiple diseases may have several advantages compared to independently introgressing of the different genes into a base inbred line.

To combine 7 genes from 7 different resistant donor lines conferring increased resistance to 4 different diseases the number of populations that need to be developed to combine these QTL into a single inbred lines, is large and the different crosses that eventually are needed to move all loci containing the resistance gene into the same background are numerous and would take a long time. In addition, selecting for and maintaining 7 independent loci together in new crosses developed as part of a regular breeding programs is commercially impractical and limits the number traits introduced in any given product cycle. One would need to backcross the independent QTL regions into the same base inbred line that needs improvement for resistance. A typical scenario is to backcross and then self to obtain Near Isogenic Lines (NILs) with the locus containing the resistance gene present in the Recurrent Parent background.

Markers may be used to genotype for the presence of the resistance locus in the backcross lines and the subsequent selfed lines. A typical scenario is to develop a third backcross generation and two selfing (BC3S2) generation lines. If three generations can be

grown per year, developing homozygous BC3S2 lines would take about 2 years.

Once Near Isogenic Lines for each of the individual seven loci have been developed, one would need to start making additional crosses to combine the 7 QTL regions, which will take additional generations (5 - 6 generations, which equals approximately 2 years) and large population sizes in order to be able to develop a Near Isogenic Line (NIL) with 7 homozygous resistance loci. To ensure these 7 loci are simultaneously selected for in subsequent breeding populations would require very large population sizes to ensure progeny containing seven homozygous loci would be obtained to maintain the desired level of resistance to multiple pathogens.

Theoretically, only 1 in 16384 progeny would be fixed for all seven loci in and F2 population derived from a line having all 7 resistance loci in homozygous from with a line not containing these 7 loci. This single progeny would only be selected for the presence of the 7 loci for resistance and not for any other desired traits. In a breeding program, many traits need to be considered when selecting the next generation of improved germplasm. Therefore, one may need for example 30-100 F2 progeny containing the 7 resistance loci in order to also allow for selection of other important traits that will be segregating in the F2 progeny of the two parents. This would translate to needing ~0.5 million to ~ 1.6 million progeny from one cross in order to ensure one can select a line that has both, improved agronomic traits and disease resistance at the 7 loci. Such population sizes will be impossible to develop as part of commercial breeding programs.

Besides the extended time needed for the development of lines containing resistance loci from different donor sources and the enormous populations sizes needed to ensure presence of the 7 loci in subsequent generations, the other challenge will be to minimize linkage drag from the donor sources. Even when marker assisted selection is being used, recurrent parent genome recovery will be less than 100%. Even if only 2 % of the donor source genome is retained in the recurrent parent background, this would translate into several hundreds of genes from the resistant donor parent being present in each of the Near Isogenic Lines developed for every single resistant locus.

When the resistance loci from 7 different Near Isogenic lines are brought together and assuming each of these NIL still contains 2% of their respective donor source genomes, the final Near Isogenic Line, containing the 7 resistant loci, may have up to 14% non-elite genome present in its background. Since resistant donor sources are often non-adapted lines, with good resistance but bad agronomic characteristics, the 14% derived from non-adapted donors will

very likely result in detrimental effects on traits such as e.g. maturity and yield.

In contrast, using a DSL approach, the seven genes are transferred into a defined genomic region in a current elite germplasm line (or a select set of elite germplasm lines) selected for good agronomics. There will be no extra donor genome present in this line besides the genomc fragment sequences for the seven disease resistant genes. In addition, this approximately 5 cM DSL region is identical or substantially identical in many commercially relevant elite lines and therefore introgression of this region into other elite lines will improve resistance to multiple pathogens.

The time frame for inserting the seven native resistance genes from different resistant maize donors into this elite line and developing the homozygous resistant lines is shorter using a DSL approach. Once such an initial resistant line, with exactly the same genomic background as the base inbred, besides the seven inserted genes within the 5 cM DSL region has been developed, it may be used as the resistant, elite bridge donor line for subsequent introgression of the DSL into other elite germplasm.

Such an introgression process may be finalized in a 2 year time frame and since the resistant bridge donor line is in an elite background, even if 2% of the genome of this resistant bridge donor line will still be present in the new introgressed line, there should be no negative effects on agronomic traits, since the bridge donor line is an elite line developed through many years of breeding for good agronomic characteristics.

Opportunities for breeding programs utilizing a DSL region

Having the option to introgress or forward breed with the DSL region which confers resistance to multiple important pathogens, also allows breeding programs to utilize the rest of the genome for selection of favorable traits besides disease resistance. In otherwords, once the DSL region is fixed, breeders are free to choose, deselect, and/or select other linked or unliked traits to the previously located disease resistant loci without risking the loss of the resistance alleles due to segregation of desirable alleles. In the current breeding process, one always needs to select for a baseline of resistance for multiple diseases. Some of the regions involved with disease resistance may be linked to negative alleles for agronomic traits. If high levels of resistance to multiple pathogens can be brought in via introgression of, or forward breeding with the DSL, breeding programs can focus on selection for best agronomic traits utilizing all of the genomic regions outside of the DSL and will not have to compromise for disease resistance and putative linkage with negative effects in the rest of the genome. The opportunity

to select desired agronomic charactersistics utilizing all of the maize genome without being restricted to simultanously select for a desired level resistance to multiple diseases, since the DSL provides such resistance, may result in quicker progress in breeding for traits such as for example yield, drought tolerance as well as other agronomic traits.

Improved Agronomic Traits with Multiple Disease Resistance with Reduced Yield Drag from Breeding

With the opportunity to select for positive agronomic traits across the genome, without the constraints of needing multiple different loci throughout the genome to confer a base level of resistance to multiple diseases, there is the potential to make additional progress in order to develop better yielding lines with better overall agronomics.

Replacing one or more resistance genes in the DSL of an elite lines containing such DSL may be necessary when the pathogen community in the field changes over the years, either due to a race shift that can overcome the resistance gene(s) or due to increasing problems with a new pathogen that was not a problem before.

Traditional crossing and selections to bring new QTL regions from non-adapted donor lines into elite germplasm is likely to be commercially costly due to the challenges mentioned around number of crosses, population size needed, timeline to develop inbred lines containing the combination of multiple QTLs in homozygous form as a disease control option. Keeping multiple QTL regions together in subsequent line germplasm development in the future is not currently feasible in regular breeding programs due to the same challenges.

In contrast, removing, replacing or adding new resistance genes to the DSL in an elite inbred line via the targeted gene editing technology is quicker and with reduced linkage drag around the gene of interest or due to background genetics coming from the resistant, non-adapted donor lines. One would be able to develop an identical or a near identical line compared to the initial DSL containing inbred line but now with either new disease resistance genes replacing non-functional disease resistance genes, newly added disease resistance genes in the Disease Super Locus, or a new swapped DSL or a remodified DSL.

Insertion of multiple copies of the same allele to optimize trait expression and eliminating biparental presence

In contrast to traditional crosses and selection procedures, one can also combine multiple desired alleles of the same gene together in the DSL (i.e., in the same chromosomal

arm/region) of one inbred line, as sometimes is desirable to confer the desired level of resistance. If two copies of a desired allele are present per chromosome at the DSL in the inbred line, then the hybrid resulting from a cross of this inbred line, with another inbred line (not having such allele) will result in a hybrid progeny with two copies of the allele. This would not be possible with traditional hybrid development, where one would need to introgress the gene of interest on both sides of the pedigree to develop a hybrid with two copies of the desired allele.

Stacking of genetically linked resistance genes from multiple sources

One may also insert alleles of resistance genes to a DSL originating from different donor sources, but which are located in exactly the same region on the maize genome in those different donor lines. Using traditional crosses, combining such genes coming from different donor sources into one elite recurrent parent will be challenging or not practical for a commercial product development cycle due the fact that obtaining the correct recombination between genes in the same location on the genome from independent donor lines only occurs in very low frequencies. It would take large number of crosses and progeny to have a chance to identify a progeny line with the desired recombinations.

Stacking of resistance genes from multiple sources with structural variation impeding homologous recombination

For example, maize contains disease resistance genes clusters, such as on the short arm of chromosome 10 (c10). These clusters can present significant structural variation, hindering homologous recombination during breeding crosses due to lack of sequence homology with other breeding lines.

If for example one would like to combine adisease resistance gene from donor line A on c10 with a disease resistance gene from donor line B that is located in the same genomic region on c10 and move both disease resistance genes into elite inbred line C, several challenges can occur. Since such a region may be genetically quite different between the three lines due to differences in gene content and intergenic sequence differences, it can potentially be difficult to obtain progeny (in a commercially relevant breeding cycle), that has any recombination in such regions since highly divergent regions will recombine less. This would hamper the opportunity to develop progeny that will have the desired recombination allowing the move of the two disease resistance genes of two different donor lines into an elite inbred

line. In addition, even if one can successfully generate such a unique recombination, there is likely a large region from the donor lines that will still be present in the elite inbred line due to lack of recombination frequency resulting in linkage drag of donor line genome around the disease resistance genes into the elite inbred line. In such a resistance gene cluster of an inbred line, it may be possible that there are genes present with a resistant allele for certain diseases and other genes that harbour a susceptible allele to other diseases. Combining only the resistance alleles of different genes from several inbred lines via recombination and simultanously avoiding recombination between the inbred lines that result in genes with desirable resistant alleles to be linked with undesirable susceptible alleles is often very difficult. A Disease Super Locus will allow for such stacking of resistance alleles from multiple maize lines without being hampered by the chance of introducing undesirable susceptible alleles through recombinantion, since a Disease Super Locus is not relying on recombination and creation of desired recombination, but allows for precise and targeted stacking of only the alleles that will confer disease resistance.

Insertion of DSL locus in proximity to another trait or region of interest

Another advantage of the development of a Disease Super Locus is that one may have this DSL be located immediately next to the genetic region in which an insect resistance locus (IRL) has been developed. In one embodiment, the IRL may be an Insect Super Locus (ISL). This will allow for simultaneous introgression of multiple insect resistance traits and disease resistance traits at the same time. The trait introgression process will be cost effective, since these multiple traits will be introgressed as one locus, it will be faster since there will be no need to introgress different loci in a recurrent parent and then make final crosses and self for several generations, to develop homozygous lines for both the insect resistance locus (IRL) and Disease Super Locus; and lastly, it will limit the presence of donor line genetics in the genomic background of the converted recurrent parent since only one Super Locus instead of two different Super Loci will be introgressed from a donor parent, which would result in a lower percentage linkage drag and lower percentage background genome from the donor parent present in the final introgressed line.

If one would need to separate the Insect Super Locus from the Disease Super Locus in the future, this will be possible by identifying recombinants between the two Super Loci (SL). A current line was created with a DSL about 0.6 cM genetic distance from an IRL, and since these SL have been developed in elite germplasm, the sequence similarity in this 0.6 cM region

between the line containing the two SL and a large portion of our inbred lines is exactly the same. Therefore the recombination frequency is expected to be normal and one should be able to identify recombinant progeny lines in an F2 populations at a frequency of 1 in 165 progeny.

Thus, if there is a need to separate the IRL from the Disease Resistance Trait Package in the DSL this may be done. Having the opportunity to introgress such combined trait packages as one locus, being able to separate the different trait packages as needed and being able to replace or add new disease resistance genes to the DSL region via gene editing, allow the development of hybrids that are best suited for specific environments.

Developing a distinct single SL that contains trait packages that allow for control of multiple diseases, or different insects or a combination of both will also simplify the process of combining such SL together with other traits like for example herbicide tolerance in a single hybrid. One can, for example, have the DSL plus ISL introgressed on the female side of the pedigree and combine this with a herbicide tolerance trait on the male side of the pedigree. By limiting the number of loci to introgress through the development of the SL, one can also more easily combine another trait this SL in one line if so desired. The number of progeny and crosses that are needed to develop a line that combines two independent loci of interest is orders of magnitude less compared to bringing 7 or more independent loci together in homozygous state in one single inbred line.

Example 3

Defining a suitable locus for genetic engineering of disease resistance traits in soybean

Several considerations are taken into account when designing and selecting a region of the soybean genome suitable for the development of a disease super locus: ease of product assembly, molecular characteristics and regulatory and stewardship concerns.

One Disease Super Locus (DSL) is located in a region that does not display major structural variation across a range of germplasm.

Identification of target sites for seamless insertion of traits

The DSL region is scanned for target sites using a bioinformatic tool searching for protospacer adjacent motifs (PAM) and retrieving an upstream 20-base sequences. Filters are then applied to select the appropriate target sites and their corresponding guide RNAs.

Target sites are deemed unsuitable if less than 2.5kb away from any native gene

annotation. Gene annotations in the target inbred are based on a bioinformatic pipeline combining in silico predictions and in vivo evidence. For downstream analytical reasons, target sites located within 2kb of repetitive regions larger than 200bp are also deemed unsuitable.

Candidate guide RNAs targeting suitable sites are finally inspected in silico for their potential off-target activity. For each candidate guide RNA, a list of potential off-target sites is generated based on the current literature, potential off-target hits are dismissed if they presented 3 or more mismatches with the guide including at least one mismatch in the PAM proximal seed sequence.

Vector construction of guides and template

A suitable Cas gene is operably linked to a soybean ubiquitin promoter by standard molecular biology techniques.

A soybean promoter is used to express guide RNAs which direct Cas nuclease to designated genomic sites. In order for the Cas endonuclease and the guide RNA to form a protein/RNA complex to mediate site-specific DNA double strand cleavage, the Cas endonuclease and guide RNA have to be present in simultaneously. To improve their co-expression and presence, the Cas endonuclease and guide RNA expression cassettes are linked into a single DNA construct. A sequence containing the guide RNA coding sequence, a variable targeting domain from the chosen soybean genomic target site, and part of the promoter are synthesized. The sequence is then cloned to the backbone already having the cas cassette and the rest of the gRNA expression cassette.

Homology-directed repair (HDR) templates are designed to enable the insertion of disease resistance genes at the desired target sites. To optimize delivery, template sequences are synthesized and cloned on the vector backbone containing Cas endonuclease and guide RNA. In this setting, release of the template from the vector is achieved by inserting the target site sequence corresponding to the guide RNA encoded on the vector on each side of the HDR template. Template sequences includes the full genomic region(s) of the disease resistance gene(s) of interest, flanked by homologous arms corresponding to the 100-1000bp region directly adjacent to the cut site.

The plasmids comprising the soybean codon optimized Cas endonuclease expression cassette, guide RNA expression cassette and HDR template are delivered to soybean embryos by Agrobacterium mediated transformation. Upon DNA cleavage at the designated site by Cas endonuclease, templates are integrated by homology directed repair, resulting in seamless

insertion at the cut site of the genomic regions conferring resistance to one or multiple diseases.

Example 4

Insertion of soybean genomic fragments conferring resistance against diseases

One template may contain a single source of resistance or multiple sources molecularly stacked to create genomic insertions at DSL. Single and stacked insertions at different target sites within DSL may then be used individually or later combined by breeding.

For example, soybean disease resistance traits may include Soybean Cyst Nematode resistance as described in U.S. Patent No. 7,872,171), tolerance against *Fusarium Solani* (a soybean sudden death syndrome pathogen; currently named *Fusarium virguliforme*) as described in U.S. Patent No. 7,767,882, Phytophthora tolerance in soybean as described in U.S. Patent Publication No. US20140178867A1, Soybean cyst nematode resistance as described in U.S. Patent Publication No. US20160130671A1 and U.S. Patent No. 9,464,330, Soybean root-knot nematode tolerance as described in U.S. Patent Publication No. US20130047301A1, Frogeye leaf spot resistance and brown stem rot resistance as described in U.S. Patent Publication No. US20160032409A1, Charcoal rot drought complex tolerance in soybean as described in U.S. Patent No. 9,894,857 and U.S. Patent Publication No. US20180084745A1, resistance of Soybean to cyst nematode as described in U.S. Patent No. 9347105, Brown stem rot resistance in soybean as described in U.S. Patent Publication No. US20180291471A1 and U.S. Patent Publication No. US20180334728A1, Soybean cyst nematode resistance as described in U.S. Patent No. 9049822, Phytophthora resistance as discribed in U.S. Patent Publication No. 2014-0283197, Phytophthora root and stem rot in soybeans as discussed in U.S. Patent No. 10995377.

Example 5

Chromosomal engineering

Chromosomal region or segments, including a DSL associated with one or more diseases in crop plants such as corn, soybean, cotton, canola, wheat, rice, sorghum, or sunflower are rearranged (e.g., inversion, translocation) such that those chromosomal regions are in a preferred chromosomal configuration that enables faster trait introgression, reduced linkage drag, optimal linkage disequilibrium compared to control and other breeding enhancements. In an embodiment, a preferred chromosomal configuration is a DSL

chromosomal segment is translocated to a preexisting transgenic locus containing one or more insect and/or herbicide tolerant traits, optionally, transgenic traits. In another embodiment, a first DSL is translocated with a second DSL, wherein the second DSL contains at least one different gene from the first DSL. In a further embodiment, a DSL is translocated to a telomeric region where trait introgression into other elite germplasm is made more efficient by relying on a single cross-over instead of two.

Example 6

Optimizing fungicide use on plants that have multiple disease resistant genes

Use of crop plants with DSL may allow for a reduced fungicide use or delayed fungicide use because these plants display multiple modes of resistance against a plurality of pathogens. Therefore, optimizing fungicide use on such plants help systems agriculture and farming operations. Fungicide use has become prevalent over the past few years due to increase pest pressure. In the US, two thirds of growers make at least one fungicide application during the growing season on their corn or soybean crop. Other geographies require additional applications to adequately protect yields, such as in Brazil and Argentina. These practices add to a farmer's cost and also inconvenient, while also increasing the use of pesticides. In addition, timing of the application is highly relevant to treatment outcome and is one of the key challenges encountered during the season. Multi-disease resistant hybrids comprising a Disease super locus can alleviate the need for fungicide use and allow flexibility in the timing application. In addition, when fungicide treatment is still advised, such hybrids are expected to require lower rates of applications, therefore increasing the durability of the fungicide and reducing the impact on the environment and increasing sustainability.

Example 7

Insertion of non-coding sequences

Disease Super Locus (DSL) may contain source of resistance from genes or sequences that don't encode polypeptides. Instead, the genes or sequences may be transcribed into non-coding transcripts or non-coding RNAs, which may regulate gene expression and function as a source of resistance against plant pathogens.

A DSL may contain one or more polynucleotide sequence that don't encode a polypeptide, transposons, repetitive sequences that may transcribe into non-coding transcripts of various sizes such as long non-coding RNAs (lncRNAs), for example. One non-coding transcript may be processed into small RNAs such as microRNA (miRNA), short-interfering RNA (siRNA), trans-acting siRNA (tasiRNA), and phased siRNA (phasiRNA). The non-coding genes and sequences in a DSL may share nucleotide sequence homology to specific sequences in plant pathogens or pests, such as viruses, bacteria, oomycetes, fungus, insects, and parasitic plants. A non-coding transcript or processed products such as small RNAs like this may regulate or interfere with the expression of specific genes or sequences in plant

pathogens or pests, resulting in reduce pathogen pathogenicity and providing improved host plant's resistance.

In an aspect, a susceptible allele may be knocked out in a plant comprising a DSL either directly – e.g., by inserting the resistant allele and replacing the susceptible allele, when such location already is part of a DSL. In other embodiments, the susceptible allele may be knocked out or knocked down by RNA interference, homologous recombination, genome modification including CRISPR and TALENS, or by inserting the DSL within the susceptible allele locus.

Example 8

DSL plants provide flexibility in crop management practices to growers

Conservation tillage practices such as no-till or strip-till are often desired in farming systems because of their positive impact on the environment. These practices contribute to limiting soil erosion and improving soil quality. In addition, they offer another advantage by reducing the fuel and labor requirement. However, increased disease pressure due to crop residue from the previous growing season is often prohibitive especially in environments prone to outbreaks. In those cases multi-disease resistant hybrids comprising a DSL would enable a wider adoption of these practices in a larger range of environments.

Hybrid plants comprising a DSL and therefore rendered more resistant to multiple diseases allow more flexibility in certain farming practices that may not have been possible or considered too risky using standard hybrids. The severity of many diseases affecting above ground parts of the plant such as leaf and/or stem is in part determined by the amount of inoculum present on the soil surface. Residue from the previous growing season is one of the possible sources of this inoculum, as many pathogens can survive on debris and other plant parts that remain in the field from the previous crop. Management practices such as crop rotation and tillage have a direct impact on the type and amount of residue left in the field after a growing season and therefore have the potential to alleviate or exacerbate disease pressure at the beginning of the next growing season.

For example, *Helminthosporium turcicum*, the pathogen responsible for Northern Leaf Blight overwinters primarily on corn residue. Besides specific weather conditions, outbreaks of the disease have been associated with corn-on-corn and conservation tillage practices. Susceptible hybrids are especially at risk of developing lesions under those practices. Hybrids comprising a DSL and rendered resistant to multiple diseases including NLB, as well as

multiple races of the NLB disease, for example, are expected to not only leave residue with a reduced pathogen load, but also show resistance to this inoculum especially early in the season.

Weed management primarily protects crops against competition for resources, such as nutrients, water and light. Because weeds can also serve as reservoirs for plant diseases and insect vectors of plant diseases, weed management can also impact plant health and protect crops from disease. For example grassy weeds such as witchgrass can harbor *Colletotrichum graminicola*, the fungal pathogen responsible for Anthracnose in corn. It is expected that the use of hybrids comprising a DSL and rendered resistant to multiple diseases including Anthracnose can alleviate the need and especially the strict timing for weed control when disease pressure is a concern. This can enable more flexibility on the farm when making management and weed treatment decisions.

Example 9

Increased disease resistance durability in crop plants – both for genetic traits and crop protection agents

Analyses of field monitoring data in studies indicate that the pyramiding of disease resistance genes within a plant is a most powerful approach to provide durable resistance to plant pathogens. Such pyramiding or stacking strategy allows for longer period of effectiveness of the resistance genes.

A Disease Super Locus (DSL) allows for such stacking of several genes conferring resistance to a pathogen and it also allows for adjustments of the DSL locus (swapping, adding genes/alleles) in case pathogen communities in the field shift over time.

Disease management such as deploying a DSL, keeps pathogen population sizes small which will assist in controlling the total number of mutation or recombinations in such smaller population and limit the occurrence of mutations or recombinations that are favorable to the pathogen for overcoming the host resistance. In other words, by limiting the population size of pests, the chance that a resistance avoiding mutation may appear in such a pest population is reduced by the presence of DSL in crop plants grown in field conditions subject to pest pressure in a crop growing environment.

The combination of disease resistance genes with other practices for pathogen control (pesticides, farming practices) is a relevant management strategy to slow down the evolution of virulent pathogen genotypes and various means of pest control can synergistically increase each other's durability.

As such, deploying a DSL in combination with a suitable pesticide management strategy, may not only extend the durability of the resistance genes in the DSL, but may also extend the durability of a pesticide utilized to control the pathogen by limiting mutations in the pathogen genes that are targeted by the pesticide.

Example 10

Increased modularity of a DSL approach compared to traditional pyramiding of traits by breeding

A Disease Super Locus approach provides an easier way to modulate the set of genes necessary to provide adequate resistance to disease in specific environments, or in specific germplasm. For example, the set of diseases that are likely to affect a corn crop depends largely on the geography: the risk of developing Corn Southern Rust is higher in the South East than in other areas in the US, while the risk of developing Gray Leaf Spot is higher in the US corn belt and the Atlantic states. In addition, race evolution in certain areas may lead to new races becoming prevalent in specific geographies and spare other areas. It is also known that specific hybrid combinations are more or less susceptible to specific diseases or races, due to the underlying combination of native traits present in the inbred parents germplasms. Under these circumstances, it may be desirable to modulate the package of disease resistance traits that are delivered through the DSL and adapt it to specific geographies and germplasm susceptibilities. A super locus approach lends itself well to this need for flexibility that a traditional breeding approach can only achieve with significant time and dedicated effort. For example, a corn hybrid may present agronomic characteristics that make it well suited to multiple geographies with varying degrees of disease pressure. Using a DSL approach, one can readily insert the desired set of disease resistance genes in one inbred parent providing adequate resistance to disease most likely to occur in one area and a slightly different set of disease resistance genes in the same inbred parent providing adequate resistance to disease most likely to occur in another area. As a result, hybrids that present similar agronomic characteristics but disease resistance profiles that are adapted to distinct geographies can be produced using this approach. This outcome could be achieved using a super locus approach by inserting two different sets of genes at DSL target sites. It could also be achieved by creating a first DSL insertion comprised of disease resistance genes against disease 1 “set 1” and then crossing with another inbred comprised of disease resistance genes against disease 2 “set 2”, while also crossing “set 1” with an inbred comprised of a third set of genes against disease 3 “set 3”, creating two

inbreds each with different sets of resistance genes (sets 1 and 2, or sets 1 and 3). The same outcome could be achieved by creating a first inbred comprised of set 1, and re-transforming this inbred to create insertions of sets 2 or 3. It could also be achieved by creating a first inbred comprised of sets 1 and 2, and swapping set 2 with set 3. If one of the genes or sets of genes in an inbred created in one of these possible manners becomes obsolete because of shifting disease pressure for example, one could directly delete the unwanted gene or sets of genes, or swap it to replace with a more relevant gene or set of genes. In comparison, achieving the same outcome using traditional breeding methods would be impractical due to the cost and time required, as well as the potential for linkage drag occurring for each of the new genes introgressed. Such modularity can also be achieved by built-in, unique recombination linking ("URL") sequences that are interspaced within a plurality of the disease resistance genes in a given DSL. For example, such a DSL can include a signature comprising "Resistance Gene A – URL1-Resistance Gene B-URL2-Resistance Gene C and so on and so forth. Such URLs can be designed to be targeted by specific recombination enhancing agents such as CRISPR-Cas endonucleases or any other site directed agent including for example, FLP/FRT recombinase based systems.

Example 11

Planting density of DSL plants

Pathogens are generally very sensitive to weather conditions. In addition, some pathogens are especially sensitive to the micro-environment in the plant canopy. This is the case of *Cercospora maydis*, which is responsible for Gray Leaf Spot. Humidity on and around the leaf surface is conducive for the development of this disease. It is expected that plant density and row spacing for example have a direct impact on this micro-environment. Higher density creates conditions where moisture is increased and ventilation is decreased, both amenable to pathogen development. The use of hybrids comprising DSL and resistant to GLS, for example, can mitigate this issue, and in turn enable higher planting densities (e.g., 40,000-80,000 or more maize plants per acre) which may otherwise not have been considered due to a higher risk of disease outbreak.

Example 12

Maturity and planting date of DSL plants

It is recognized that later maturity hybrids and delayed plantings are at higher risk of developing disease late in the season and incurring significant yield losses. Hybrids comprising a DSL and resistant to multiple diseases including those developing later in the growing season are expected to perform better when disease pressure is high during grain fill. Multi disease resistance brought by the presence of a disease super locus in the germplasm may provide more flexibility in planting date and enhanced yield protection for later maturity hybrid classes.

Example 13

Combining a knockdown of susceptibility native locus with a DSL

In addition to inserting disease resistance alleles at a Disease Super Locus, it is known in the field that knocking out or down regulating the expression of susceptibility genes can enhance the durability and spectrum of pathogen resistance. Thus combining a DSL approach with knock outs of known disease susceptibility genes can be desirable. For example, it is known that genes involved in nutrient transport and availability are sometimes activated during pathogen infection and used at the plants' expense to sustain pathogen infection. In one embodiment, several methods may be envisioned that would enable combining both modes of resistance. One approach is to create an inbred that is comprised of one or several susceptibility genes knock outs obtained by gene editing, classical mutagenesis or breeding of natural variation, and combining this material with an inbred comprised of a DSL by breeding crosses. Another approach is to create the same by inserting disease resistance genes at a DSL in an inbred that is comprised of one or several susceptibility genes knocks by direct transformation. A third approach is to create a similar outcome by inserting at the DSL both disease resistance genes as well as non-coding transcripts acting in trans to down-regulate or knock out the expression of susceptibility genes located in the genome.

Example 14

Using native enhancers to change expression of disease resistance genes in a DSL for desired phenotype in crops

Genes or QTLs can be recessive or semi-dominant and require two copies of the gene or QTL to obtain the desired trait. Two or more copies of a gene or QTL may be introduced into a DSL. In hybrid crops this requires that the gene or QTL is introgressed in both the male

and female parents. This introgressed region can bring additional genomic regions that results in linkage drag. If the causal gene is known, then a plasmid vector carrying the gene necessary for the desired trait can be used as a template to add an additional copy to a parent using CRISPR or transgenic approaches. When using a transgenic approach, different regulatory element combinations, such as promoters, introns and terminators, can be used to express the causal gene appropriately for the desired phenotype. However, if two copies of a QTL are needed, a plasmid template is not possible. The expression of a QTL region can be altered by native enhancers or super enhancers using CRISPR-Cas. One possibility of altering the expression of the causal gene or group of genes within the QTL is to use CRISPR to move a native enhancer near the QTL or another part of the genome, which changes the expression level or expression pattern of genes within the QTL, leading to the desired phenotype. An alternative approach is to move the QTL to a new chromosomal region in which a native enhancer or super enhancer changes the temporal, spatial or level of expression of the causal gene within the QTL. If similar expression changes are needed for multiple QTLs, these QTLs could be co-located in a super locus in which a native enhancer affects multiple genes and QTLs.

Example 15

Short stature maize plants containing genetic modifications that impact plant height

In some embodiments, maize plants comprising DSL are of short stature. See US20200199609A1, incorporated herein by reference in its entirety, for enabling methods and compositions to generate short stature plants and agronomic management solutions involving short stature plants. DSL maize plants comprise one or more genetic modifications that target more than one distinct genomic loci that are involved in plant height reduction. In an embodiment, the plant height is reduced by about 5% to about 30% compared to the control plant. In an embodiment, the plant comprises an average leaf length to width ratio reduced at V6-V8 growth stages. In an embodiment, the plant height reduction does not substantially affect flowering time. In an embodiment, the flowering time does not change by more than about 5-10 CRM or plus or minus 10% GDU or 125-250 GDU, compared to a control plant not comprising the modifications.

In an embodiment, DSL maize plants as shown herein comprise a Br2 genomic locus that comprises an edit in a polynucleotide that encodes a Br2 polypeptide comprising an amino acid sequence that is at least 95% identical to SEQ ID NO: 43 of US20200199609A1,

such that the edit results in results in (a) reduced expression of a polynucleotide encoding the Br2 polypeptide; (b) reduced activity of the Br2 polypeptide; (c) generation of one or more alternative spliced transcripts of a polynucleotide encoding the Br2 polypeptide; (d) deletion of one or more domains of the Br2 polypeptide; (e) frameshift mutation in one or more exons of a polynucleotide encoding the Br2 polypeptide; (f) deletion of a substantial portion of the polynucleotide encoding the Br2 polypeptide or deletion of the polynucleotide encoding the Br2 polypeptide; (g) repression of an enhancer motif present within a regulatory region encoding the Br2 polypeptide; (h) modification of one or more nucleotides or deletion of a regulatory element operably linked to the expression of the polynucleotide encoding the Br2 polypeptide, wherein the regulatory element is present within a promoter, intron, 3′UTR, terminator or a combination thereof.

In an embodiment, DSL maize plants as shown herein comprise a D8 genomic locus that comprises a gibberellic acid biosynthesis or signaling pathway that is modulated by one or more introduced nucleotide changes at D8 genetic loci selected from the group consisting of: (a) reduced expression of a polynucleotide encoding the D8 polypeptide (as represented by SEQ ID NO: 76 of US20200199609A1, incorporated herein by reference in its entirety; (b) reduced activity of the D8 polypeptide; (c) generation of one or more alternative spliced transcripts of a polynucleotide encoding the D8 polypeptide; (d) deletion of one or more domains of the D8 polypeptide; (e) frameshift mutation in one or more exons of a polynucleotide encoding the D8 polypeptide; (f) deletion of a substantial portion of the polynucleotide encoding the D8 polypeptide or deletion of the polynucleotide encoding the Br2 polypeptide; (g) repression of an enhancer motif present within a regulatory region encoding the D8 polypeptide; (h) modification of one or more nucleotides or deletion of a regulatory element operably linked to the expression of the polynucleotide encoding the D8 polypeptide, wherein the regulatory element is present within a promoter, intron, 3′UTR, terminator or a combination thereof.

In certain embodiments, maize DSL plants of the present disclosure are planted at a higher planting density. This includes providing corn plants wherein the expression and/or activity of a polynucleotide involved in plant height is modulated resulting in a substantial height reduction or stature modification when compared to a control plant (i.e., reducing plant height by introducing a genetic modification that results in reduced stature of the corn plants); and planting the corn plants at a planting density of about 30,000 to about 75,000 plants per acre.

In certain embodiments, the planting density is at least 50,000 plants; 55,000 plants; 58,000 plants; 60,000 plants; 62,000 plants; 64,000 plants. In certain aspects, the corn plants comprise a mutation in a genomic region encoding D8 polypeptide or reduced expression of the polynucleotide encoding D8 polypeptide. In certain aspects, the corn plants are planted in a plurality of rows having a row width of about 8 inches to about 30 inches.

CLAIMS

What is claimed is:

1. A method of generating a heterologous genomic locus in a crop plant cell that comprises a plurality of intraspecies polynucleotide sequences, the method comprising introducing two or more intraspecies polynucleotide sequences to a predetermined heterologous genomic locus in the plant cell, wherein

(a) the introducing step does not result in integration of a transgene or a foreign polynucleotide that is not native to the plant;

(b) the intraspecies polynucleotides confer one or more agronomic characteristics to the plant;

(c) at least one or more of the intraspecies polynucleotides are from different chromosomes or the intraspecies polyucleotides are not located in the same chromosome in their native configuration compared to the heterologous genomic locus, prior to their integration into the heterologous genomic locus; and

(d) the introducing step comprises at least one site-directed genome modification that is not traditional breeding.

2. The method of claim 1, wherein the genomic locus is adjacent to a genomic locus that comprises one or more transgenic traits, the transgenic traits comprising a plurality of polynucleotides that are not from the same plant species.

3. The method of claim 2, wherein the transgenic traits comprise one or more traits conferring resistance to one or more insects.

4. The method of claim 2, wherein the transgenic trait comprises a herbicide tolerance trait.

5. The method of claim 1, wherein the genomic locus is defined by a chromosomal region that is about 1 to about 5 cM or an equivalent physical chromosomal map distance for the crop plant species.

6. The method of claim 5, wherein the chromosomal region is about 10Kb to about 50Mb.

7. The method of claim 1, wherein the polynucleotide sequences comprises at least two alleles of a gene.

8. The method of claim 1, wherein the plant is a corn, soy, canola, or cotton plant.

9. A method of generating a disease super locus in an elite crop plant genome to increase trait introgression efficiency in the elite crop plant, the method comprising

introducing a plurality of disease resistance traits at a predetermined genomic locus of the crop plant chromosome by engineering (a) insertion of two or more disease resistant genes, (b) genomic translocation of one or more disease resistant genes through targeted chromosomal engineering, (c) duplication of one or more disease resistant genes at the genomic locus by targeted genome modification, (d) modifying the genomic locus by introducing one or more insertions, (e) deletions or substitions of nucleotides in the genome, or a combination of the foregoing.

10. The method of claim 9, wherein the disease super locus is present in linkage disequilbrium with a transgenic trait.

11. The method of claim 10, wherein the transgenic trait is selected from the group consisting of insect resistance, herbicide tolerance, and an agronomic trait.

12. The method of claim 10, wherein the transgenic trait is a pre-existing commercial trait.

13. The method of claim 9, wherein the trait introgression efficiency is increased by reducing the backcrosses by at least 50% or by reducing the backcrosses by at least two or three generations.

14. The method of claim 9, wherein the plant is a corn, soy, canola, or cotton plant.

15. A method for obtaining a plant cell with a modified genomic locus comprising at least two heterologous polynucleotide sequences that confer enhanced disease resistance to at least one plant disease, or at least two traits resulting in resistance to at least one disease through two different modes of action, wherein said at least two polynucleotide sequences are heterologous to the corresponding genomic locus and are from the same plant species, the method comprising:

a. introducing a site-specific modification at at least one target site in a genomic locus in a plant cell;

b. introducing at least two polynucleotide sequences that confer enhanced disease resistance to the target site; and

c. obtaining the plant cell having a genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance.

16. The method of claim 15 wherein the at least one target site comprises a target site selected from Table 2.

17. The method of claim 15 wherein at least one of the two heterologous polynucleotides further comprise a site-specific modification.

18. The method of claim 17, wherein the site-specific modification is genetic or epigenetic modification.

19. The method of claim 15, wherein the polynucleotide sequence encodes a polypeptide sequence that confers enhanced disease resistance.

20. The method of claim 19, wherein the polynucleotide sequence encodes a polypeptide sequence that confers enhanced disease resistance, wherein the polypeptide sequence has at least 90% identity to a polypeptide sequence selected from the group consisting of RppK (SEQ ID NO: 11), Ht1 (SEQ ID NO: 8), NLB18 (SEQ ID NOs: 3 or 5), NLR01 (SEQ ID No: 29), NLR02 (SEQ ID No: 26), RCG1 (SEQ ID Nos: 31), and RCG1b (SEQ ID Nos: 33).

21. The method of claim 19, wherein the polynucleotide sequence encodes a polypeptide sequence that confers enhanced disease resistance, wherein the polypeptide sequence has at least 90% identity to a polypeptide sequence selected from the group consisting of PRR03 (SEQ ID No: 36), PRR01 (SEQ ID No: 38), NLR01 (SEQ ID No: 41), and NLR04 (SEQ ID No: 44).

22. The method of claim 15, wherein the at least two polynucleotide sequences comprise non-coding RNA or dsRNA.

23. The method of claim 15, wherein the plant is a corn, soy, canola, or cotton plant.

24. A method for obtaining a plant cell with a modified genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance to at least one plant disease, or at least two traits resulting in resistance to at least one disease through two different modes of action, wherein said at least two polynucleotide sequences are heterologous to the corresponding genomic locus, the method comprising:

a. introducing a double-strand break or site-specific modification at one or more target sites in a genomic locus in a plant cell;

b. introducing at least two polynucleotide sequences that confer enhanced disease resistance; and

c. obtaining a plant cell having a genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance.

25. The method of claim 24 wherein the at least one target site comprises a target site selected from Table 2.

26. The method of claim 24, wherein the polynucleotide sequence encodes a polypeptide sequence that confers enhanced disease resistance.

27. The method of claim 26, wherein the polypeptide sequence has at least 90% identity to a polypeptide sequence selected from the group consisting of RppK (SEQ ID NO: 11), Ht1 (SEQ ID NO: 8), NLB18 (SEQ ID NOs: 3 or 5), NLR01 (SEQ ID No: 29), NLR02 (SEQ ID No: 26), RCG1 (SEQ ID Nos: 31), and RCG1b (SEQ ID Nos: 33).

28. The method of claim 26, wherein the polypeptide sequence has at least 90% identity to a polypeptide sequence selected from the group consisting of PRR03 (SEQ ID No: 36), PRR01 (SEQ ID No: 38), NLR01 (SEQ ID No: 41), and NLR04 (SEQ ID No: 44).

29. The method of claim 24, wherein the plant is a corn, soy, canola, or cotton plant.

30. A corn plant comprising a modified genomic locus, the locus comprising at least a first modified target site and second modified target site, wherein the first modified target site comprises a first polynucleotide sequence that confers enhanced disease resistance to a first plant disease, and wherein the second modified target site comprises a second polynucleotide sequence that confers enhanced disease resistance to the first plant disease or to a second plant disease, wherein the first and the second polynucleotide sequences are heterologous to the modified genomic locus and are present within a genomic window of less than about 1 cM.

31. The plant of claim 30, wherein the plant comprises a first or a second polynucleotide sequence selected from a polypeptide, non-coding RNA, or dsRNA.

32. The plant of claim 31, wherein the polypeptide has at least 90% sequence identity to a sequence selected from the group consisting of RppK (SEQ ID NO: 11), Ht1 (SEQ ID NO: 8), NLB18 (SEQ ID NOs: 3 or 5), NLR01 (SEQ ID No: 29), NLR02 (SEQ ID No: 26), RCG1 (SEQ ID Nos: 31), and RCG1b (SEQ ID Nos: 33).

33. The plant of claim 31, wherein the polypeptide has at least 90% sequence identity to a sequence selected from the group consisting of PRR03 (SEQ ID No: 36), PRR01 (SEQ ID No: 38), NLR01 (SEQ ID No: 41), and NLR04 (SEQ ID No: 44).

34. The method of claim 30, wherein the plant is a corn, soy, canola, or cotton plant.

35. A method for obtaining a plant cell with an modified genomic locus comprising at least two polynucleotide sequences that confer enhanced disease resistance to at least one plant disease, or at least two traits resulting in resistance to at least one

disease through two different modes of action, wherein said at least two polynucleotide sequences are heterologous to the corresponding genomic locus, wherein the genomic locus is located in the distal region of chromosome 1.

36. The method of claim 35, wherein the genomic locus is located in the telomeric region.

37. A method of breeding transgenic and native disease traits at a single locus in a plant comprising:

a. inserting at a single locus in a plant a first heterologous polynucleotide sequence that confers enhanced disease resistance to a first plant disease, and second heterologous polynucleotide sequence that confers enhanced disease resistance to the first plant disease or to a second plant disease;

b. inserting at least one heterologous polynucleotide sequence encoding an insecticidal polypeptide, agronomic trait polypeptide, or a herbicide resistance polypeptide at the single locus;

c. crossing the plant with the single locus with a different plant; and

d. obtaining a progeny plant comprising the single locus; and

wherein the single locus allows for fewer backcrosses compared to a plant with traits at more than one locus.

38. The method of claim 37, wherein the different plant comprises a second locus comprising at least one heterologous polynucleotide sequence encoding an insecticidal or herbicide resistance polypeptide.

39. The method of claim 37, wherein the plant is a corn, soy, canola, or cotton plant.

40. A modified plant comprising a first heterologous polynucleotide encoding a first polypeptide that confers enhanced disease resistance to a first plant disease, and a second heterologous polynucleotide encoding a second polypeptide that confers enhanced disease resistance to a second plant disease; and a third heterologous polynucleotide encoding an insecticidal polypeptide or a herbicide resistance polypeptide; wherein the first heterologous polynucleotide, second heterologous polynucleotide, and third heterologous polynucleotide are located at a single locus in a plant.

41. The modified plant of claim 40, wherein the single locus comprises about 1cM, 5cM, or 10cM.

42. The method of claim 40, wherein the plant is a corn, soy, canola, or cotton plant.

43. A method of introgressing or forward breeding multiple disease resistance loci into an elite germplasm, wherein the timeframe for inserting two or more heterologous polynucltotides from different donor plants into the elite line and developing the homozygous resistant lines is shorter.

44. The method of claim 43, further comprising improving agronomic traits with multiple disease resistance with reduced yield drag from breeding.

45. The method of claim 43, wherein the plant is a corn, soy, canola, or cotton plant

46. The method of claim 1, wherein the polynucleotide sequences comprise at least two copies of the same gene.

47. The method of claim 1, wherein the genomic locus is stable through generations.

48. The method of claim 1, wherein the two or more intraspecies polynucletide sequences are genetically linked.

49. A modified crop plant comprising at least two, at least three, or at least four trait genes stacked in a single genomic locus, wherein the trait stack in a single locus allows for increased breeding efficiency and wherein the trait stack comprises at least two or more non-transgenic native traits introduced through genome modification, the native traits comprising polynucleotides from the same crop plant.

50. The plant of claim 49, wherein the trait genes are native traits.

51. The plant of claim 49, wherein the trait genes are selceted from the group consisting of herbicide tolerance, insect resistance, output traits, or disease resistance.

52. The method of claim 49, wherein the plant is a corn, soy, canola, or cotton plant.

53. A method for obtaining a plant cell with a modified genomic locus comprising at least two polynucleotide sequences, wherein said at least two polynucleotide sequences are heterologous to the corresponding genomic locus, the method comprising:

a. introducing a site-specific modification at at least one target site in a genomic locus in a plant cell;

b. introducing at least two heterologous polynucleotide sequences to the target site; and

c. obtaining the plant cell having a genomic locus comprising at least two heterologous polynucleotide sequences.

54. The method of claim 53, wherein at least one of the two heterologous polynucleotide sequences comprises a polynucleotide sequences that confer enhanced disease resistance to at least one plant disease.

55. The method of claim 53, wherein the plant is a corn, soy, canola, or cotton plant.

56. The method of claim 53, wherein the plant is a monocot or dicot.

57. The method of claim 53, wherein the plant is a maize plant, and wherein the at least two polynucleotide sequences are selected from the group consisting of NLB18, Ht1, RppK, PRR03, NLR01, NLR02, RCG1, RCG1b, PRR03, PRR01, NLR01, and NLR04.

58. A modified plant comprising at least three disease resistance genes selected from the group consisting of NLB18, Ht1, and RppK, wherein the at least three disease resistence genes are located in the same genomic locus.

59. The method of claim 58, wherein the plant is a maize plant.

60. The modified plant of claim 58, wherein the plant further comprises PRR03.

61. The modified plant of claim 58, wherein the plant further comprises at least one gene selected from NLR01, NLR02, RCG1, RCG1b, PRR03, PRR01, NLR01, and NLR04.

FIG. 1

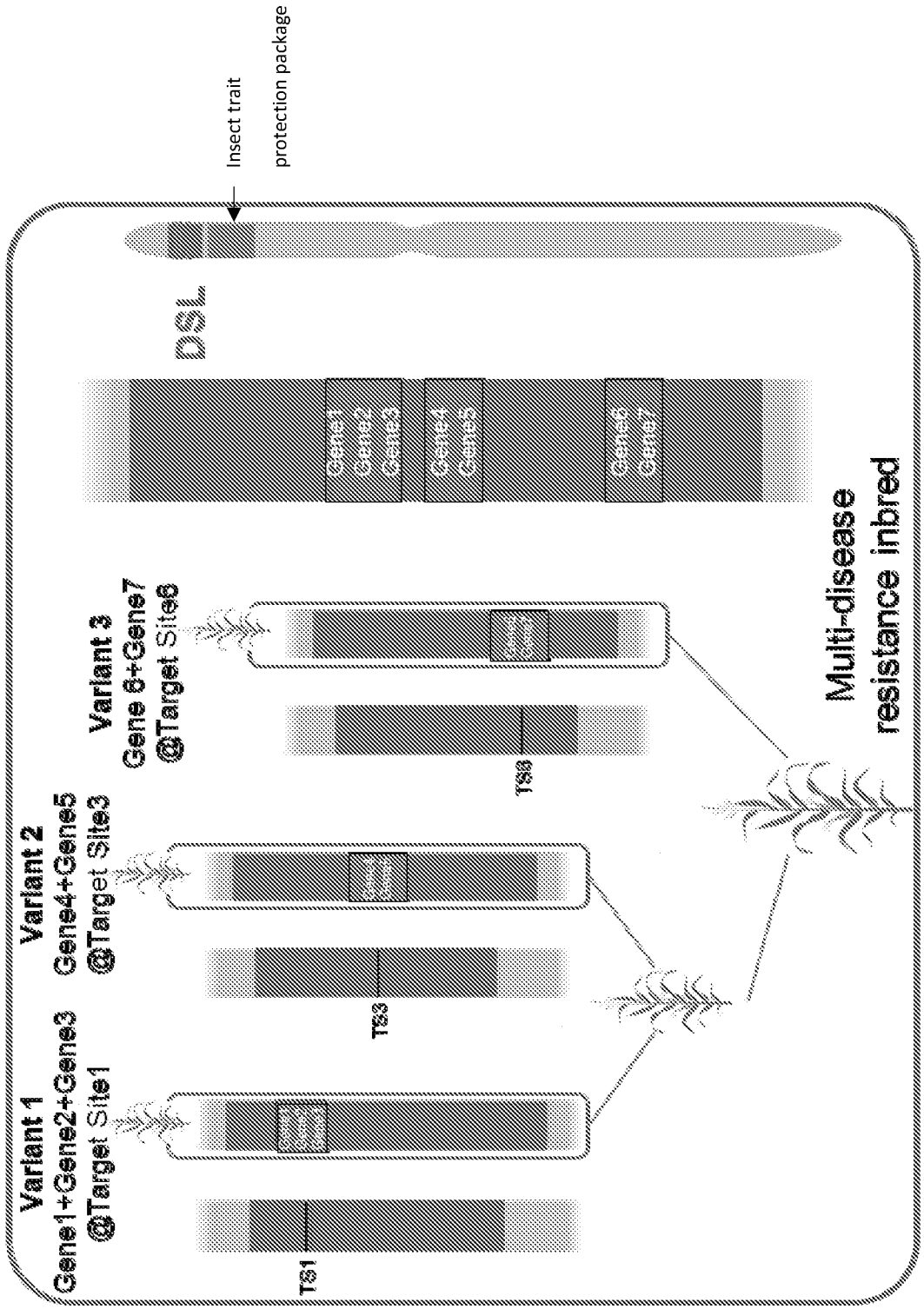

Insect trait
protection package
DSL
Gene1
Gene2
Gene3
Gene4
Gene5
Gene6
Gene7
Variant 1
Gene1+Gene2+Gene3
@Target Site1
Variant 2
Gene4+Gene5
@Target Site3
Variant 3
Gene 6+Gene7
@Target Site6
TS1
TS3
TS6
Multi-disease
resistance inbred

FIG. 2a

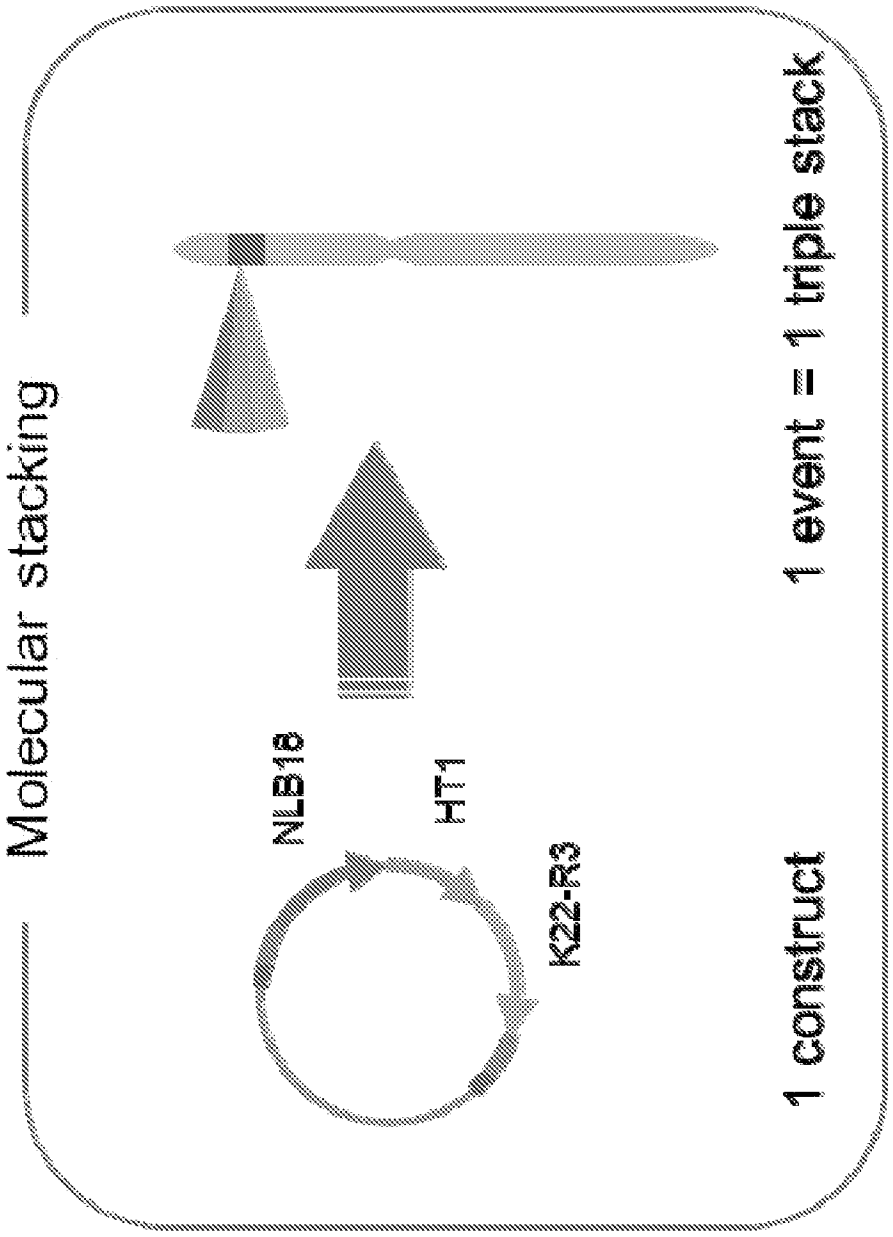

Molecular stacking
NLB18
HT1
K22-R3
1 construct
1 event = 1 triple stack

FIG. 2b

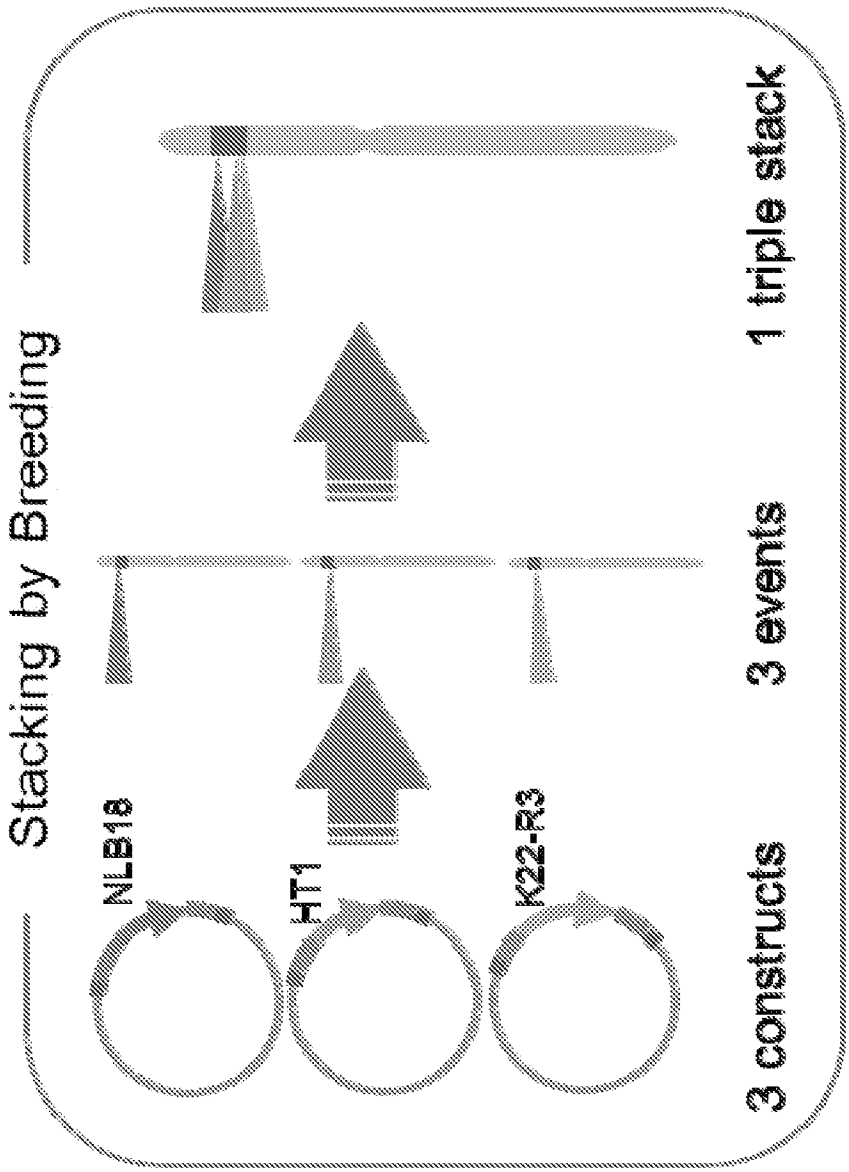
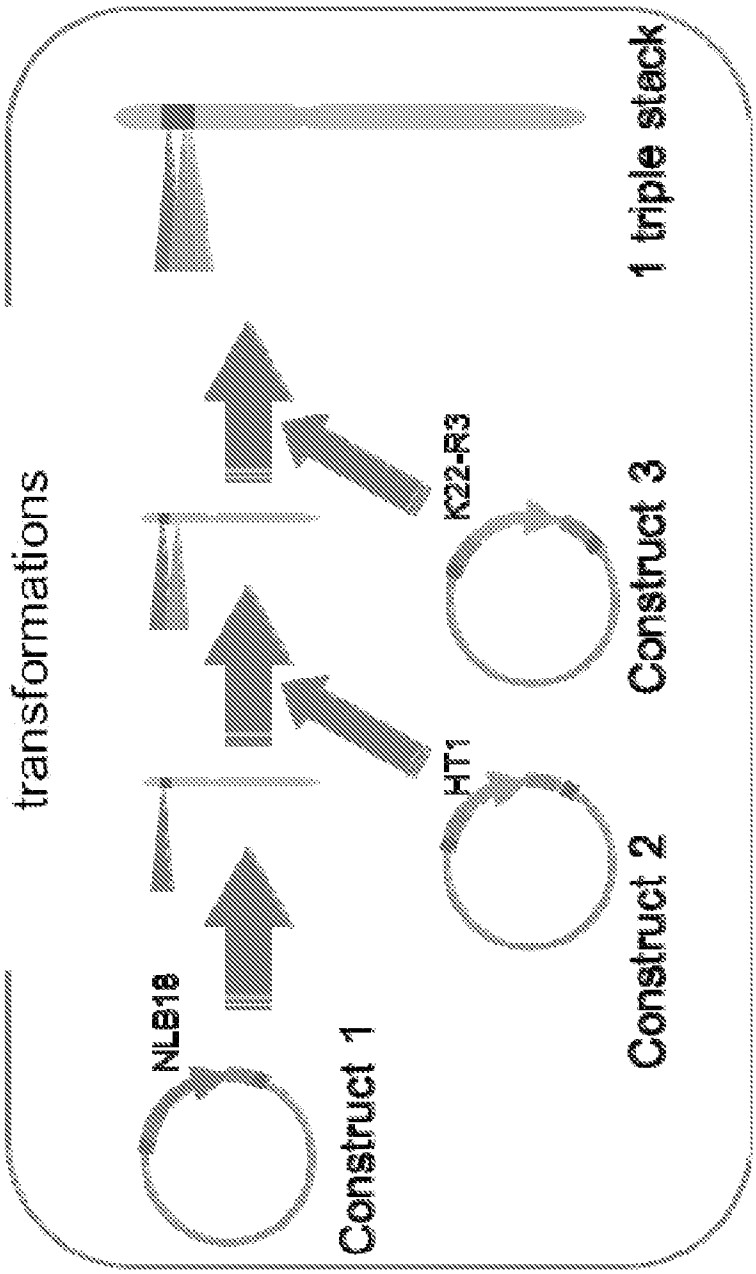

Stacking by Breeding
NLB18
HT1
K22-R3
3 constructs
3 events
1 triple stack

FIG. 2c


Stacking by successive transformations
NLB18
Construct 1
HT1
Construct 2
K22-R3
Construct 3
1 triple stack

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/046227

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 111 197 056 A (SOUTH CHINA BOTANICAL GARDEN CAS) 26 May 2020 (2020-05-26)	9,13,14, 24,26, 29,53-56
Y	example 2	10-12, 20-22, 27,28, 30-48, 57-61

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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

"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
26 November 2021	10/12/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kania, Thomas

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/071362 A1 (PIONEER HI BRED INT [US]) 19 April 2018 (2018-04-19)	1-9, 13-15, 17-24, 26,29, 49-56
Y	claims 38-74 page 44, line 6 - page 46, line 20	10-12, 20-22, 27,28, 30-48, 57-61
X	GAO HUIRONG ET AL: "Complex Trait Loci in Maize Enabled by CRISPR-Cas9 Mediated Gene Insertion", FRONTIERS IN PLANT SCIENCE, vol. 11, 5 May 2020 (2020-05-05), XP55864822, CH ISSN: 1664-462X, DOI: 10.3389/fpls.2020.00535 the whole document	30,35, 36,40-45
A	CHEN WEIQIANG ET AL: "Precise, flexible and affordable gene stacking for crop improvement", BIOENGINEERED, vol. 8, no. 5, 3 September 2017 (2017-09-03), pages 451-456, XP55864832, US ISSN: 2165-5979, DOI: 10.1080/21655979.2016.1276679	1-61
A	WO 2019/074841 A1 (PIONEER HI BRED INT [US]) 18 April 2019 (2019-04-18) paragraph [0602]	1-61
A	LUO MING ET AL: "Applications of CRISPR/Cas9 technology for targeted mutagenesis, gene replacement and stacking of genes in higher plants", PLANT CELL REPORTS, SPRINGER BERLIN HEIDELBERG, BERLIN/HEIDELBERG, vol. 35, no. 7, 4 May 2016 (2016-05-04), pages 1439-1450, XP035976276, ISSN: 0721-7714, DOI: 10.1007/S00299-016-1989-8 [retrieved on 2016-05-04] page 1445, paragraph 2	1-61

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WILLIAM M. AINLEY ET AL: "Trait stacking via targeted genome editing", PLANT BIOTECHNOLOGY JOURNAL, vol. 11, no. 9, 19 August 2013 (2013-08-19), pages 1126-1134, XP055218224, ISSN: 1467-7644, DOI: 10.1111/pbi.12107 -----	1-61
A	Yang Zhao ET AL: "Generation of a selectable marker free, highly expressed single copy locus as landing pad for transgene stacking insugarcane", Plant Molecular Biology, vol. 100 27 March 2019 (2019-03-27), pages 247-263, XP55864829, Retrieved from the Internet: URL:https://link.springer.com/content/pdf/10.1007/s11103-019-00856-4.pdf [retrieved on 2021-11-23] -----	1-61

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2021/046227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 111197056 A	26-05-2020	NONE	
WO 2018071362 A1	19-04-2018	AR 109941 A1	06-02-2019
		BR 112019007327 A2	02-07-2019
		CA 3037560 A1	19-04-2018
		CN 109844121 A	04-06-2019
		EA 201990924 A1	30-09-2019
		EP 3525578 A1	21-08-2019
		WO 2018071362 A1	19-04-2018
		ZA 201901854 B	27-11-2019
WO 2019074841 A1	18-04-2019	CA 3078845 A1	18-04-2019
		EP 3694992 A1	19-08-2020
		US 2020332306 A1	22-10-2020
		WO 2019074841 A1	18-04-2019