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(54) Title: ENGINEERING PLANT GENOMES USING CRISPR/CAS SYSTEMS

(57) Abstract: Materials and methods for gene targeting using Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas) systems are provided herein.

ENGINEERING PLANT GENOMES USING CRISPR/Cas SYSTEMS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority from U.S. Provisional Application Serial No. 61/790,694, filed on March 15, 2013.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made with government support under GM 834720 awarded by the National Institutes of Health, and DBI0923827 awarded by the National Science Foundation. The government has certain rights in the invention.

TECHNICAL FIELD

This document relates to materials and methods for gene targeting in plants, and particularly to methods for gene targeting that include using CRISPR/Cas systems.

BACKGROUND

Technologies enabling the precise modification of DNA sequences within living cells can be valuable for both basic and applied research. Precise genome modification – either targeted mutagenesis or gene targeting (GT) – relies on the DNA-repair machinery of the target cell. With respect to targeted mutagenesis, sequence-specific nuclease (SSN)-mediated DNA double-strand breaks (DSBs) are frequently repaired by the error-prone non-homologous end joining (NHEJ) pathway, resulting in mutations at the break site. On the other hand, if a donor molecule is co-delivered with a SSN, the ensuing DSB can stimulate homologous recombination (HR) of sequences near the break site with sequences present on the donor molecule. Consequently, any modified sequence carried by the donor molecule will be stably incorporated into the genome (referred to as GT). Attempts to implement GT in plants often are plagued by extremely low HR frequencies. The majority of the time, donor DNA molecules integrate illegitimately via NHEJ. This process occurs regardless of the size of the homologous "arms;" increasing the length of homology to approximately 22 kb results in no significant enhancement in GT (Thykjaer

et al., *Plant Mol Biol*, 35:523-530, 1997). However, introducing a DSB with a SSN can greatly increase the frequency of GT by HR (Shukla et al., *Nature* 459:437-441, 2009; and Townsend et al., *Nature* 459:442-445, 2009).

SUMMARY

This document is based in part on the discovery that the Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas) system can be used for plant genome engineering. The CRISPR/Cas system provides a relatively simple, effective tool for generating modifications in genomic DNA at selected sites. CRISPR/Cas systems can be used to create targeted DSBs or single-strand breaks, and can be used for, without limitation, targeted mutagenesis, gene targeting, gene replacement, targeted deletions, targeted inversions, targeted translocations, targeted insertions, and multiplexed genome modification through multiple DSBs in a single cell directed by co-expression of multiple targeting RNAs. This technology can be used to accelerate the rate of functional genetic studies in plants, and to engineer plants with improved characteristics, including enhanced nutritional quality, increased resistance to disease and stress, and heightened production of commercially valuable compounds.

In one aspect, this document features a method for modifying the genomic material in a plant cell. The method can include (a) introducing into the cell a nucleic acid comprising a crRNA and a tracrRNA, or a chimeric cr/tracrRNA hybrid, where the crRNA and tracrRNA, or the cr/tracrRNA hybrid, is targeted to a sequence that is endogenous to the plant cell; and (b) introducing into the cell a Cas9 endonuclease molecule that induces a double strand break at or near the sequence to which the crRNA and tracrRNA sequence is targeted, or at or near the sequence to which the cr/tracrRNA hybrid is targeted. The introducing steps can include delivering to the plant cell a nucleic acid encoding the Cas9 endonuclease and a nucleic acid encoding the crRNA and tracrRNA or the cr/tracrRNA hybrid, where the delivering is by a DNA virus (e.g., a geminivirus) or an RNA virus (e.g., a tobravirus). The introducing steps can include delivering to the plant cell a T-DNA containing a nucleic acid sequence encoding the Cas9 endonuclease and a nucleic acid sequence encoding the crRNA and tracrRNA or the

cr/tracrRNA hybrid, where the delivering is via *Agrobacterium* or *Ensifer*. The nucleic acid sequence encoding the Cas9 endonuclease can be operably linked to a promoter that is constitutive (e.g., a cauliflower mosaic virus 35S promoter), cell specific, inducible, or activated by alternative splicing of a suicide exon. The introducing steps can include microprojectile bombardment of nucleic acid encoding Cas9 and the crRNA and tracrRNA or the cr/tracrRNA hybrid. The nucleic acid sequence encoding the Cas9 endonuclease can be operably linked to a promoter that is constitutive, cell specific, inducible, or activated by alternative splicing of a suicide exon. The plant cell can be from a monocotyledonous plant (e.g., wheat, maize, rice, or Setaria), or from a dicotyledonous plant (e.g., tomato, soybean, tobacco, potato, cassava, or *Arabidopsis*). The method can further include screening the plant cell after the introducing steps to determine if a double strand break has occurred at or near the sequence targeted by the crRNA and tracrRNA or the cr/tracrRNA hybrid. The method also can include regenerating a plant from the plant cell, and in some embodiments, the method can include cross breeding the plant to obtain a genetically desired plant lineage.

In another aspect, this document features a plant cell containing a nucleic acid encoding a polypeptide having at least 80% sequence identity with SEQ ID NO:12, as well as a plant cell containing a nucleic acid encoding a polypeptide that includes an amino acid sequence having at least 80% sequence identity with amino acids 810 to 872 of SEQ ID NO:12.

In another aspect, this document features a virus vector containing a nucleotide sequence that encodes a Cas9 polypeptide. The virus vector can contain a nucleotide sequence encoding a polypeptide with an amino acid sequence having at least 90% identity to SEQ ID NO:12. The virus vector can be from a tobravirus or a geminivirus.

In another aspect, this document features a T-DNA containing a nucleic acid sequence encoding a polypeptide that has an amino acid sequence having at least 80% sequence identity with amino acids 810 to 872 of SEQ ID NO:12. This document also features an *Agrobacterium* strain containing the T-DNA.

In yet another aspect, this document features a method for expressing a Cas protein in a plant cell. The method can include providing an *Agrobacterium* or *Ensifer*

vector containing a T-DNA that includes a nucleic acid sequence encoding a polypeptide having an amino acid sequence with at least 80% sequence identity to amino acids 810 to 872 of SEQ ID NO:12, where the polypeptide-encoding sequence is operably linked to a promoter; bringing the *Agrobacterium* or *Ensifer* vector into contact with the plant cell; and expressing the nucleic acid sequence in the plant cell. The promoter can be an inducible promoter (e.g., an estrogen inducible promoter). The method can further include contacting the plant cell with a nucleic acid encoding a guide RNA that associates with the Cas protein. The plant cell can be a protoplast.

Unless otherwise defined, 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 invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 is a schematic of a pMDC32 plasmid (a standard T-DNA expression plasmid) containing a Cas9 coding sequence and a cr/tracrRNA hybrid sequence. The nucleotide sequence of the plasmid is set forth in SEQ ID NO:6.

FIG. 2 is a schematic of a pFZ19 plasmid (an estrogen-inducible T-DNA expression vector) containing a Cas9 coding sequence and a cr/tracrRNA hybrid sequence. The nucleotide sequence of the plasmid is set forth in SEQ ID NO:7.

FIG. 3 is a schematic of a pNJB121 plasmid (a geminivirus-replicon T-DNA vector) containing a Cas9 coding sequence and a cr/tracrRNA hybrid. The nucleotide sequence of the plasmid is set forth in SEQ ID NO:8.

FIGS. 4A-4D provide evidence of CRISPR/Cas function in plant cells in which a Cas9 coding sequence and a cr/tracrRNA hybrid were delivered by *Agrobacterium* or geminivirus replicons. FIG. 4A is an illustration of a T-DNA harboring a plant codon-optimized Cas9 sequence. The cr/tracrRNA hybrid (designated sgRNA) was placed downstream of the *Arabidopsis* AtU6-26 promoter (PU6). The "lollypops" indicate the long intergenic region (LIR) that is important for replication mediated by replicase (Rep). The gray box represents the short intergenic region (SIR) that also is important for replicon function. The unlabeled gray arrow is a 35S promoter that can drive Cas9 expression upon circularization of the replicon. Cas9 expression also can be driven by the LIR, which functions as a promoter. The entire construct depicted is referred to as an LSL T-DNA. FIG. 4B is a picture of an agarose gel containing PCR products, demonstrating circularization of the geminivirus replicon in plant cells. PCR primers (small arrows in FIG. 4A) were used to amplify DNA from cells infected with *Agrobacterium* T-DNA carrying the replicon. Only in the presence of the plasmid encoding the geminivirus replicase (pRep) did circularization and amplification of the replicon occur. FIG. 4C shows detection of Cas9-induced mutations at the *Nicotiana tabacum SurA/SurB* loci. Tobacco leaf tissue was syringe infiltrated with two strains of *Agrobacterium* containing pREP and the LSL T-DNA depicted in FIG. 4A; this was done to test for CRISPR/Cas9-mediated mutagenesis using geminivirus replicons. Alternatively, leaf tissue was infiltrated with single strain of *Agrobacterium* containing only the LSL T-DNA; this was done to test for CRISPR/Cas9-mediated mutagenesis by standard *Agrobacterium* T-DNA delivery. Five days post infiltration, genomic DNA was isolated and used as a template in a PCR reaction designed to amplify the Cas9 target site within *SurA/SurB*. The resulting amplicons were digested with AlwI, and bands were separated by gel electrophoresis. FIG. 4D shows sequences (SEQ ID NOS:1-5) that resulted from cleavage-resistant amplicons in the sample transformed with the LSL T-DNA and pREP T-DNA. PAM, protospacer adjacent motif.

FIG. 5 is a schematic of a reporter plasmid encoding a non-functional yellow fluorescent protein (YFP).

FIG. 6 is a graph plotting fluorescence levels as evidence of CRISPR/Cas function in protoplasts using a YFP reporter plasmid. Tobacco protoplasts were prepared and transformed with various constructs to test for targeted cleavage by CRISPR/Cas9, and YFP fluorescence was measured by flow cytometry. Column 1 shows levels of fluorescence observed from cells transformed with the YFP reporter and constructs expressing Cas9 and the cr/tracr RNA expressed from the AtU6-26 promoter. Column 2 shows levels of fluorescence observed from cells transformed with the reporter, Cas9 and the cr/tracr RNA expressed from the At7SL2-2 promoter. Column 3 shows fluorescence observed in cells transformed with the reporter only (negative control); column 4 shows fluorescence in cells transformed with a construct that expresses YFP (positive control).

DETAILED DESCRIPTION

Efficient genome engineering in plants can be enabled by introducing targeted double-strand breaks (DSBs) in a DNA sequence to be modified. The DSBs activate cellular DNA repair pathways, which can be harnessed to achieve desired DNA sequence modifications near the break site. Targeted DSBs can be introduced using sequence-specific nucleases (SSNs), a specialized class of proteins that includes transcription activator-liked (TAL) effector endonucleases, zinc-finger nucleases (ZFNs), and homing endonucleases (HEs). Recognition of a specific DNA sequence is achieved through interaction with specific amino acids encoded by the SSNs. Prior to the development of TAL effector endonucleases, a challenge of engineering SSNs was the unpredictable context dependencies between amino acids that bind to DNA sequence. While TAL effector endonucleases greatly alleviated this difficulty, their large size (on average, each TAL effector endonuclease monomer contains 2.5-3 kb of coding sequence) and repetitive nature may hinder their use in applications where vector size and stability is a concern (Voytas, *Annu Rev Plant Biol*, 64: 327-350, 2013).

This document is based in part on the discovery that the CRISPR/Cas system can be used as a simple, effective tool for plant genome engineering. CRISPR/Cas molecules are components of a prokaryotic adaptive immune system that uses RNA base pairing to direct DNA cleavage. Directing DNA DSBs requires two components: the Cas9 protein,

which functions as an endonuclease, and CRISPR RNA (crRNA) and tracer RNA (tracrRNA) sequences that aid in directing the Cas9/RNA complex to target DNA sequence (Makarova et al., *Nat Rev Microbiol*, 9(6):467-477, 2011). The modification of a single targeting RNA can be sufficient to alter the nucleotide target of a Cas protein. In some cases, crRNA and tracrRNA can be engineered as a single cr/tracrRNA hybrid to direct Cas9 cleavage activity (Jinek et al., *Science*, 337(6096):816-821, 2012). The CRISPR/Cas system can be used in bacteria, yeast, humans, and zebrafish, as described elsewhere (*see*, e.g., Jiang et al., *Nat Biotechnol,* 31(3):233-239, 2013; Dicarlo et al., *Nucleic Acids Res*, doi:10.1093/nar/gkt135, 2013; Cong et al., *Science*, 339(6121):819-823, 2013; Mali et al., *Science*, 339(6121):823-826, 2013; Cho et al., *Nat Biotechnol*, 31(3):230-232, 2013; and Hwang et al., *Nat Biotechnol*, 31(3):227-229, 2013).

The utility of the CRISPR/Cas system in plants has not previously been demonstrated. The CRISPR/Cas system originates from prokaryotic cells with relatively small genomes, in which Cas9 is stably expressed in cells in the presence of significant RNAse III activity. Thus, when the plant cell work described herein was initiated, there was uncertainty as to whether expression of a Cas9 transgene would be possible in plant cells, and whether Cas9 would properly cooperate with RNA-guides and RNAse III activity in the plant context. In addition, expression of heterologous proteins in plant cells is generally challenging due to different codon usage. Further, some toxicity from Cas9 expression in plants was expected, as the large size of plant genomes increases the probability that nonspecific cleavage of genomic DNA may induce genotoxicity to the cells. The CRISPR/Cas9 system is reported to operate with specific recognition sequences comprising 10-20 nucleotides, which is less specific than most other rare-cutting endonuclease systems such as TAL effector endonucleases, meganucleases, and zinc finger nucleases.

As described herein, CRISPR/Cas systems can be used to create targeted DSBs or single-strand breaks, and can be used for, without limitation, targeted mutagenesis, gene targeting, gene replacement, targeted deletions, targeted inversions, targeted translocations, targeted insertions, and multiplexed genome modification through multiple DSBs in a single cell directed by co-expression of multiple targeting RNAs. This

technology can be used to accelerate the rate of functional genetic studies in plants, and to engineer plants with improved characteristics, including enhanced nutritional quality, increased resistance to disease and stress, and heightened production of commercially valuable compounds. Proof-of-concept experiments can be performed in plant leaf tissue by targeting DSBs to reporter genes and endogenous loci. The technology then can be adapted for use in protoplasts and whole plants, and in viral-based delivery systems. Finally, multiplex genome engineering can be demonstrated by targeting DSBs to multiple sites within the same genome.

In general, the systems and methods described herein include at least two components: the RNAs (crRNA and tracrRNA, or a single cr/tracrRNA hybrid) complementary (and thus targeted) to a particular sequence in a plant cell (e.g., in a plant genome, or in an extrachromosomal plasmid, such as a reporter), and a Cas9 endonuclease that can cleave the plant DNA at the target sequence. A representative Cas9 coding sequence is shown in nucleotides 9771 to 14045 of SEQ ID NO:6 (also nucleotides 4331 to 8605 of SEQ ID NO:7, and nucleotides 9487 to 13761 of SEQ ID NO:8). In some cases, a system also can include a nucleic acid containing a donor sequence targeted to a plant sequence. The endonuclease can to create targeted DNA double-strand breaks at the desired locus (or loci), and the plant cell can repair the double-strand break using the donor DNA sequence, thereby incorporating the modification stably into the plant genome.

The Cas9 protein includes two distinct active sites – a RuvC-like nuclease domain and a HNH-like nuclease domain, which generate site-specific nicks on opposite DNA strands (Gasiunus et al., *Proc Natl Acad Sci USA* 109(39):E2579-E2586, 2012). The RuvC-like domain is near the amino terminus of the Cas9 protein and is thought to cleave the target DNA noncomplementary to the crRNA, while the HNH-like domain is in the middle of the protein and is thought to cleave the target DNA complementary to the crRNA. A representative Cas9 sequence from *Streptococcus thermophilus* is set forth in SEQ ID NO:11 (*see*, also, UniProtKB number Q03JI6), and a representative Cas9 sequence from *S. pyogenese* is set forth in SEQ ID NO:12 (*see*, also, UniProtKB number Q99ZW2). Thus, the methods described herein can be carried out using a nucleotide

sequence encoding a Cas9 polypeptide having the sequence of SEQ ID NO:11 or SEQ ID NO:12. In some embodiments, however, the methods described herein can be carried out using a nucleotide sequence encoding a Cas9 functional variant having at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) sequence identity with SEQ ID NO:11 or SEQ ID NO:12. Further, Cas9 can be split into two portions, with one portion including the HNH domain and the other including the RuvC domain. The HNH domain may have some cleavage activity by itself in association with the RNA-guide, so this document also contemplates the use of Cas9 polypeptides containing an HNH domain with at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) sequence identity with the HNH domain within SEQ ID NO:11 (e.g., amino acids 828 to 879 of SEQ ID NO:11) or SEQ ID NO:12 (e.g., amino acids 810 to 872 of SEQ ID NO:12).

The percent sequence identity between a particular nucleic acid or amino acid sequence and a sequence referenced by a particular sequence identification number is determined as follows. First, a nucleic acid or amino acid sequence is compared to the sequence set forth in a particular sequence identification number using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTN version 2.0.14 and BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained online at fr.com/blast or at ncbi.nlm.nih.gov. Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. To compare two nucleic acid sequences, the options are set as follows: -i is set to a file containing the first nucleic acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second nucleic acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastn; -o is set to any desired file name (e.g., C:\output.txt); -q is set to -1; -r is set to 2; and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastn -o c:\output.txt -q -1 -r 2. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first

amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

Once aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence (e.g., SEQ ID NO:11), or by an articulated length (e.g., 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, an amino acid sequence that has 1300 matches when aligned with the sequence set forth in SEQ ID NO:11 is 93.7 percent identical to the sequence set forth in SEQ ID NO:11 (i.e., 1300 ÷ 1388 x 100 = 93.7). It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 is rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 is rounded up to 75.2. It also is noted that the length value will always be an integer.

As used herein, the term "functional variant" is intended to refer to a catalytically active mutant of a protein or a protein domain. Such a mutant can have the same level of activity, or a higher or lower level of activity as compared to the parent protein or protein domain.

The construct(s) containing the crRNA, tracrRNA, cr/tracrRNA hybrid, endonuclease coding sequence, and, where applicable, donor sequence, can be delivered to a plant, plant part, or plant cell using, for example, biolistic bombardment. Alternatively, the system components can be delivered using *Agrobacterium*-mediated

transformation. In some embodiments, the system components can be delivered in a viral vector (e.g., a vector from a DNA virus such as, without limitation, geminivirus (e.g., cabbage leaf curl virus, bean yellow dwarf virus, wheat dwarf virus, tomato leaf curl virus, maize streak virus, tobacco leaf curl virus, or tomato golden mosaic virus) or nanovirus (e.g., Faba bean necrotic yellow virus), or a vector from an RNA virus such as, without limitation, tobravirus (e.g., tobacco rattle virus, tobacco mosaic virus), potexvirus (e.g., potato virus X), or hordeivirus (e.g., barley stripe mosaic virus).

After a plant, plant part, or plant cell is infected or transfected with an endonuclease encoding sequence and a crRNA and a tracrRNA, or a cr/tracrRNA hybrid (and, in some cases, a donor sequence), any suitable method can be used to determine whether GT or targeted mutagenesis has occurred at the target site. In some embodiments, a phenotypic change can indicate that a donor sequence has been incorporated into the target site. PCR-based methods also can be used to ascertain whether a genomic target site contains targeted mutations or donor sequence, and/or whether precise recombination has occurred at the 5' and 3' ends of the donor. One method to detect targeted mutations, referred to herein as "PCR digest," is described by Zhang et al. (*Proc Natl Acad Sci USA* 107:12028-12033, 2010). Methods to detect precise recombination include southern blotting using a probe with homology to the donor sequence.

In some embodiments, the methods provided herein can include introducing into a plant, plant part, or plant cell a nucleic acid that includes a crRNA and a tracrRNA, or a chimeric cr/tracrRNA hybrid, where the crRNA and tracrRNA, or the cr/tracrRNA hybrid, is targeted to a nucleotide sequence that is endogenous to the plant cell, and also introducing into the plant, plant part, or plant cell a Cas9 endonuclease molecule (e.g., a Cas9 polypeptide or a portion thereof, such as a portion of a Cas9 polypeptide that includes the HNH domain, or a nucleic acid encoding a Cas9 polypeptide or a portion thereof), where the Cas9 endonuclease molecule induces a double strand break at or near the sequence to which the crRNA and tracrRNA sequences (or the cr/tracrRNA hybrid) are targeted.

The plants, plant parts, and plant cells used in the methods provided herein can be from any species of plant. In some embodiments, for example, the methods provided herein can utilize monocotyledonous plants, portions thereof, or cells therefrom. Exemplary monocotyledonous plants include, without limitation, wheat, maize, rice, orchids, onion, aloe, true lilies, grasses (e.g., *Setaria*), woody shrubs and trees (e.g., palms and bamboo), and food plants such as pineapple and sugar cane. Exemplary dicotyledonous plants include, without limitation, tomato, cassava, soybean, tobacco, potato, *Arabidopsis*, rose, pansy, sunflower, grape, strawberry, squash, bean, pea, and peanut.

In some embodiments, the methods described herein can include screening the plant, plant part, or plant cell to determine if a DSB has occurred at or near the sequence targeted by the crRNA and tracrRNA or the cr/tracrRNA hybrid. For example, the PCR-digest assay described by Zhang et al. (*supra*) can be used to determine whether a DSB has occurred. Other useful methods include, without limitation, the T7 assay, the Surveyor assay, and southern blotting (if a restriction enzyme binding sequence is present at or near the predicted cleavage site).

In addition, in some embodiments in which a plant part or plant cell is used, the methods provided herein can include regenerating a plant from the plant part or plant cell. The methods also can include breeding the plant (e.g., the plant into which the nucleic acids were introduced, or the plant obtained after regeneration of the plant part or plant cell used as a starting material) to obtain a genetically desired plant lineage. Methods for regenerating and breeding plants are well established in the art.

Also provided herein are plants, plant parts, and plant cells containing a nucleic acid that encodes a Cas9 polypeptide with an amino acid sequence that is at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) identical to the amino acid sequence set forth in SEQ ID NO:11 or SEQ ID NO:12, or a nucleic acid that encodes a Cas9 polypeptide containing an amino acid sequence that is at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) identical to amino acids 828 to 879 of SEQ ID NO:11, or amino acids 810 to 872 of SEQ ID NO:12.

This document also provides virus vectors that contain nucleotide sequences encoding Cas9 polypeptides. For example, a virus vector can include a nucleotide sequence encoding a polypeptide having an amino acid sequence that is at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) identical to the amino acid sequence set forth in SEQ ID NO:11 or SEQ ID NO:12. In some embodiments, a virus vector can have a nucleotide sequence encoding a Cas9 polypeptide that includes an amino acid sequence with at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) sequence identity to amino acids 828 to 879 of SEQ ID NO:11, or amino acids 810 to 872 of SEQ ID NO:12. The vector can be from any suitable type of virus, such as a tobravirus or a geminivirus, for example.

Also provide herein are T-DNA molecules that contain a nucleic acid sequence encoding a Cas9 polypeptide having an amino acid sequence that is at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) identical to the amino acid sequence set forth in SEQ ID NO:11 or SEQ ID NO:12. In some embodiments, a T-DNA can include a nucleotide sequence encoding a Cas9 polypeptide that includes an amino acid sequence with at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) sequence identity to amino acids 828 to 879 of SEQ ID NO:11, or amino acids 810 to 872 of SEQ ID NO:12.

This document also provides *Agrobacterium* strains comprising a T-DNA as described herein.

In addition, this document provides methods for expressing a Cas protein in a plant, a plant part, or a plant cell. Such methods can include, for example, (a) providing an *Agrobacterium* or *Ensifer* vector containing a T-DNA that includes a nucleic acid sequence encoding a Cas9 polypeptide having an amino acid sequence with at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 98%) sequence identity to SEQ ID NO:11 or SEQ ID NO:12, where the Cas9-encoding sequence is operably linked to a promoter, (b) bringing the *Agrobacterium* or *Ensifer* vector into contact with a plant, plant part, or plant cell, and (c) expressing the nucleic acid sequence in the plant, plant part, or plant cell. The promoter can be, for example, a constitutive promoter (e.g., a CaMV 35S promoter), an inducible promoter (e.g., an estradiol-induced XVE promoter;

Zuo et al., *Plant J* 24:265-273, 2000), a cell specific promoter, or a promoter that is activated by alternative splicing of a suicide exon. In some embodiments, such methods also can include contacting the plant, plant part, or plant cell with a nucleic acid encoding a guide RNA that associates with the Cas protein, and expressing the guide RNA.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – Plasmids for expressing CRISPR/Cas components

To demonstrate functionality of the CRISPR/Cas systems for genome editing in plants, plasmids were constructed to encode Cas9, crRNA and tracrRNA, the cr/tracrRNA hybrid, and RNA polymerase III promoters (e.g., AtU6-26 or At7SL-2) from which to express the crRNA, tracrRNA, or cr/tracrRNA hybrid. Plant codon-optimized Cas9 coding sequence was synthesized and cloned into a MultiSite Gateway entry plasmid. Additionally, crRNA and tracrRNA, or cr/tracrRNA hybrid, driven by the RNA polymerase III (PolIII) promoters AtU6-26 and At7SL2-2, were synthesized and cloned into a second MultiSite Gateway entry plasmid. To enable efficient reconstruction of the crRNA sequences (serving to redirect CRISPR/Cas-mediated DSBs), inverted type-IIS restriction enzyme sites (e.g., *Bsa*I and *Esp*3I) were inserted within the crRNA nucleotide sequence. By digesting with the appropriate type-IIS restriction enzyme, target sequences can be efficiently cloned into the crRNA sequence using oligonucleotides. Entry plasmids for both Cas9 and the expression of the crRNA and tracrRNA or the cr/tracrRNA hybrid, from a RNA polymerase III promoter (AtU6-26 or At7SL2-2), were recombined into pMDC32 (a standard T-DNA expression plasmid with a 2x35S promoter; FIG. 1 and SEQ ID NO:6), pFZ19 (an estrogen-inducible T-DNA expression vector; FIG. 2 and SEQ ID NO:7; Zuo et al., *Plant J.* 24(2):265-273, 2000), and pNJB121 (a geminivirus-replicon T-DNA vector; FIG. 3 and SEQ ID NO:8).

Example 2 – CRISPR/Cas activity in somatic plant tissue

To demonstrate the capacity for CRISPR/Cas systems to function as SSNs, the geminivirus-replicon T-DNA vector, pNJB121, was modified to encode both Cas9 and cr/tracrRNA hybrid sequences (FIG. 4A). Targeting RNA sequences (encoded by nucleotide sequence within the crRNA; responsible for directing Cas9 cleavage) were designed to be homologous to sequences within the endogenous *SuRA* and *SuRB* genes. The sequence of the targeting portion of the crRNA that matched the *SuR* loci was 5'-GUGGGAGGAUCGGUUCUAUA (SEQ ID NO:9; the 5' G does not match the *SuR* loci, but is needed for transcription by RNA polymerase III). Although pNJB121 is a geminivirus-replicon, in the absence of replicase (Rep), no amplification occurs. Therefore, pNJB121 in the absence of Rep is a standard T-DNA vector and no replicons are formed. The modified pNJB121 plasmid delivered to *Nicotiana tabacum* leaf tissue by syringe infiltration with *Agrobacterium tumefaciens*. Five days after infiltration, *SuRA/SuRB* sequences were assessed for Cas9-mediated mutations using PCR-digest (FIG. 4C). The presence of mutations at the corresponding target sequences indicated functionality of CRISPR/Cas systems in plant leaf cells.

Example 3 – CRISPR/Cas activity in protoplasts

To further demonstrate the activity of CRISPR/Cas systems in plants, targeted mutagenesis of DNA sequence within *Arabidopsis thaliana* and *Nicotiana tabacum* protoplasts is assessed. Targeting crRNA sequences are redesigned to be homologous to sequences present within the endogenous *ADH1* or *TT4* genes (*Arabidopsis*), or the integrated *gus:npt*II reporter gene or *SuRA/SuRB* (*Nicotiana*). Protoplasts are isolated from *Arabidopsis* and *Nicotiana* leaf tissue and transfected with plasmids encoding Cas9 and the *ADH1*- or *TT4*-targeting crRNAs, or Cas9 and the *gus:npt*II- or *SuRA/SuRB*-targeting crRNA, respectively. Genomic DNA is extracted 5-7 days post transfection and assessed for mutations at the corresponding target sequences. Detecting mutations within the *ADH1*, *TT4*, *gus:nptII* or *SuRA/SuRB* genes indicates the functionality of CRISPR/Cas systems to target endogenous genes in plant protoplasts.

In initial studies, the CRISPR/Cas system was assessed for the ability to cleave an extrachromosomal reporter plasmid, using methods similar to those described by Zhang et al. (*Plant Physiol* 161:20-27, 2013). The reporter plasmid encodes a non-functional yellow fluorescent protein (YFP; FIG. 5 and SEQ ID NO:10). YFP expression is disrupted by a direct repeat of internal coding sequence that flanks a target sequence for the Cas9/crRNA complex. The generation of targeted DSBs at the Cas9/crRNA target sequence results in recombination of the direct repeat sequences, thereby restoring YFP gene function. A sequence from the tobacco *SuRA/SuRB* loci was cloned into the YFP reporter between the direct repeats. A cr/tracrRNA hybrid construct that targets this site was then generated. The sequence of the portion of the crRNA that targets the *SuR* loci was 5'- GUGGGAGGAUCGGUUCUAUA (SEQ ID NO:9; again, the 5' G does not match the *SuR* loci, but it is needed for transcription by RNA polymerase III). *Nicotiana tabacum* protoplasts were transformed with plasmids encoding Cas9, a cr/tracrRNA hybrid, and the YFP reporter, and restoration of YFP expression as a result of CRISPR/Cas nuclease activity was monitored by flow cytometry. Using a positive control plasmid that encodes YFP, 94.7% of the cells were transformed and expressed YFP (FIG. 6, column 4). Cells transformed with the reporter alone gave activity levels barely above background (FIG. 6, column 3). When cells were transformed with constructs expressing Cas9 and a cr/tracr RNA, significant activity was observed, indicating the Cas9/crRNA complex cleaved the target. For the cr/tracrRNA expressed from the AtU6-26 promoter, 18.8% of the cells fluoresced (FIG. 6, column 1). When the cr/tracr RNA was expressed from the At7SL2-2 promoter, 20.7% of the cells were YFP positive (FIG. 6, column 2). Detection of YFP-expressing cells indicated the functionality of CRISPR/Cas systems in plant protoplasts.

Example 4 – Multiplex genome engineering in protoplasts using CRISPR/Cas systems

The ability of CRISPR/Cas systems to create multiple DSBs at different DNA sequences is assessed using plant protoplasts. To direct Cas9 nuclease activity to *TT4*, *ADH1*, and the extrachromosomal YFP reporter plasmid (within the same *Arabidopsis* protoplast), crRNA and tracrRNA or cr/tracrRNA hybrid plasmid is modified to express

multiple crRNA targeting sequences. These sequences are designed to be homologous to sequences present within *TT4, ADH1* and the YFP reporter plasmid. Following transfection with Cas9, crRNA, tracrRNA, or the cr/tracrRNA hybrid, and YFP reporter plasmids into *Arabidopsis* protoplasts, YFP-expressing cells are quantified and isolated, and genomic DNA is extracted. Observing mutations within the *ADH1* and *TT4* genes in YFP-expressing cells suggests that CRISPR/Cas can facilitate multiplex genome engineering in *Arabidopsis* cells.

To demonstrate multiplex genome engineering in *Nicotiana* protoplasts, plasmids containing multiple crRNA are modified to encode sequences that are homologous to the integrated *gus:npt*II reporter gene, *SuRA/SuRB*, and the YFP reporter plasmid. Similar to the methods described in *Arabidopsis* protoplasts, *Nicotiana* protoplasts are transfected with Cas9, crRNA, tracrRNA, or the cr/tracrRNA hybrid, and YFP reporter plasmids. YFP-expressing cells are quantified and isolated, and genomic DNA is extracted. Observing mutations within the integrated *gus:npt*II reporter gene and *SuRA/SuRB* in YFP-expressing cells suggests that CRISPR/Cas can facilitate multiplex genome engineering in tobacco cells.

Example 5 – CRISPR/Cas activity *in planta*

To demonstrate CRISPR/Cas activity *in planta*, pFZ19 T-DNA is modified to encode both Cas9 and the crRNA and tracrRNA, or the cr/tracrRNA hybrid sequences. Target DNA sequences are present within the endogenous *ADH1* or *TT4* genes. The resulting T-DNA is integrated into the *Arabidopsis thaliana* genome by floral dip using *Agrobacterium*. Cas9 expression is induced in primary transgenic plants by direct exposure to estrogen. Genomic DNA from somatic leaf tissue is extracted and assessed for mutations at the corresponding genomic locus by PCR-digest. Observing mutations within the *ADH1* or *TT4* genes demonstrates CRISPR/Cas activity *in planta*. Alternatively, CRISPR/Cas activity can be assessed by screening T2 seeds (produced from induced T1 patents) for heterozygous or homozygous mutations at the corresponding genomic locus. Furthermore, the capacity for CRISPR/Cas to carry out multiplex genome engineering is assessed by modifying plasmids containing multiple

crRNAs with homologous sequences to both *ADH1* and *TT4*. The resulting T-DNA plasmid is integrated into the *Arabidopsis* genome, Cas9 expression is induced in primary transgenic plants, and CRISPR/Cas activity is assessed by evaluating the *ADH1* and *TT4* genes in both T1 and T2 plants. Observing mutations in both the *ADH1* and *TT4* genes suggests CRISPR/Cas can facilitate multiplex genome engineering in *Arabidopsis* plants.

Example 6 – Viral delivery of CRISPR/Cas components

Plant viruses can be effective vectors for delivery of heterologous nucleic acid sequence, such as for RNAi reagents or for expressing heterologous proteins. Useful plant viruses include both RNA viruses (e.g., tobacco mosaic virus, tobacco rattle virus, potato virus X, and barley stripe mosaic virus) and DNA viruses (e.g., cabbage leaf curl virus, bean yellow dwarf virus, wheat dwarf virus, tomato leaf curl virus, maize streak virus, tobacco leaf curl virus, tomato golden mosaic virus, and Faba bean necrotic yellow virus; Rybicki et al., *Curr Top Microbiol Immunol*, 2011; and Gleba et al., *Curr Opin Biotechnol* 2007, 134-141). Such plant viruses can be modified for the delivery of CRISPR/Cas9 components. Proof-of-concept experiments were performed in *Nicotiana tabacum* leaf cells using DNA viruses (geminivirus replicons; Baltes et al., *Plant Cell* 26:151-163, 2014). To this end, crRNA sequences were modified to contain homology to the endogenous *SuRA/SuRB* loci. The resulting plasmids were cloned into pNJB121 (a T-DNA destination vector with *cis*-acting elements required for geminivirus replication (LSL T-DNA)) along with Cas9 (FIG. 4A). Co-delivery of LSL T-DNA along with T-DNA encoding replicase protein (Rep; REP T-DNA) by *Agrobacterium* resulted in the replicational release of geminiviral replicons (FIG. 4B). The T-DNA was delivered to tobacco leaf tissue by syringe infiltration with *Agrobacterium*. Five to seven days after infiltration, *SuRA/SuRB* sequences were assessed for Cas9-mediated mutations using PCR-digest (FIG. 4C). Digestion-resistant PCR amplicons were cloned and sequenced. The presence of mutations at the corresponding target sequences indicates that plant viruses are effective vectors for delivery of CRISPR/Cas components (FIG. 4D).

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

CLAIMS

1. A method for modifying the genomic material in a plant cell, comprising:

(a) introducing into the cell a nucleic acid comprising a clustered regularly interspersed short palindromic repeats- (CRISPR-) associated RNA(crRNA) and a trans-activating crRNA (tracrRNA), or a chimeric cr/tracrRNA hybrid, wherein the crRNA and tracrRNA, or the cr/tracrRNA hybrid, is targeted to a sequence that is endogenous to the plant cell; and

(b) introducing into the cell a CRISPR-associated (Cas9) endonuclease molecule that induces a double strand break at or near the sequence to which the crRNA and tracrRNA sequence is targeted, or at or near the sequence to which the cr/tracrRNA hybrid is targeted.

2. The method of claim 1, wherein the introducing steps comprise delivering to the plant cell a nucleic acid encoding the Cas9 endonuclease and a nucleic acid encoding the crRNA and tracrRNA or the cr/tracrRNA hybrid, and wherein the delivering is by a DNA or RNA virus.

3. The method of claim 2, wherein the delivering is by a DNA virus, and wherein the DNA virus is a geminivirus.

4. The method of claim 2, wherein the delivering is by an RNA virus, and wherein the RNA virus is a tobravirus.

5. The method of claim 1, wherein the introducing steps comprise delivering to the plant cell a T-DNA containing a nucleic acid sequence encoding the Cas9 endonuclease and a nucleic acid sequence encoding the crRNA and tracrRNA or the cr/tracrRNA hybrid, and wherein the delivering is via *Agrobacterium* or *Ensifer*.

6. The method of claim 5, wherein the nucleic acid sequence encoding the Cas9 endonuclease is operably linked to a promoter that is constitutive, cell specific, inducible, or activated by alternative splicing of a suicide exon.

7. The method of claim 1, wherein the introducing steps comprise microprojectile bombardment of nucleic acid encoding Cas9 and the crRNA and tracrRNA or the cr/tracrRNA hybrid.

8. The method of claim 1, wherein the plant cell is from a monocotyledonous plant.

9. The method of claim 8, wherein the monocotyledonous plant is wheat, maize, rice, or Setaria.

10. The method of claim 1, wherein the plant cell is from a dicotyledonous plant.

11. The method of claim 10, wherein the dicotyledonous plant is tomato, soybean, tobacco, potato, cassava, or *Arabidopsis*.

12. The method of claim 1, further comprising screening the plant cell after the introducing steps to determine if a double strand break has occurred at or near the sequence targeted by the crRNA and tracrRNA or the cr/tracrRNA hybrid.

13. The method of claim 1, further comprising regenerating a plant from the plant cell.

14. The method of claim 13, further comprising cross breeding the plant to obtain a genetically desired plant lineage.

15. A plant cell comprising a nucleic acid encoding a polypeptide having at least 80% sequence identity with SEQ ID NO:12.

16. A plant cell comprising a nucleic acid encoding a polypeptide that comprises an amino acid sequence having at least 80% sequence identity with amino acids 810 to 872 of SEQ ID NO:12.

17. A virus vector comprising a nucleotide sequence that encodes a Cas9 polypeptide wherein the virus is a tobravirus or a geminivirus.

18. The virus vector of claim 17, wherein the vector comprises a nucleotide sequence encoding a polypeptide with an amino acid sequence having at least 90% identity to SEQ ID NO:12.

19. A T-DNA comprising a nucleic acid sequence encoding a polypeptide that comprises an amino acid sequence having at least 80% sequence identity with amino acids 810 to 872 of SEQ ID NO:12.

20. An *Agrobacterium* strain comprising the T-DNA of claim 19.

21. A method for expressing a Cas protein in a plant cell, comprising:

providing an *Agrobacterium* or *Ensifer* vector containing a T-DNA that comprises a nucleic acid sequence encoding a polypeptide having an amino acid sequence with at least 80% sequence identity to amino acids 810 to 872 of SEQ ID NO:12, wherein the polypeptide-encoding sequence is operably linked to a promoter;

bringing the *Agrobacterium* or *Ensifer* vector into contact with the plant cell; and

expressing the nucleic acid sequence in the plant cell.

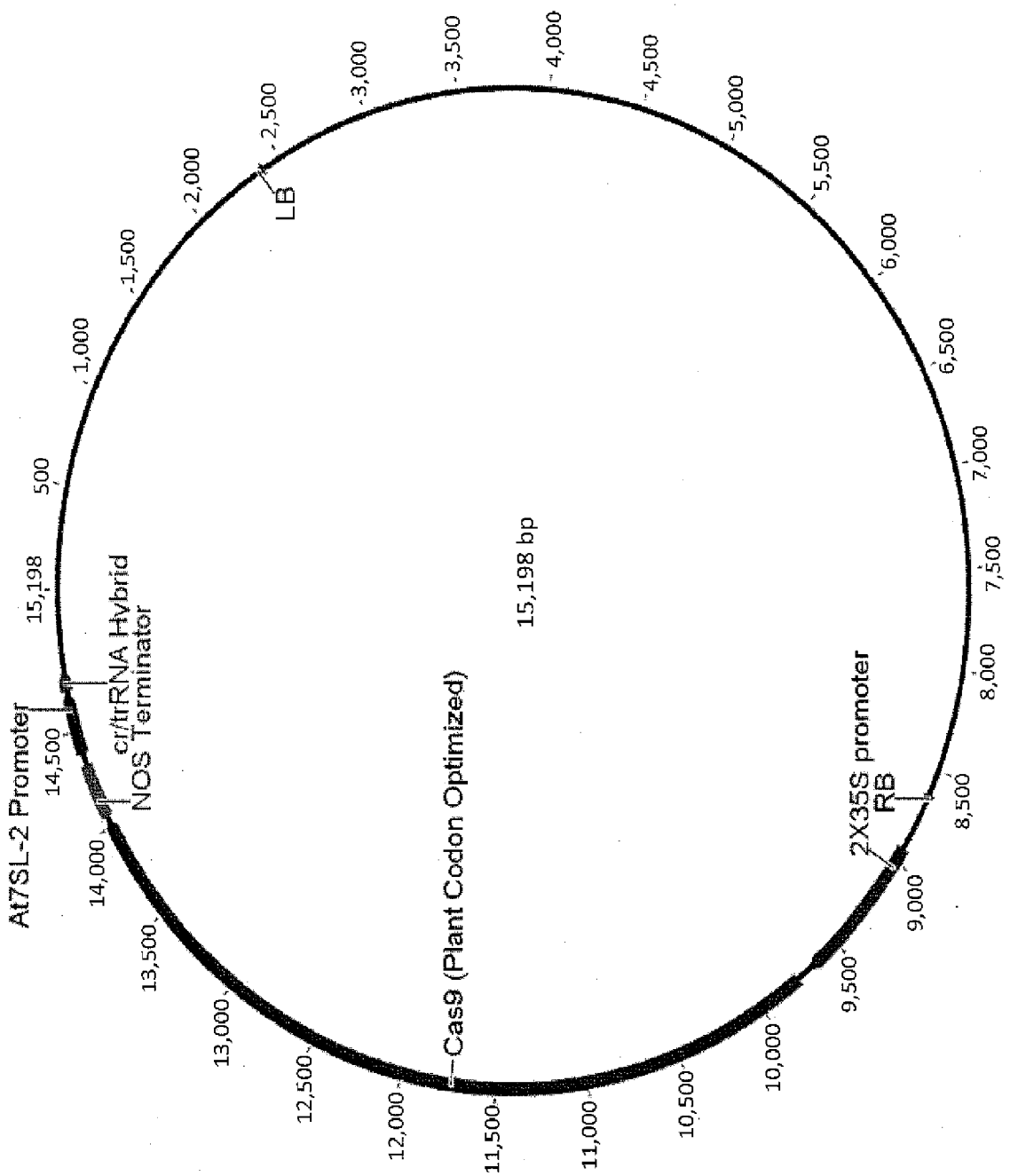
22. The method of claim 21, wherein the promoter is an inducible promoter.

23. The method of claim 22, wherein the inducible promoter is an estrogen inducible promoter.

24. The method of claim 21, further comprising contacting the plant cell with a nucleic acid encoding a guide RNA that associates with the Cas protein.

25. The method of claim 21, wherein the plant cell is a protoplast.

FIG. 1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RB
Cas9 (Plant Codon Optimized)
cr/trRNA Hybrid
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At7SL-2 Promoter
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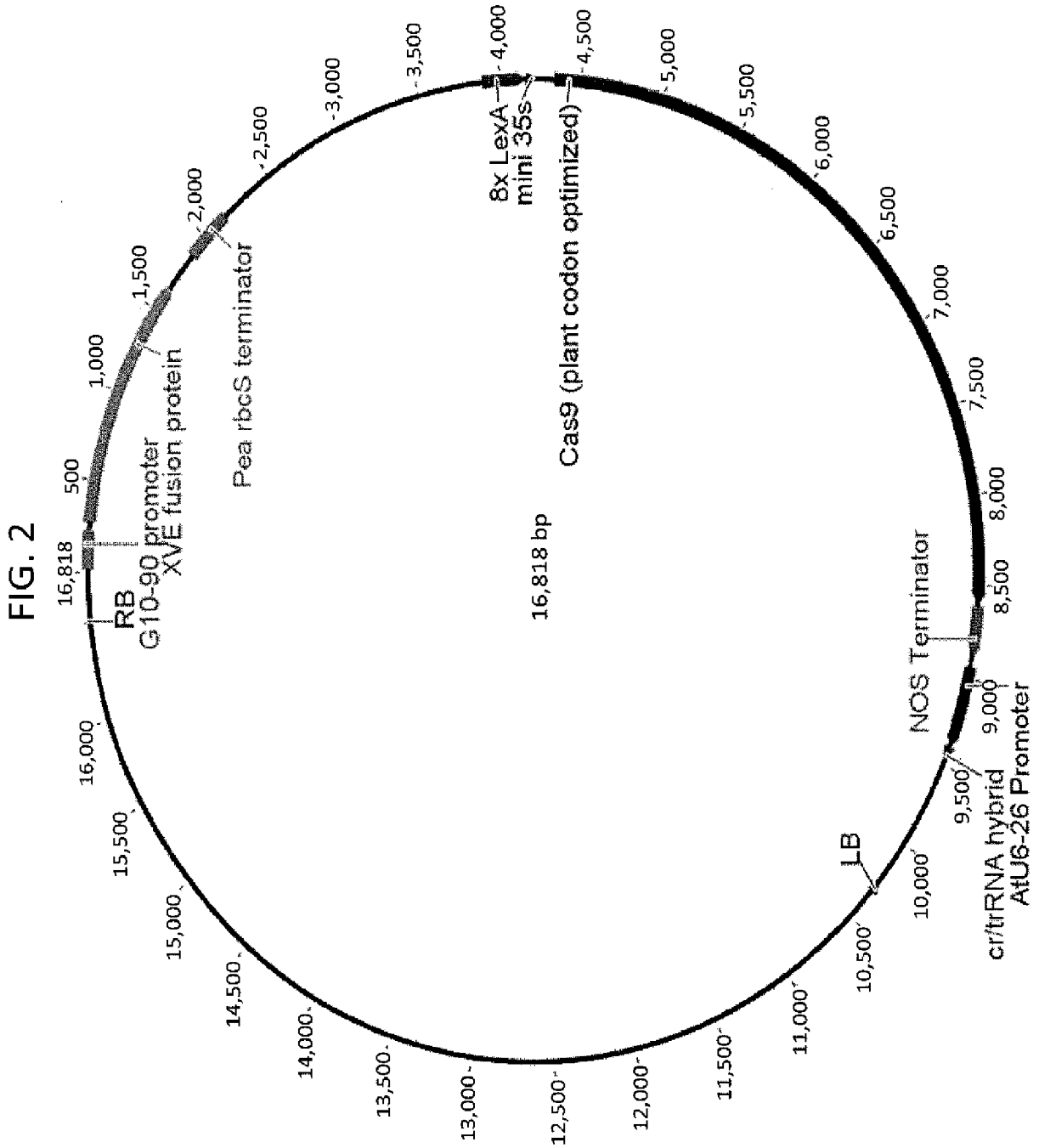
FIG. 2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XVE fusion protein
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cr/trRNA hybrid
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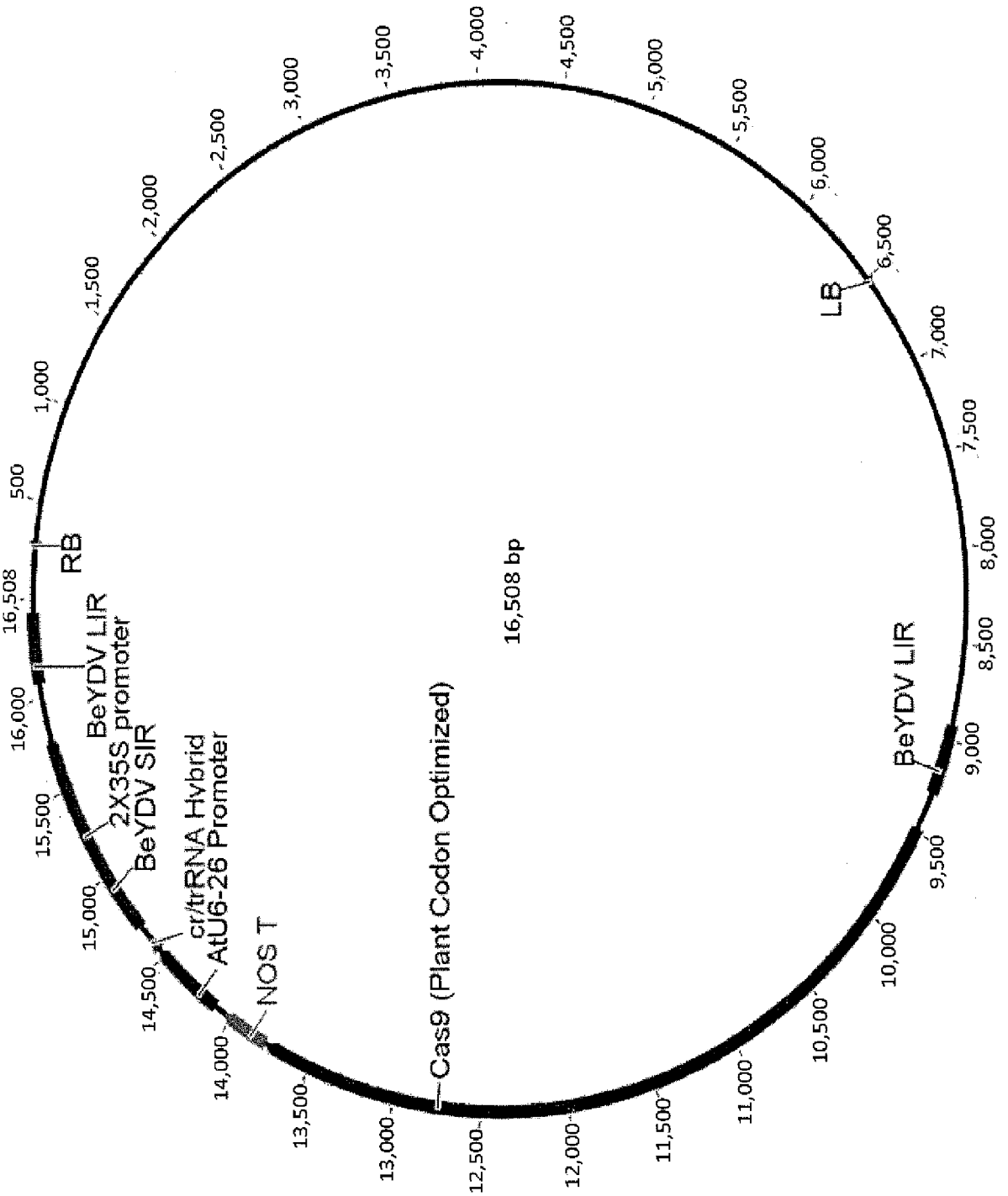
FIG. 3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2X35S promoter
BeYDV SIR
cr/trRNA Hybrid
AtU6-26 Promoter
NOS T
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BeYDV LIR
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FIG. 4A

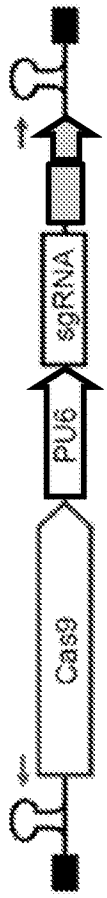

FIG. 4B

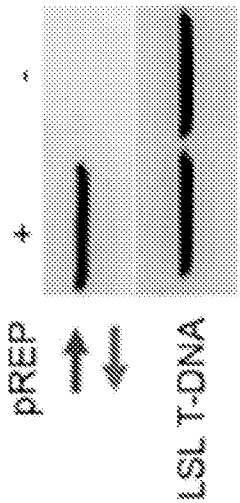

FIG. 4C

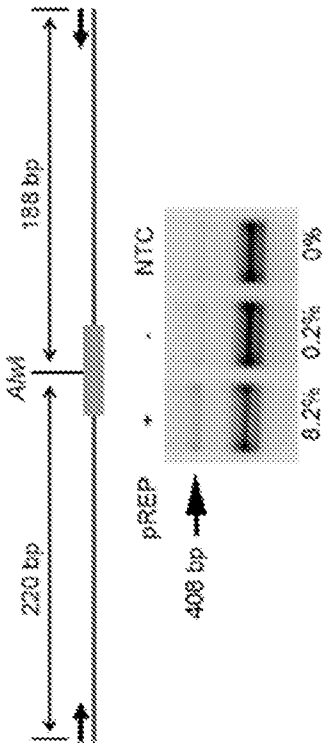

FIG. 4D

PAM

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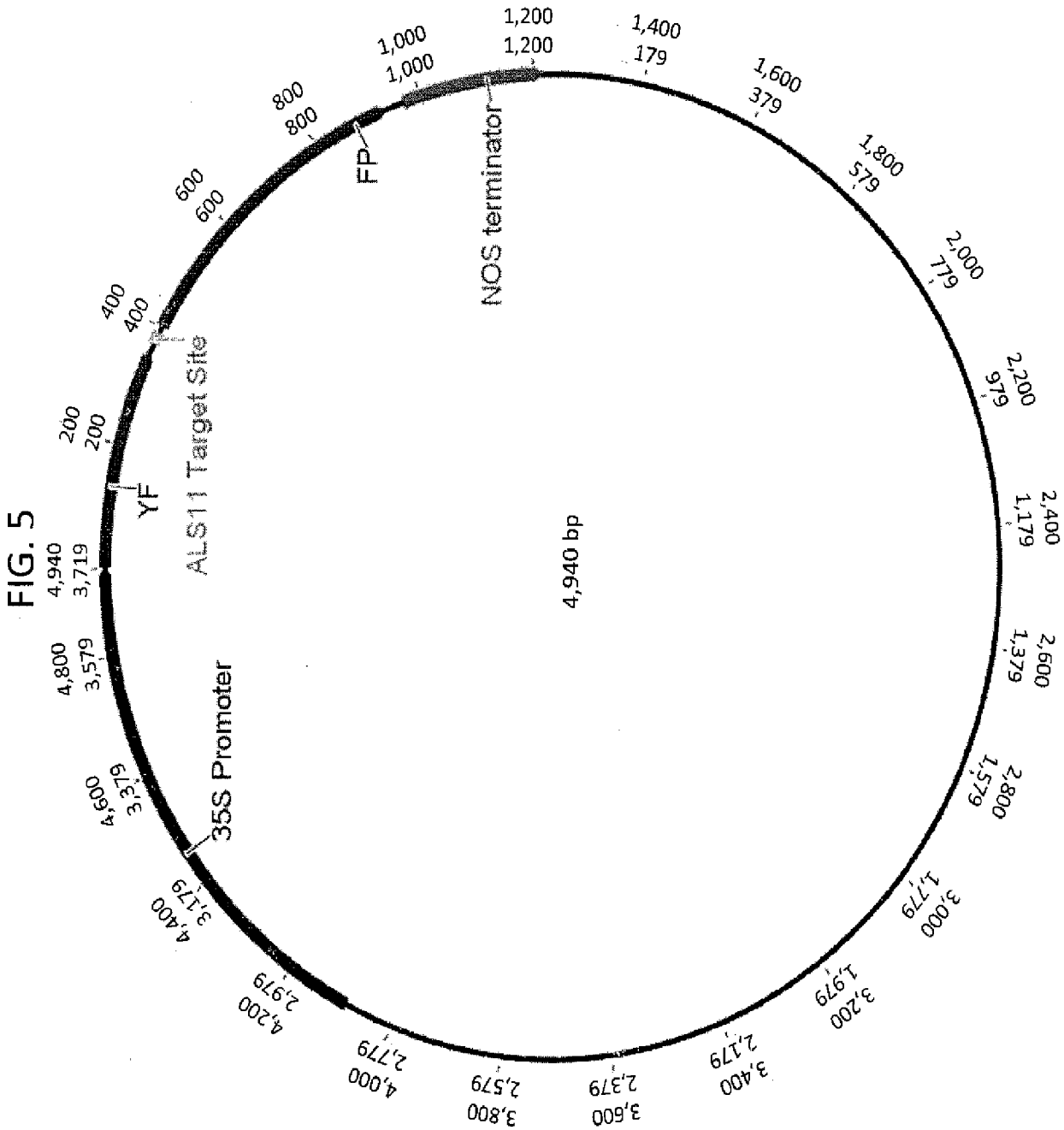
GGTTCAATGGGAGGATCGGTT----AAGGCTAACAGA -4 (SEQ ID NO:2)
GGTTCAATGGGAGGAT----------ATAAGGCTAACAGA -7 (x2) (SEQ ID NO:3)
GGT----------ATAAGGCTAACAGA -20 (SEQ ID NO:4)
GGTTCAATGGGAGGAT---------- -31 (SEQ ID NO:5)

FIG. 5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FP
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YF
35S Promoter
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779
2,200
979
2,400
1,179
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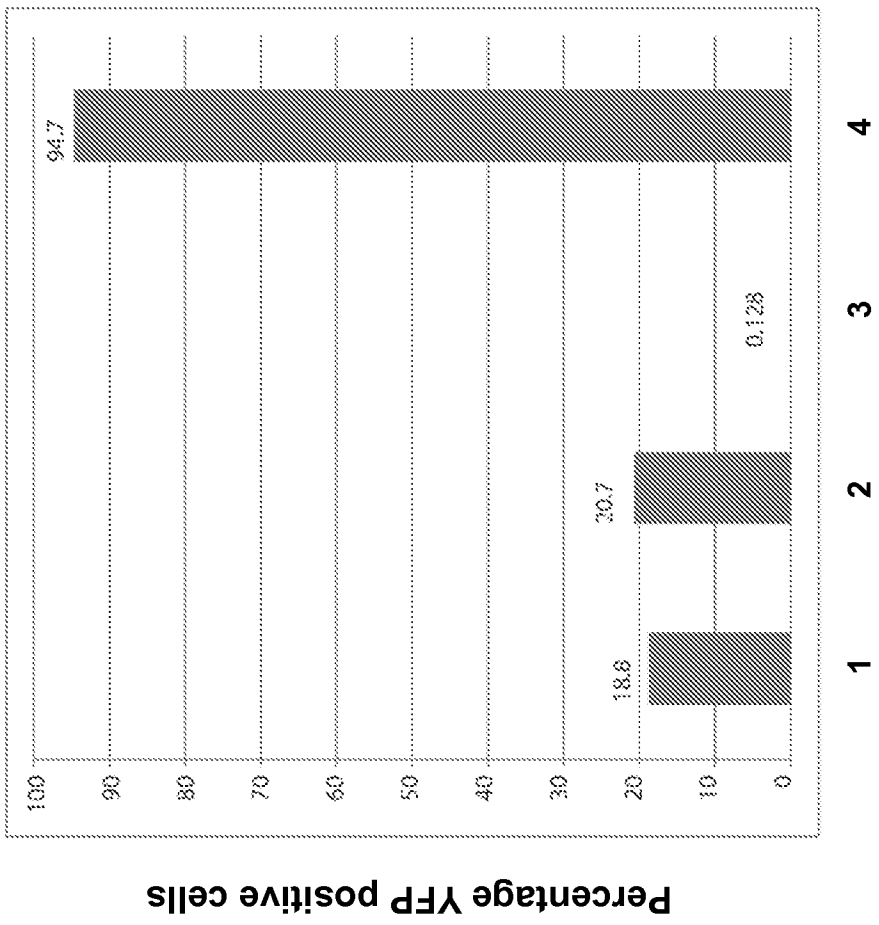
FIG. 6


Percentage YFP positive cells
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30
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10
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30.7
18.6
0.128
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2
3
4

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CRISPR/Cas SYSTEMS

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attaatgaat cggccaacgc gcggggagag gcggtttgcg tattggctag agcagcttgc 240
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Glu Tyr Ile Arg Asn Ile Ser Leu Lys Thr Tyr Asn Glu Val Phe Lys
            340   345   350
Asp Asp Thr Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Lys Thr Asn
        355   360   365
Gln Glu Asp Phe Tyr Val Tyr Leu Lys Lys Leu Leu Ala Glu Phe Glu
    370   375   380
Gly Ala Asp Tyr Phe Leu Glu Lys Ile Asp Arg Glu Asp Phe Leu Arg
385   390   395   400
Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro Tyr Gln Ile His Leu
                405   410   415
Gln Glu Met Arg Ala Ile Leu Asp Lys Gln Ala Lys Phe Tyr Pro Phe
            420   425   430
Leu Ala Lys Asn Lys Glu Arg Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435   440   445
Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Asp Phe Ala Trp
    450   455   460
Ser Ile Arg Lys Arg Asn Glu Lys Ile Thr Pro Trp Asn Phe Glu Asp
465   470   475   480
Val Ile Asp Lys Glu Ser Ser Ala Glu Ala Phe Ile Asn Arg Met Thr

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                485                 490                 495
Ser Phe Asp Leu Tyr Leu Pro Glu Glu Lys Val Leu Pro Lys His Ser
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Leu Leu Tyr Glu Thr Phe Asn Val Tyr Asn Glu Leu Thr Lys Val Arg
        515                 520                 525
Phe Ile Ala Glu Ser Met Arg Asp Tyr Gln Phe Leu Asp Ser Lys Gln
    530                 535                 540
Lys Lys Asp Ile Val Arg Leu Tyr Phe Lys Asp Lys Arg Lys Val Thr
545                 550                 555                 560
Asp Lys Asp Ile Ile Glu Tyr Leu His Ala Ile Tyr Gly Tyr Asp Gly
                565                 570                 575
Ile Glu Leu Lys Gly Ile Glu Lys Gln Phe Asn Ser Ser Leu Ser Thr
            580                 585                 590
Tyr His Asp Leu Leu Asn Ile Ile Asn Asp Lys Glu Phe Leu Asp Asp
        595                 600                 605
Ser Ser Asn Glu Ala Ile Ile Glu Glu Ile Ile His Thr Leu Thr Ile
    610                 615                 620
Phe Glu Asp Arg Glu Met Ile Lys Gln Arg Leu Ser Lys Phe Glu Asn
625                 630                 635                 640
Ile Phe Asp Lys Ser Val Leu Lys Lys Leu Ser Arg Arg His Tyr Thr
                645                 650                 655
Gly Trp Gly Lys Leu Ser Ala Lys Leu Ile Asn Gly Ile Arg Asp Glu
            660                 665                 670
Lys Ser Gly Asn Thr Ile Leu Asp Tyr Leu Ile Asp Asp Gly Ile Ser
        675                 680                 685
Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ala Leu Ser Phe Lys
    690                 695                 700
Lys Lys Ile Gln Lys Ala Gln Ile Ile Gly Asp Glu Asp Lys Gly Asn
705                 710                 715                 720
Ile Lys Glu Val Val Lys Ser Leu Pro Gly Ser Pro Ala Ile Lys Lys
                725                 730                 735
Gly Ile Leu Gln Ser Ile Lys Ile Val Asp Glu Leu Val Lys Val Met
            740                 745                 750
Gly Gly Arg Lys Pro Glu Ser Ile Val Val Glu Met Ala Arg Glu Asn
        755                 760                 765
Gln Tyr Thr Asn Gln Gly Lys Ser Asn Ser Gln Gln Arg Leu Lys Arg
    770                 775                 780
Leu Glu Lys Ser Leu Lys Glu Leu Gly Ser Lys Ile Leu Lys Glu Asn
785                 790                 795                 800
Ile Pro Ala Lys Leu Ser Lys Ile Asp Asn Asn Ala Leu Gln Asn Asp
                805                 810                 815
Arg Leu Tyr Leu Tyr Tyr Leu Gln Asn Gly Lys Asp Met Tyr Thr Gly
            820                 825                 830
Asp Asp Leu Asp Ile Asp Arg Leu Ser Asn Tyr Asp Ile Asp His Ile
        835                 840                 845
Ile Pro Gln Ala Phe Leu Lys Asp Asn Ser Ile Asp Asn Lys Val Leu
    850                 855                 860
Val Ser Ser Ala Ser Asn Arg Gly Lys Ser Asp Asp Val Pro Ser Leu
865                 870                 875                 880
Glu Val Val Lys Lys Arg Lys Thr Phe Trp Tyr Gln Leu Leu Lys Ser
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Lys Leu Ile Ser Gln Arg Lys Phe Asp Asn Leu Thr Lys Ala Glu Arg
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Gly Gly Leu Ser Pro Glu Asp Lys Ala Gly Phe Ile Gln Arg Gln Leu
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Val Glu Thr Arg Gln Ile Thr Lys His Val Ala Arg Leu Leu Asp Glu
    930                 935                 940
Lys Phe Asn Asn Lys Lys Asp Glu Asn Asn Arg Ala Val Arg Thr Val
945                 950                 955                 960
Lys Ile Ile Thr Leu Lys Ser Thr Leu Val Ser Gln Phe Arg Lys Asp
                965                 970                 975
Phe Glu Leu Tyr Lys Val Arg Glu Ile Asn Asp Phe His His Ala His
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Asp Ala Tyr Leu Asn Ala Val Val Ala Ser Ala Leu Leu Lys Lys Tyr
        995                 1000                1005
Pro Lys Leu Glu Pro Glu Phe Val Tyr Gly Asp Tyr Pro Lys Tyr Asn
    1010                1015                1020
Ser Phe Arg Glu Arg Lys Ser Ala Thr Glu Lys Val Tyr Phe Tyr Ser
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Asn Ile Met Asn Ile Phe Lys Lys Ser Ile Ser Leu Ala Asp Gly Arg
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Val Ile Glu Arg Pro Leu Ile Glu Val Asn Glu Glu Thr Gly Glu Ser
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Val Trp Asn Lys Glu Ser Asp Leu Ala Thr Val Arg Arg Val Leu Ser
1075 1080 1085
Tyr Pro Gln Val Asn Val Val Lys Lys Val Glu Glu Gln Asn His Gly
1090 1095 1100
Leu Asp Arg Gly Lys Pro Lys Gly Leu Phe Asn Ala Asn Leu Ser Ser
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Lys Pro Lys Pro Asn Ser Asn Glu Asn Leu Val Gly Ala Lys Glu Tyr
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Leu Asp Pro Lys Lys Tyr Gly Gly Tyr Ala Gly Ile Ser Asn Ser Phe
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Thr Val Leu Val Lys Gly Thr Ile Glu Lys Gly Ala Lys Lys Lys Ile
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Tyr Arg Lys Asp Lys Leu Asn Phe Leu Leu Glu Lys Gly Tyr Lys Asp
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Ile Glu Leu Ile Ile Glu Leu Pro Lys Tyr Ser Leu Phe Glu Leu Ser
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Arg Gly Glu Ile His Lys Gly Asn Gln Ile Phe Leu Ser Gln Lys Phe
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Val Lys Leu Leu Tyr His Ala Lys Arg Ile Ser Asn Thr Ile Asn Glu
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Asn His Arg Lys Tyr Val Glu Asn His Lys Lys Glu Phe Glu Glu Leu
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Phe Tyr Tyr Ile Leu Glu Phe Asn Glu Asn Tyr Val Gly Ala Lys Lys
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Asn Gly Lys Leu Leu Asn Ser Ala Phe Gln Ser Trp Gln Asn His Ser
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Lys Gly Leu Phe Glu Leu Thr Ser Arg Gly Ser Ala Ala Asp Phe Glu
1330 1335 1340
Phe Leu Gly Val Lys Ile Pro Arg Tyr Arg Asp Tyr Thr Pro Ser Ser
1345 1350 1355 1360
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Glu Thr Arg Ile Asp Leu Ala Lys Leu Gly Glu Gly
1380 1385

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35 40 45
Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
50 55 60
Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
65 70 75 80
Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
85 90 95
Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
100 105 110
His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr

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        115                 120                 125
His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
    130                 135                 140
Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
145                 150                 155                 160
Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
                165                 170                 175
Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr
            180                 185                 190
Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala
        195                 200                 205
Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn
    210                 215                 220
Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn
225                 230                 235                 240
Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe
                245                 250                 255
Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp
            260                 265                 270
Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp
        275                 280                 285
Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp
    290                 295                 300
Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser
305                 310                 315                 320
Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys
                325                 330                 335
Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe
            340                 345                 350
Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser
        355                 360                 365
Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp
    370                 375                 380
Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg
385                 390                 395                 400
Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu
                405                 410                 415
Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe
            420                 425                 430
Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile
        435                 440                 445
Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp
    450                 455                 460
Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu
465                 470                 475                 480
Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr
                485                 490                 495
Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser
            500                 505                 510
Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys
        515                 520                 525
Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln
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Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr
545                 550                 555                 560
Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp
                565                 570                 575
Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly
            580                 585                 590
Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp
        595                 600                 605
Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr
    610                 615                 620
Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala
625                 630                 635                 640
His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr
                645                 650                 655
Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp
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Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe
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Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr Phe
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Lys Glu Asp Ile Gln Lys Ala Gln Val Ser Gly Gln Gly Asp Ser Leu
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His Glu His Ile Ala Asn Leu Ala Gly Ser Pro Ala Ile Lys Lys Gly
725 730 735
Ile Leu Gln Thr Val Lys Val Val Asp Glu Leu Val Lys Val Met Gly
740 745 750
Arg His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln
755 760 765
Thr Thr Gln Lys Gly Gln Lys Asn Ser Arg Glu Arg Met Lys Arg Ile
770 775 780
Glu Glu Gly Ile Lys Glu Leu Gly Ser Gln Ile Leu Lys Glu His Pro
785 790 795 800
Val Glu Asn Thr Gln Leu Gln Asn Glu Lys Leu Tyr Leu Tyr Tyr Leu
805 810 815
Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg
820 825 830
Leu Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys
835 840 845
Asp Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg
850 855 860
Gly Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys
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Asn Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys
885 890 895
Phe Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp
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Lys Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr
915 920 925
Lys His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp
930 935 940
Glu Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser
945 950 955 960
Lys Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg
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Glu Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val
980 985 990
Val Gly Thr Ala Leu Ile Lys Lys Tyr Pro Lys Leu Glu Ser Glu Phe
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Val Tyr Gly Asp Tyr Lys Val Tyr Asp Val Arg Lys Met Ile Ala Lys
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Ser Glu Gln Glu Ile Gly Lys Ala Thr Ala Lys Tyr Phe Phe Tyr Ser
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Asn Ile Met Asn Phe Phe Lys Thr Glu Ile Thr Leu Ala Asn Gly Glu
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Ile Arg Lys Arg Pro Leu Ile Glu Thr Asn Gly Glu Thr Gly Glu Ile
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Val Trp Asp Lys Gly Arg Asp Phe Ala Thr Val Arg Lys Val Leu Ser
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Phe Ser Lys Glu Ser Ile Leu Pro Lys Arg Asn Ser Asp Lys Leu Ile
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Ala Arg Lys Lys Asp Trp Asp Pro Lys Lys Tyr Gly Gly Phe Asp Ser
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Pro Thr Val Ala Tyr Ser Val Leu Val Val Ala Lys Val Glu Lys Gly
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Lys Ser Lys Lys Leu Lys Ser Val Lys Glu Leu Leu Gly Ile Thr Ile
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Met Glu Arg Ser Ser Phe Glu Lys Asn Pro Ile Asp Phe Leu Glu Ala
1170 1175 1180
Lys Gly Tyr Lys Glu Val Lys Lys Asp Leu Ile Ile Lys Leu Pro Lys
1185 1190 1195 1200
Tyr Ser Leu Phe Glu Leu Glu Asn Gly Arg Lys Arg Met Leu Ala Ser

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Ala Gly Glu Leu Gln Lys Gly Asn Glu Leu Ala Leu Pro Ser Lys Tyr
            1220                1225                1230
Val Asn Phe Leu Tyr Leu Ala Ser His Tyr Glu Lys Leu Lys Gly Ser
        1235                1240                1245
Pro Glu Asp Asn Glu Gln Lys Gln Leu Phe Val Glu Gln His Lys His
    1250                1255                1260
Tyr Leu Asp Glu Ile Ile Glu Gln Ile Ser Glu Phe Ser Lys Arg Val
1265                1270                1275                1280
Ile Leu Ala Asp Ala Asn Leu Asp Lys Val Leu Ser Ala Tyr Asn Lys
                1285                1290                1295
His Arg Asp Lys Pro Ile Arg Glu Gln Ala Glu Asn Ile Ile His Leu
            1300                1305                1310
Phe Thr Leu Thr Asn Leu Gly Ala Pro Ala Ala Phe Lys Tyr Phe Asp
        1315                1320                1325
Thr Thr Ile Asp Arg Lys Arg Tyr Thr Ser Thr Lys Glu Val Leu Asp
    1330                1335                1340
Ala Thr Leu Ile His Gln Ser Ile Thr Gly Leu Tyr Glu Thr Arg Ile
1345                1350                1355                1360
Asp Leu Ser Gln Leu Gly Gly Asp
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