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(54) Title: THE TELOMERATOR-A TOOL FOR CHROMOSOME ENGINEERING

(57) Abstract: The present invention relates to the field of chromosome engineering. More specifically, the present invention provides methods and compositions useful for inducibly linearizing circular DNA molecules in vivo in yeast. In one embodiment, a comprises a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises an intron comprising an endonuclease recognition site flanked by telomere seed sequences.

THE TELOMERATOR—A TOOL FOR CHROMOSOME ENGINEERING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/773,206, filed March 6, 2013, which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENTAL INTEREST

This invention was made with government support under grant number MCB1026068, awarded by the National Science Foundation. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to the field of chromosome engineering. More specifically, the present invention provides methods and compositions useful for inducibly linearizing circular DNA molecules *in vivo* in yeast.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

This application contains a sequence listing. It has been submitted electronically via EFS-Web as an ASCII text file entitled "P12280-02_Sequence_Listing.txt." The sequence listing is 8,462 bytes in size, and was created on March 6, 2014. It is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Chromosome engineering is the study of genetic modifications that affect large segments of chromosomes. Top down approaches start with pre-existing chromosomes and modify them *in vivo* by introducing, for instance, large deletions, inversions, or duplications. Bottom up approaches, on the other hand, attempt to design and build chromosomes *de novo*. In either case, we need a strong understanding not only of chromosomal features that confer mitotic stability, including centromeres, telomeres, and origins of replication, but also the effect of the spatial relationships of these elements with one another and with other chromosomal features like genes.

The *Saccharomyces cerevisiae* genome is an excellent platform to develop tools for chromosome engineering given the ease of genetic manipulation and similarity to higher eukaryotes. The *S. cerevisiae* genome is composed of 12Mb of DNA inherited via 16 linear chromosomes ranging in size from 270kb to over 1Mb (*1*). Two important cis elements are required to maintain chromosome stability through mitosis and meiosis: compact point centromeres (~125bp) ensure faithful segregation of sister chromatids (*2*), and conserved

telomere sequences protect the ends of each chromosome and guarantee the maintenance of chromosome length during replication (*3, 4*). With these elements intact, many lines of evidence indicate budding yeast tolerates a high degree of chromosomal modifications without affecting viability, for instance: (a) the largest yeast chromosome (*IV*) can be sub-divided into 11 separate mini-chromosomes (*5*); (b) more than 500kb, including 247 non-essential genes, can be deleted in a single haploid strain (*6*); (c) any of the 16 chromosomes can be individually destabilized in a diploid cell to generate a chromosomal complement of 2n-1 (*7*); (d) a designer, synthetic chromosome arm, *synIXR*, was shown to power growth of budding yeast in the absence of the native chromosome sequence (*8*).

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the development of compositions and methods to inducibly convert circular DNA molecules in yeast into stably maintained linear chromosomes. We call this genetic system "The Telomerator." In brief, we can introduce a "telomerator cassette" by homologous recombination into any circular DNA intended to be linearized, in principle, at any position. The cassette encodes a selectable marker gene sequence interrupted by an intron that harbors a homing endonuclease recognition site such as I-SceI. The recognition site is flanked by telomere seed sequences (TeSS), short stretches of minimal yeast telomere sequences. When the endonuclease is inducibly expressed, circular DNA is efficiently linearized and subsequently stably maintained due to the presence of the terminal TeSSs.

Accordingly, in one aspect, the present invention provides a telomerator cassette. In one embodiment, a telomerator cassette comprises a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises an intron comprising an endonuclease recognition site flanked by telomere seed sequences. In a specific embodiment, the intron is the ACT1 intron. A telomerator cassette can also be referred to as a cassette, an expression cassette, a nucleic acid construct, a construct or simply, a composition.

In another embodiment, a telomerator cassette comprises a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises an endonuclease recognition site flanked by telomere seed sequences. The site and flanking sequences are inserted in-frame into the nucleic acid sequence encoding the selectable marker to allow expression. In a specific embodiment, the endonuclease recognition site flanked by telomere seed sequences comprises SEQ ID NO:4. In another embodiment, a telomerator cassette comprises a nucleic acid encoding a selectable marker, wherein the nucleic acid

encoding the selectable marker comprises an endonuclease recognition site flanked by telomere seed sequences such that the selectable marker retains its function. In a further embodiment, a telomerator cassette comprises a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises the original marker sequence operably linked as a protein fusion with an endonuclease recognition site flanked by telomere seed sequences.

In one embodiment, the marker is an auxotrophic marker. Auxotrophic markers can include, but are not limited to, LEU2, URA3, TRP1, MET15, LYS1, and HIS3. In a specific embodiment, the auxotrophic marker is the URA3 gene. In another embodiment, the marker is a drug selection marker and may include, but is not limited to, nourseothrecin (NAT), geneticin, and hygromycin

In particular embodiments, the selectable marker is both selectable and counterselectable. Examples in yeast include URA3, LYS2 and TRP1, counterselectable using 5-fluoro-orotic acid, alpha-amino-adipate, and 5-fluoro-anthranilic acid, respectively. In other embodiments, a counterselectable marker, e.g., CYH2 (counterselectable on cycloheximide), can be fused to any other selectable marker by joining the two open reading frames in such a way that both moieties are functional.

In another embodiment, the endonuclease recognition site is specific for I-SceI. In a more specific embodiment, the I-SceI endonuclease recognition site comprises SEQ ID NO:2. In other embodiments, the endonuclease may comprise restriction endonucleases, Zinc finger nucleases, TALENs or other meganucleases.

In certain embodiments, the telomere seed sequences comprise minimal telomere seed sequences. In general, the telomere seed sequences for yeast comprise $[(TG_{(1-3)})_{16} \ldots (C_{(1-3)}A)_{16}]$. In a specific embodiment, the telomere seed sequences comprise SEQ ID NO:1 and SEQ ID NO:3.

In particular embodiments, a circular chromosome comprises a telomerator cassette described herein. In other embodiments, a eukaryotic host cell comprises a circular chromosome of the present invention. In a specific embodiment, the eukaryote is yeast. In a more specific embodiment, the yeast is *S. cerevisiae.* In other embodiments, the yeast is *Hansenula polymorpha*, *Pichia pastoris* or *Schizosaccharomyces pombe.*

In specific embodiments, the eukaryotic host cell comprises a vector encoding the endonuclease under the control of an inducible promoter. In a particular embodiment, the inducible promoter is the GAL1 promoter. Other promoters can include, but are not limited to, pMET15, pCUP2, other pGAL sequences, and TetON/TetOFF systems.

In a specific embodiment, a telomerator cassette of the present invention comprises a nucleic acid that encodes the auxotrophic marker URA3, wherein the nucleic acid comprises an intron that comprises the I-SceI endonuclease recognition site flanked by telomere seed sequences. In a more specific embodiment, the telomere seed sequences comprise SEQ ID NO:1 and SEQ ID NO:3. In another embodiment, the I-SceI endonuclease recognition site comprises SEQ ID NO:2. A circular chromosome can comprise a telomerator cassette described herein. In another embodiment, a yeast host cell comprises a circular chromosome described herein. The yeast host cell may comprise a vector encoding the I-SceI endonuclease under the control of an inducible promoter. More specifically, the inducible promoter can be the GAL1 promoter.

In one embodiment, a yeast host cell comprises a circular chromosome comprising a telomerator cassette encoding a selectable marker, wherein the nucleic acid encoding the selectable marker comprises an intron that comprises an endonuclease recognition site flanked by telomere seed sequences; and a vector encoding the endonuclease under the control of an inducible promoter. In a specific embodiment, the selectable marker is an auxotrophic marker. In a more specific embodiment, the auxotrophic marker is the URA3 gene. In the yeast host the endonuclease recognition site is specific for I-SceI. More specifically, the I-SceI endonuclease recognition site comprises SEQ ID NO:2. Moreover, the telomere seed sequences can comprise SEQ ID NO:1 and SEQ ID NO:3.

In another aspect, the present invention provides methods for chromosome engineering. In one embodiment, a method for engineering a circular chromosome capable of being inducibly linearized comprises the step of integrating a telomerator cassette into a circular chromosome, wherein the telomerator cassette comprises a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises an intron comprising an endonuclease recognition site flanked by telomere seed sequences. In a specific embodiment, the integrating step is accomplished by transforming a telomerator cassette into a host cell comprising the circular chromosome. In another embodiment, the host cell comprises a vector encoding the endonuclease under the control of an inducible promoter.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: Telomerator Design and Function. (A) The telomerator encodes a selectable *URA3* gene sequence interrupted by an intron (*ACT1* intron) that harbors a homing endonuclease recognition site (*I-SceI*). The recognition site is flanked by telomere seed sequences (*TeSS*). The telomerator cassette is encoded on a circular DNA molecule yielding

cells that grow on medium lacking uracil (Ura^+) but are sensitive to the uracil counterselection drug Foa, assuming the circular DNA molecule encodes at least one essential genetic part. Expression of I-SceI, under the control of the *GAL1* promoter (*pGAL1*), is induced by growth in galactose medium. Cells harboring newly linearized molecules should no longer grow on plates lacking uracil (Ura^-) and moreover should be resistant to Foa (Foa^R). (B) Sequence of the *TeSS-I-SceI-TeSS* region of the telomerator.

FIG. 2: The telomerator complements yeast cell growth on medium lacking uracil. Plasmids encoding either a native *URA3* gene (left), a *URA3* gene interrupted by the intron of *ACT1* (middle), or a *URA3* gene interrupted by a telomerator sequence encoded within the *ACT1* intron (right), were transformed into a yeast strain (BY4741) incapable of growing on medium lacking uracil (SC-Ura). Growth of the resulting transformed colonies on SC-Ura was indistinguishable indicating normal expression of the *URA3* gene product from the telomerator-encoding construct.

FIG. 3: The telomerator drives *in vivo* linearization of a synthetic yeast chromosome arm, *synIXR* BAC. (A) *synIXR* BAC is a ~100kb circular chromosome arm that can power a yeast cell. In independent integration experiments, the telomerator cassette was introduced by homologous recombination at two gene-free regions of *synIXR* BAC as indicated (Integration Site 1, ~40kb; Integration Site 2, ~80kb). (B) Following 24 hours of growth in galactose-containing medium to induce expression of I-SceI, single cells from Integration Site 1 (left) and Integration Site 2 (right) were plated on YPD medium, and then replica plated onto synthetic complete medium supplemented with 5'-fluoroorotic acid (Foa), and synthetic complete medium lacking uracil or leucine (SC-Ura and SC-Leu, respectively). Only in cell populations encoding an intact I-SceI recognition sequence as part of the telomerator cassette (+ I-SceI, top row) did linearization occur, as assayed by growth on Foa and non-growth on SC-Ura.

FIG. 4: Circular permutation of a synthetic eukaryotic chromosome, *synIXR* BAC. (A) Strategy to integrate the telomerator cassette downstream of 54 genes on *synIXR BAC*. (B) Characterization of permuted strains. The telomerator cassette was integrated downstream of each indicated gene, and growth of strains, pre- (circular) and post-linearization (linear), was assessed at 30°C (top) or 37°C (bottom) on rich medium. Shown here are 10-fold serial dilutions after two days growth. Permuted strains not pictured here did not exhibit growth defects. (C) Pulsed field gel showing appearance of linear *synIXR* after galactose induction in six representative strains (the telomerator was integrated downstream of *YIR039C, YIR040C, YIR041W, YIR042C, YIR043C, YIR044C*), as compared to a wild type

strains encoding a native chromosome *IX* (BY4741), or the parental strain used for telomerator integrations (*synIXR BAC*). The circular form of *synIXR BAC* is not detectable on pulsed field gel. O = circular; | = linear.

FIG. 5: Deletion of *SIR2* rescues growth defects of *synIXR BAC* circular permutations. The *SIR2* gene was deleted in the each of the 54 unique telomerator-containing *synIXR* BAC strains. Following transformation of the pGAL1-I-SceI construct and growth in galactose, linear permutations were isolated on Foa medium. Shown here are 10-fold serial dilutions after two days growth on rich medium (YPD) at the indicated temperatures. Only the permuted strains corresponding to those pictured in 4b are shown.

FIG. 6: PCR confirmation of integration and circular/linear status. All permutations were tested, pre- and post-linearization (circular and linear, respectively), by PCR using two primer sets. First, integration was confirmed using a gene-specific primer in combination with a primer that annealed within the telomerator (green arrows). These primers confirmed the presence of this region in either the linear or circular conformation of *synIXR* in all strains. Secondly, a primer pair designed to anneal to the *ACT1* intron (red arrows) was used. This primer pair amplifies a product from the endogenous *ACT1* intron at its native locus on chromosome six, as well as from an intact telomerator cassette. Following growth in galactose, absence of this band is consistent with linearization.

FIG. 7: Position effect of genes expected to impact cell fitness. Left panel: Fitness defects in linearized strains (as in FIG. 3b). Right panel: Orientation and approximate location on linearized *synIXR* of essential ORFs and ORFs required for fast growth.

DETAILED DESCRIPTION OF THE INVENTION

It is understood that the present invention is not limited to the particular methods and components, etc., described herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to a "protein" is a reference to one or more proteins, and includes equivalents thereof known to those skilled in the art and so forth.

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 invention belongs. Specific methods, devices, and materials are described, although any methods and

materials similar or equivalent to those described herein can be used in the practice or testing of the present invention.

All publications cited herein are hereby incorporated by reference including all journal articles, books, manuals, published patent applications, and issued patents. In addition, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided. The definitions are not meant to be limiting in nature and serve to provide a clearer understanding of certain aspects of the present invention.

Chromosome engineering is an emerging focus in the fields of systems biology, genetics, synthetic biology, and the functional analysis of genomes. Here we describe the 'telomerator', a new synthetic biology device designed to inducibly linearize circular DNA molecules in vivo in *Saccharomyces cerevisiae*. To demonstrate the functionality and utility of the telomerator, we generate linear variants of a synthetic yeast chromosome originally built as a circular molecule, synIXR BAC. By circularly permuting synIXR BAC using the telomerator, we generated an array of 53 different linearized chromosome structures, many of which confer novel phenotypic properties. This tool offers a new way to study the effect of gene placement on chromosomes (i.e. telomere proximity), the essentiality of 3' non-coding regions of genes, and the plasticity of gene order and chromosome structure on cell fitness. The telomerator will be an important tool to generate artificial, linear chromosomes in yeast and the concept could be extended to other eukaryotes including mammals and human cells.

The term "nucleic acid" or "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides and/or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the nucleic acid can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the nucleic acid can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate (P-NH_2) or a mixed phosphoramidate-phosphodiester oligomer. In addition, a double-stranded nucleic acid can be obtained from the single stranded nucleic acid product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

The following are non-limiting examples of nucleic acids: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A nucleic acid may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A nucleic acid may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the nucleic acid to proteins, metal ions, labeling components, other nucleic acids, or a solid support.

As used herein, the term "operably linked" means that nucleic acid sequences or proteins are operably linked when placed into a functional relationship with another nucleic acid sequence or protein. For example, a promoter sequence is operably linked to a coding sequence if the promoter promotes transcription of the coding sequence. As a further example, a repressor protein and a nucleic acid sequence are operably linked if the repressor protein binds to the nucleic acid sequence. Additionally, a protein may be operably linked to a first and a second nucleic acid sequence if the protein binds to the first nucleic acid sequence and so influences transcription of the second, separate nucleic acid sequence. Generally, "operably linked" means that the DNA sequences being linked are contiguous, although they need not be, and that a gene and a regulatory sequence or sequences (e.g., a promoter) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins--transcription factors--or proteins which include transcriptional activator domains) are bound to the regulatory sequence or sequences.

The term "plasmid" refers to an extrachromosomal circular DNA capable of autonomous replication in a given cell. In certain embodiments, the plasmid is designed for amplification and expression in bacteria. Plasmids can be engineered by standard molecular biology techniques. *See* Sambrook et al., Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989), N.Y. The term "expression vector" is used interchangeably herein with the term "plasmid" and refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for expression of the operably linked coding sequence (e.g. an insert sequence that codes for a product) in a particular host cell. Nucleic acid sequences necessary for expression in

prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences.

The term "promoter" refers to the DNA region, usually upstream of the coding sequence of a gene or operon, which binds RNA polymerase and directs the enzyme to the correct transcriptional start site.

The terms "endonuclease" refers to any wild-type or variant enzyme capable of catalyzing the hydrolysis (cleavage) of bonds between nucleic acids within a DNA or RNA molecule, preferably a DNA molecule. Endonucleases do not cleave the DNA or RNA molecule irrespective of its sequence, but recognize and cleave the DNA or RNA molecule at specific polynucleotide sequences, further referred to as "target sequences" or "target sites". Endonucleases can for example be a homing endonuclease (Paques et al., Curr Gen Ther. 2007 7:49-66), a chimeric Zinc-Finger nuclease (ZFN) resulting from the fusion of engineered zinc-finger domains with the catalytic domain of a restriction enzyme such as FokI (Porteus et al., Nat Biotechnol. 2005 23:967-973) or a chemical endonuclease (Arimondo et al., Mol Cell Bioi. 2006 26:324-333; Simon et al., NAR 2008 36:3531-3538; Eisenschmidt et al., NAR 2005 33 :7039-7047; Cannata et al., PNAS 2008 105:9576-9581). In chemical endonucleases, a chemical or peptidic cleaver is conjugated either to a polymer of nucleic acids or to another DNA recognizing a specific target sequence, thereby targeting the cleavage activity to a specific sequence. Chemical endonucleases also encompass synthetic nucleases like conjugates of orthophenanthroline, a DNA cleaving molecule, and triplex-forming oligonucleotides (TFOs), known to bind specific DNA sequences (Kalish and Glazer Ann NY Acad Sci 2005 1058: 151-61). Such chemical endonucleases are considered to fall within the scope of the term "endonuclease" according to the present invention. Also within the scope of the present invention is intended any fusion between molecules able to bind DNA specific sequences and agent/reagent/chemical able to cleave DNA or interfere with cellular proteins implicated in the double strand break (DSB) repair (Majumdar et al., J. Bioi. Chern 2008 283, 17:11244-11252; Liu et al. NAR 2009 37:6378-6388); as a non-limiting example such a fusion can be constituted by a specific DNA-sequence binding domain linked to a chemical inhibitor known to inhibit re-ligation activity of a topoisomerase after DSB cleavage. Endonucleases can comprise a homing endonuclease, also known under the name of meganuclease. The term "meganuclease" refers to an endonuclease having a double-stranded DNA target sequence of about 12 to about 45 base pairs (bp). Such homing endonucleases are well-known to the art (see e.g. Stoddard, Quarterly Reviews of Biophysics, 2006, 38:49-95). Homing endonucleases recognize a DNA target sequence and generate a

single- or double-strand break. Homing endonucleases are highly specific, recognizing DNA target sites ranging from 12 to 45 base pairs (bp) in length, usually ranging from about 14 to about 40 bp in length. The homing endonuclease according to the invention may for example correspond to a LAGLIDADG endonuclease, to a HNH endonuclease, or to a GIY-YIG endonuclease. In certain embodiments, a meganuclease is either a dimeric enzyme, wherein each domain is on a monomer or a monomeric enzyme comprising the two domains on a single polypeptide.

Endonucleases according to the invention can also be derived from TALENs, a new class of chimeric nucleases using a Fokl catalytic domain and a DNA binding domain derived from Transcription Activator Like Effector (TALE), a family of proteins used in the infection process by plant pathogens of the Xanthomonas genus (Boch, Scholze et al. 2009; Moscou and Bogdanove 2009; Christian, Cermak et al., 201 0; Li, Huang et al., 2011) (Boch, Scholze et al., 2009; Moscou and Bogdanove 2009; Christian, Cermak et al., 201 0; Li, Huang et al., 2010). The functional layout of a Fokl-based TALE-nuclease (TALEN) is essentially that of a ZFN, with the Zinc-finger DNA binding domain being replaced by the TALE domain. As such, DNA cleavage by a TALEN requires two DNA recognition regions flanking an unspecific central region. Endonucleases encompassed in the present invention can also be derived from TALENs. An endonuclease according to the present invention can be derived from a TALE- nuclease (TALEN), i. e. a fusion between a DNA-binding domain derived from a Transcription Activator Like Effector (TALE) and one or two catalytic domains. In other embodiments, CRISPR/Cas systems may be used. See Mali P, Yang L, Esvelt KM, Aach J, Guell M, Dicarlo JE, Norville JE, Church GM: RNA-guided human genome engineering via Cas9. Science 2013; Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F:Multiplex genome engineering using CRISPR/Cas systems. Science 2013; Cho SW, Kim S, Kim JM, Kim JS: Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 2013; Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK:Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 2013; and Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA: RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat Biotechnol 2013.

As used herein, a "selectable marker" refers to a phenotypic trait conferred on transformed cells that protects them from a selective agent in their environment, i.e., the growth media. Examples of selectable markers include, but are not limited to, antibiotic resistance markers (e.g., genes encoding resistance to kanamycin, ampicillin,

chloramphenicol, gentamycin, or tetracycline), metabolic markers (e.g., amino acid synthesis genes or transfer RNA genes) and auxotrophic markers.

As used herein, the term "vector" refers to a nucleic acid construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors" which are designed for isolation, propagation and replication of inserted nucleotides, "expression vectors" which are designed for expression of a nucleotide sequence in a host cell. The term "replication" means duplication of a vector.

Any yeast cell can be used in the methods of the present invention. In particular embodiments, a yeast species within the genus of *Saccharomyces*, particularly *Saccharomyces cerevisiae*, are used. Other examples of suitable yeast species include, but are not limited to, *Hansenula polymorpha*, *Pichia pastoris*, and *Schizosaccharomyces pombe*. Indeed, numerous yeast strains or derivative strains are known in the art. The application and optional modification of such strains for purposes of the present invention should be apparent to a skilled artisan apprised of the present disclosure.

Our goal was to systematically perturb order and orientation of genetic elements on linear chromosomes in *S. cerevisiae*. To achieve this, we have developed the telomerator, a genetic tool that can inducibly linearize circular DNA molecules *in vivo*. We use the telomerator to circularly permute the *synIXR* synthetic chromosome, which is encoded as a bacterial artificial chromosome (BAC, herein refered to *synIXR* BAC). Our results support that telomerator-induced linearization generates linear chromosomes with functional telomeres on which heterochromatin is fully established.

At least two previous methods have been used to construct artificial linear chromosomes in yeast. First, non-native chromosomes were generated by *in vitro* ligation of synthetic telomere sequences onto linear DNA followed by transformation and selection in yeast (*20, 21*). This approach overcomes the complicating factor that linear DNA molecules cannot be replicated in *E. coli* and can therefore be difficult to construct. Secondly, a circular derivative of chromosome three (*22*) was linearized by integrating into it synthetic telomere sequences; over time these sequences resolved *in vivo* to generate a linear molecule (*23*). Together these studies shed light on important features about the mitotic stability of chromosomes. First, the overall length of linear DNA molecules is a major contributing factor to mitotic stability (*16, 20, 21, 23*). Second, the spatial relationship between the centromere and telomeres has very little impact on mitotic stability (*16, 23*). Finally, X and Y' elements located in subtelomeric regions of native S. cerevisiae chromosomes have little or no impact on mitotic stability (*23*).

The telomerator represents a new and convenient way to linearize circular DNA molecules *in vivo* in *S. cerevisiae* and can be applied to the construction of artificial chromosomes. Here we demonstrate its utility by circularly permuting a pre-existing circular synthetic chromosome in yeast - *synIXR*. In terms of chromosome engineering, the telomerator presents four major advantages over the approaches described above: (i) the use of telomere seed sequences that correspond to native yeast telomeres; (ii) the ease with which a telomerator construct can be PCR amplified and thus integrated at multiple unique loci; (iii) the method gives virtually 100% correct clones with no requirement for cumbersome screening approaches; and (iv) the inducible control over timing of linearization *in vivo*.

Linear Permutations of synIXR. In our work, we linearized circular chromosomes to generate minimal telomere sequences. Based on the *SIR2*-dependent phenotypes observed, we conclude that heterochromatin is forming on the linearized chromosomes. In native chromosomes, subtelomeric sequences can buffer these effects, as can insulators. Future telomerator designs could incorporate such elements.

With the exception of effects on gene expression, the panel of permuted chromosomes displays surprisingly little variation in behavior, suggesting that the relative placement of telomeres and centromeres in *S. cerevisiae* is quite flexible. For instance, linearization at either *YIL001W* or *YIR001C* produces telocentric versions of *synIXR* with only ~1kb separating the centromere from a telomere; that these permutations exhibit no apparent growth defect is consistent with previous work suggesting telocentric chromosomes in budding yeast are mitotically stable (*16, 23*).

Other Applications for Telomerator. The telomerator provides a flexible new strategy to aid in the construction and expression of supernumerary chromosomes encoding non-native pathways that give a multitude of new functions to the cell. In principle, this technology can be extended to other eukaryotic organisms, especially those with point/small centromeres like budding yeast. Applying the telomerator to chromosome engineering in mammalian cells will be more challenging as 'regional' mammalian centromeres can extend for hundreds of kilobases and include repetitive sequences. Artificially generated human minichromosomes, existing as either linear or circular constructs, have been described and they may serve as a starting point for mammalian artificial chromosome engineering (*24-30*). We ultimately envision such artificial chromosomes as valuable platforms for gene targeting that will allow delivery of large DNAs to recipient cells and provide a means by which to investigate the incorporation of complex segments of DNA encoding networks and pathways.

Prospects for Telomerators in Other Systems/Organisms. The linearization function of the telomerator can obviously be achieved with a wide variety of restriction endonucleases, Zinc finger nucleases, TALENs or meganucleases besides I-SceI. One important consideration is that the recognition sequence of the enzyme must be sufficiently rare within the sequence of the host genome (i.e., no or few existing sites) to work without causing "collateral damage" in the rest of the genome. In this regard the I-SceI site is not present in yeast nuclear genome (*31*). A wide variety of selectable/counterselectable markers could also be employed.

Another approach to introduce the Telomerator sequence into a circular DNA molecule for linearization is to include the TeSS-I-SceI-TeSS sequence as part of the protein coding sequence of the selectable marker. In such embodiments, the TeSS-I-SceI-TeSS sequence is "in-frame" with the selectable marker sequence (i.e., a multiple of 3) such that the resulting protein translation would not be truncated. The TeSS-I-SceI-Tess sequence is inserted into the selectable marker in a position that would not disrupt function of the resulting protein product. Shorter TeSSs have been described in the literature (Yamagishi 2008 Appl Microbiol Biotechnol 79:699-706), and could be employed in this so-called Telomerator-fusion protein model in order to increase the stability of the highly repetitive TeSS sequences.

This work should be easily expandable to other yeast species. This is relevant in the academic setting, where fungal species other than *S. cerevisiae* are becoming increasingly commonplace (i.e., *Ashbya gossypii*, *Candida glabrata, Pichia pastoris* etc.). Furthermore, many yeast species are used industrially, exploiting unique properties beneficial to the process of interest (e.g., *P. pastoris*, high-level secreted protein production; *A. gossypii*, riboflavin production). Similarly, the approach could almost certainly be expanded to other eukaryotic microorganisms such as algae, and potentially to various tissue culture cells.

Without further elaboration, it is believed that one skilled in the art, using the preceding description, can utilize the present invention to the fullest extent. The following examples are illustrative only, and not limiting of the remainder of the disclosure in any way whatsoever.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices, and/or methods described and claimed herein are made and evaluated, and are intended to be purely illustrative and are not intended to limit the scope of what the inventors

regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for herein. Unless indicated otherwise, parts are parts by weight, temperature is in degrees Celsius or is at ambient temperature, and pressure is at or near atmospheric. There are numerous variations and combinations of reaction conditions, e.g., component concentrations, desired solvents, solvent mixtures, temperatures, pressures and other reaction ranges and conditions that can be used to optimize the product purity and yield obtained from the described process. Only reasonable and routine experimentation will be required to optimize such process conditions.

Materials and Methods

Construction of the Telomerator. The TeSS-I-SceI sequence was introduced by one-step isothermal assembly(*32*) into the unique *Xho*I site encoded centrally within the *ACT1* intron that had been previously transplanted into *URA3*(*12*). Specifically, the TeSS-I-SceI-TeSS sequence (5'TGTGTGGTGTGTGGGTGTGTGTGGGTGTGTGGGTGTGTGGGTAGGGATAACAG GGTAATCACCCACCACACACACCCACACACACCACACACCCACCCA3') (SEQ ID NO:4) flanked by 40bp corresponding to sequence on either side of the XhoI site was ordered from IDT as an Ultramer. This sequence was exponentially amplified using external primers (F 5'ATCCCATTTAACTGTAAGAAGAATTGC3' (SEQ ID NO:5); R 5'GGAGAGTGAAAAATAGTAAAAAAAGGT3' (SEQ ID NO:6)). Expression of the *URA3-intronACT1-TeSS-I-Sce-I-TeSS-intronACT1-URA3* cassette was verified by functional complementation assay as described in the text.

Integration of Telomerator into synIXR BAC. Primers were designed to bind upstream of the *URA3* promoter (5'CCCGGGGGATCCGGTGATTG3') (SEQ ID NO:7) and downstream of the *URA3* terminator (5'CCAAAGCTGGAGCTCCACCG3') (SEQ ID NO:8) on the telomerator construct. 50bp corresponding to either side of each unique integration site was then appended to these two primers. Standard PCR conditions were used to amplify the telomerator, which was then transformed into a *synIXR* BAC containing strain (yJS587 (*8*)). Integration was confirmed with a location specific primer in combination with a primer internal to the *URA3* promoter sequence (FIG. 6).

Linearization of synIXR. The *pGAL1-I-SceI* sequence was subcloned from a pre-existing plasmid (*33*) into the yeast shuttle vector pRS413 (*14*) at the *Sal*I recognition site. This construct was transformed into strains to be linearized and selected on medium lacking histidine (SC-His). Linearization was induced in liquid culture in synthetic medium lacking

histidine with 2% galactose for 24 hours. Strains were then transferred onto synthetic complete solid medium supplemented with Foa and extra uracil. Each strain was subjected to single round of colony purification on YPD medium and single isolates that had lost the *pGAL-I-Sce-I* construct, confirmed by replica-plating on SC-His, were selected for further analysis. Circular versus linear status of *synIXR* was tested by PCR using the primers originally designed to amplify the TeSS-I-Sce-I-TeSS ultramer (described above and FIG. 6).

Pulsed Field Gel Electrophoresis. Full length yeast chromosomes were prepared in agarose plugs as previously described (*34*). Chromosomes were separated by clamped homogeneous electric field (CHEF) gel electrophoresis using the CHEF-DR III Pulsed Field Electrophoresis Systems (Biorad) with the following settings: 6V/cm, switch time 60-120 seconds over 24 hours, 14°C, 0.5X TBE, 1% gel prepared with low melting point agarose (Lonza, 50100). Gels were stained with 5ug/ml ethidium bromide in water post-electrophoresis and then imaged.

SIR2 Gene Deletion. The *sir2ΔkanMX* locus was amplified from the deletion mutant collection strain (*35*) using primers ~500bp flanking the gene deletion and subsequently transformed into the array of permutable strains by selection on YP supplemented with 200µg/mL geneticin. *SIR2* gene deletion was confirmed functionally by confirming the loss of mating proficiency.

Results

Example 1: Telomerator Design and Function/Application. The design of the telomerator includes several different elements (FIG. 1A). Centrally, the telomerator encodes an 18 base pair recognition sequence for the I-SceI homing endonuclease(*9, 10*). This site is flanked by a pair of convergent telomere seed sequences (TeSS) that encode ~40bp of conserved yeast telomere repeats $[(TG_{(1\text{-}3)})_{16} \ldots (C_{(1\text{-}3)}A)_{16}]$ previously shown to serve as a minimal telomeric repeat(*11*) (FIG. 1B). The entire TeSS-I-SceI-TeSS sequence is encoded within an intron that interrupts the *URA3 S. cerevisiae* auxotrophic selectable marker coding sequence. Previous work showed that transplantation of the *ACT1* gene intron into the middle of the *URA3* coding sequence did not disrupt complementation on medium lacking uracil, indicating the mRNA was expressed and the non-native intron correctly spliced prior to translation of the *URA3* gene product (*12*). Thus, the overall design of the telomerator is as follows: 5'-*URA3exon1*-intron*ACT1*-TeSS-I-SceI-TeSS-intron*ACT1-URA3exon2*-3'.

Expression of the I-SceI homing endonuclease, which recognizes and cleaves the 18bp I-SceI recognition site, can linearize a circular DNA molecule encoding the telomerator. To facilitate inducible linearization, I-SceI expression can be placed under control of the

inducible *GAL1* promoter and thus linearization driven specifically by growth in medium containing galactose. Under standard growth conditions in glucose medium, the telomerator cassette should remain intact with cells expressing a functional Ura3 protein, complementing growth of a *ura3Δ0* strain on medium lacking uracil. Following growth in galactose, the linearization reaction can be selected on 5-fluoroorotic acid (Foa) medium, which counterselects against $URA3^{+}$ cells (*13*); a circular telomerator-containing molecule will grow on medium lacking uracil and die on FOA, but a cell harboring a linearized molecule, in which the *URA3* gene component of the telomerator is literally split in two, should display the opposite growth phenotype (Ura^{-}, Foa^{R} [uracil requiring and resistant to Foa]) (FIG. 1A).

Example 2: Telomerator Constructs Complement Growth on Medium Lacking Uracil. While expression of the *URA3* gene interrupted by the *ACT1* intron was previously shown to complement growth of *ura3* mutants on medium lacking uracil (*12*), the effect on complementation of further encoding the TeSS-I-SceI-TeSS sequence within the intron was unknown. To test this, we constructed the telomerator cassette in pRS413, a centromere-based yeast shuttle vector encoding the *HIS3* selectable marker (*14*). The telomerator-containing construct was transformed into BY4741, a yeast strain with a complete *URA3* deletion (*ura3Δ0*) (*15*), and plated on medium lacking uracil. Growth of the resulting transformants was compared to cells transformed with pRS416, another centromere-based yeast shuttle vector encoding a wild type *URA3* gene, or a plasmid encoding the *URA3exon1-intronACT1-URA3exon2* cassette (*12*), identical to the telomerator plasmid but lacking the TeSS-I-SceI-TeSS sequence. Growth of cells transformed with any of the three constructs on medium lacking uracil was indistinguishable (FIG. 2). Thus, the presence of the TeSS-I-SceI-TeSS sequence within the *ACT1* intron interrupting the *URA3* gene does not interfere with growth on medium lacking uracil.

Example 3: The Telomerator Can Inducibly Linearize Circular DNA Molecules In Vivo. Chromosome length has important consequences for mitotic stability as linear chromosomes shorter than 90 kb exhibit markedly decreased stability (*16*). Therefore, we tested the capacity of the telomerator to linearize circular DNA molecules *in vivo* using *synIXR*, a synthetic yeast chromosome arm corresponding to the right arm of chromosome nine previously constructed as a bacterial artificial chromosome (BAC) (*8, 17*) approximately 100kb long. *SynIXR* encodes all 52 genes from the right arm of chromosome 9 (*YIR001C-YIR044C*), 2 genes from the left arm (*YIL001W, YIL002C*), the native chromosome 9 centromere (*CEN9*), a yeast selectable marker (*LEU2*), plus ~10kb of BAC vector sequence including the chloramphenicol resistance gene (FIG. 3A). *synIXR*, which encodes seven

essential genes, was previously shown to be mitotically stable *in vivo* and power yeast cell growth at wild type levels in the absence of the wild type chromosome arm *IXR*(*8*). Two 'gene-free' regions of *synIXR* BAC were chosen as initial integration sites for the telomerator cassette, which was then introduced by homologous recombination to generate two new strains for linearization. Additionally, the *URA3exon1-intronACT1-URA3exon2* cassette (*12*), lacking the TeSS-I-SceI-TeSS sequence, was integrated at the same two positions to generate equivalent, but non-linearizeable control strains. All four strains were transformed with a plasmid expressing *pGAL1-I-SceI*, grown in galactose medium for 24 hours, and finally ~200 cells were plated on YPD, permissive yeast medium containing dextrose. These plates were replica printed onto medium containing Foa and uracil, or on medium lacking uracil (SC-Ura) or leucine (SC-Leu) (FIG. 3B). At either integration site 1 or 2, in cells expressing the functional telomerator cassette (+ I-SceI), ~50% of cells became resistant to growth on Foa and correspondingly lost ability to grow on SC-Ura, consistent with successful linearization (FIG. 3B, top row). The remaining half of cells that were sensitive to Foa was able to grow on SC-Ura, suggesting the *synIXR* BAC had not been linearized. When comparing cells harboring circular or linear *synIXR* molecules, there were no apparent changes to colony size or shape, suggesting the linearized construct was mitotically stable. Importantly, in cells lacking the I-SceI recognition site (- I-SceI), the Foa^R phenotype was not observed, demonstrating that linearization depends on this sequence (FIG. 3B, bottom row). Taken together, this work suggests that the telomerator efficient linearizes circular DNA molecules *in vivo* upon induction.

Example 4 Circular Permutation of a Synthetic Eukaryotic Chromosome. The telomerator enables fine control over the location at which a circular DNA molecule is linearized and thus represents a tool to probe the function of gene order, orientation, and chromosomal structure *in vivo*. For instance, if integrated between two genes, telomerator-driven linearization will yield novel telomere-promixal gene positioning that could lead to subtelomeric silencing. Furthermore, depending on the orientation of each gene with respect to the telomerator cassette, linearization could disrupt either 5' or 3' regulatory elements that drive their transcription. To test these ideas, we integrated the telomerator cassette three base pairs downstream of all 54 chromosome IX genes on *synIXR* BAC, generating an array of 54 permutable strains. We chose this position as it was previously shown to tolerate the insertion of synthetic sequences (*8*); indeed *synIXR* BAC encodes a 34 base pair site-specific recombination site (loxPsym) at this position downstream of every non-essential gene on the BAC with no effect on cell fitness (FIG. 3A). For the telomerator cassette integration, at

each of the 47 non-essential genes we "overwrote" the loxPsym site, and for the 7 essential genes that had no pre-exiting loxPsym site we inserted the cassette cleanly 3 base pairs downstream of the stop codon (FIG. 4A). Integration at the correct locus was confirmed for all 54 permutable strains by PCR using a gene-specific primer in combination with a primer that annealed within the telomerator (FIG. 6). We found that at all 54 loci integration of the telomerator caused no noticeable change in fitness (FIG. 4B and data not shown). Notably, the promoter region of the telomerator-encoded *URA3* contains a terminator sequence that would polyadenylate incoming transcripts; thus integration of the telomerator 3' to any gene, while disrupting native 3' end forming sequences, provides a built-in, non-native terminator sequence for the resulting transcript. Next, the *pGAL-I-SceI* construct was transformed into the 54 strains, which were grown in galactose medium for 24 hours to induce linearization. Foa resistant cells were selected, enabling isolation of the permuted array of linearized *synIXR*-containing strains. To investigate whether the *synIXR* BACs were linearized, full-length chromosomes from six permuted strains, pre- and post-linearization were chosen and separated by pulse-field gel electrophoresis. Only telomerator-integrated strains subjected to growth in galactose exhibited a band on the gel corresponding to the linearized form of *synIXR* (FIG. 4C). Of the 54 sites into which the telomerator had been integrated, 53 could be linearized and give rise to viable FoaR colonies. At just one position, *YIR013C*, linearization of *synIXR* was lethal (FIG. 4B). *YIR013C* encodes a non-essential, GATA family zinc finger motif containing protein with no annotated function. While not lethal, many other permuted chromosomes resulted in modest growth defects at 30°C (FIG. 4B). At 37°C, almost half of all permuted strains grew very slowly. Together, the data indicate that telomerator-induced linearization provides a new way to generate novel phenotypes.

Example 5: Telomeric Silencing Mediates Fitness Defects of Linearized synIXR Permutations. We hypothesized that telomeric silencing could underlie the fitness defects observed in many of the linear permutations of *synIXR* (FIG. 3B). Telomeric silencing, or the transcriptional repression of genes in proximity to telomeres, is an epigenetic phenomenon relying on the establishment of heterochromatin that spreads from the ends of chromosomes inwards. Linearization-dependent re-location of essential genes into sub-telomeric regions and the subsequent reduction in expression could have a major impact on impact cell fitness and/or survival. Indeed, mapping the position of the seven essential genes onto the array of linearized permutations of *synIXR* suggests that fitness defects tend to occur in strains in which one or more essential genes are re-located to within 10kb of a telomere (FIG. 7). Consequently, they might be subject to silencing, a process mediated in part by the lysine

deacetylase enzyme Sir2 (*18*), which deacetylates histone H4 (*19*). To test our hypothesis, we deleted *SIR2* in each of the 54 linearizable *synIXR* strains. Following transformation of the *pGAL1-I-SceI* construct and growth in galactose, cells harboring linearized *synIXR* molecules were selected on medium containing Foa. We discovered that disruption of telomeric silencing by *SIR2* deletion rescued nearly all growth defects associated linear permutations of *synIXR* (FIG. 5). Notably, this included the location at which we previously could not detect linearization, *YIR013C* (FIG. 3B). Importantly, these results are consistent with the conclusion that the telomerator-encoded 40bp telomere seed sequences indeed generate functional telomeres complete with the appropriate establishment of heterochromatin. Moreover, the telomerator clearly provides a new means to probe epigenetic regulation and chromatin architecture as we show that proper gene expression can depend on the chromosomal location/environment in which a gene is positioned.

Example 6: Another Telomerator Sequence. Another approach to introduce the Telomerator sequence into a circular DNA molecule for linearization is to include the TeSS-I-SceI-TeSS sequence as part of the protein coding sequence of the selectable marker. In such embodiments, the TeSS-I-SceI-TeSS sequence is "in-frame" with the selectable marker sequence (i.e., a multiple of 3) such that the resulting protein translation would not be truncated. The TeSS-I-SceI-Tess sequence is inserted into the selectable marker in a position that would not disrupt function of the resulting protein product.

SEQ ID NO:9 shows the TeSS-ISceI-TeSS sequence encoded within the native URA3 coding sequence:

atgTGTGTGGTGTGTGGGTGTGTGTGGGTGTGTGGGTGTGTGGGTAGGGATAACAG
GGTAATCACCCACCACACACACCCACACACACCACACACCCACCCAtcgaaagctacata
taaggaacgtgctgctactcatcctagtcctgttgctgccaagctatttaatatcatgcacgaaaagcaaacaaacttgtgtgcttcattgg
atgttcgtaccaccaaggaattactggagttagttgaagcattaggtcccaaaatttgtttactaaaaacacatgtggatatcttgactgattt
ttccatggagggcacagttaagccgctaaaggcattatccgccaagtacaatttttactcttcgaagacagaaaatttgctgacattggta
atacagtcaaattgcagtactctgcgggtgtatacagaatagcagaatgggcagacattacgaatgcacacggtgtggtgggcccagg
tattgttagcggtttgaagcaggcggcagaagaagtaacaaaggaacctagaggccttttgatgttagcagaattgtcatgcaagggct
ccctatctactggagaatatactaagggtactgttgacattgcgaagagcgacaaagattttgttatcggctttattgctcaaagagacatg
ggtggaagagatgaaggttacgattggttgattatgacacccggtgtgggtttagatgacaagggagaTgcattgggtcaacagtata
gaaccgtggatgatgtggtGtctacaggatctgacattattattgttggaagaggactatttgcaaagggaagggatgctaaggtagag
ggtgaacgttacagaaaagcaggctgggaagcatatttgagaagatgcggccagcaaaactaa (SEQ ID NO:9)

The TeSS-ISceI-TeSS sequence, which is 99 bp and thus should be in-frame, is inserted just past the start codon (ATG) of URA3. The lower case letters are native URA3 coding sequence and the upper case letters correspond to the TeSS-ISceI-TeSS

A construct encoding SEQ ID NO:9 was built and transformed into yeast. Plasmids were recovered from colonies that grew on SC-Ura plates and sequenced. Clone 1 has the following sequence:

atgTGTGTGGTGTGTGGGTGTGTGTGGGTGTGTGGGTGTGTGTGGGTGTGTGGGTG
TGTGGGTAGGGATAACAGGGTAATCACCCACCACACACACCCACACACACCACA
CACCCACCCAtcgaaagctacatataaggaacgtgctgctactcatcctagtcctgttgctgccaagctatttaatatcatgcac
gaaaagcaaacaaacttgtgtgcttcattggatgttcgtaccaccaaggaattactggagttagttgaagcattaggtcccaaaatttgttt
actaaaaacacatgtggatatcttgactgatttttccatggagggcacagttaagccgctaaaggcattatccgccaagtacaatttttttact
cttcgaagacagaaaatttgctgacattggtaatacagtcaaattgcagtactctgcgggtgtatacagaatagcagaatgngcagacat
tacgaatgcacacggtgtggtgggcccaggtattgttagcggtttgaagcaggcggcagaagaagtaacaaaggaacctagaggcc
ttttgatgttagcagaattgtcatgcaagggctccctatctactggagaatatactaagggtactgttgacattgcgaagagcgacaaaga
ttttgttatcggctttattgctcaaagagacatgggtggaagagatgaaggttacgattggttgattatgacacccggtgtgggtttagatg
acaagggagacgcattgggtcaacagtatagaaccgtggatgatgtggtctctacaggatctgacattattattgttggaagaggactat
ttgcaaagggaagggatgctaaggtagagggtgaacgttacagaaaagcaggctgggaagcatatttgagaagatgcggccagcaa
aactaa (SEQ ID NO:10)

Clone 2 has the following sequence:

atgTGTGTGGTGTGTGGGTGTGTGTGGGTGTGTGGGTGTGTGGGTAGGGATAACAG
GGTAATCACCCACCCAtcgaaagctacatataaggaacgtgctgctactcatcctagtcctgttgctgccaagctatttaat
atcatgcacgaaaagcaaacaaacttgtgtgcttcattggatgttcgtaccaccaaggaattactggagttagttgaagcattaggtccca
aaatttgtttactaaaaacacatgtggatatcttgactgatttttccatggagggcacagttaagccgctaaaggcattatccgccaagtac
aatttttttactcttcgaagacagaaaatttgctgacattggtaatacagtcaaattgcagtactctgcgggtgtatacagaatagcagaatg
ggcagacattacgaatgcacacggtgtggtgggcccaggtattgttagcggtttgaagcaggcggcagaagaagtaacaaaggaac
ctagaggccttttgatgttagcagaattgtcatgcaagggctccctatctactggagaatatactaagggtactgttgacattgcgaagag
cgacaaagattttgttatcggctttattgctcaaagagacatgggtggaagagatgaaggttacgattggttgattatgacacccggtgtg
ggtttagatgacaagggagacgcattgggtcaacagtatagaaccgtggatgatgtggtctctacaggatctgacattattattgttggaa
gaggactatttgcaaagggaagggatgctaaggtagagggtgaacgttacagaaaagcaggctgggaagcatatttgagaagatgc
ggccagcaaaactaa (SEQ ID NO:11)

Clone 3 has the following sequence:

atgTGTGTGGTGTGTGGGTGTGTGTGGGTGTGTGGGTGTGTGGGTAGGGATAACAG
GGTAATCACCCACCACACACACCCACACACACCACACACCCACCCAtcgaaagctacata
taaggaacgtgctgctactcatcctagtcctgttgctgccaagctatttaatatcatgcacgaaaagcaaacaaacttgtgtgcttcattgg
atgttcgtaccaccaaggaattactggagttagttgaagcattaggtcccaaaatttgtttactaaaaacacatgtggatatcttgactgattt
ttccatggagggcacagttaagccgctaaaggcattatccgccaagtacaatttttactcttcgaagacagaaaatttgctgacattggta
atacagtcaaattgcagtactctgcgggtgtatacagaatagcagaatgggcagacattacgaatgcacacggtgtggtgggcccagg
tattgttagcggtttgaagcangcggcagaagaagtaacaaaggaacctagaggccttttgatgttagcagaattgtcatgcaagggct
ccctatctactggagaatatactaagggtactgttgacattgcgaagagcgacaaagattttgttatcggctttattgctcaaagagacatg
ggtggaagagatgaaggttacgattggttgattatgacacccggtgtgggtttagatgacaagggagacgcattgggtcaacagtata
gaaccgtggatgatgtggtctctacaggatctgacattattattgttggaagaggactatttgcaaagggaagggatgctaaggtagag
ggtgaacgttacagaaaagcaggctgggaagcatatttgagaagatgcggccagcaaaactaa (SEQ ID NO:12)

Clone 4 has the following sequence:

atgTGTGTGGTGTGTGGGTGTGTGTGGGTGTGTGGGTGTGTGGGTAGGGATAACAG
GGTAATCACCCACCCAtcgaaagctacatataaggaacgtgctgctactcatcctagtcctgttgctgccaagctatttaat
atcatgcacgaaaagcaaacaaacttgtgtgcttcattggatgttcgtaccaccaaggaattactggagttagttgaagcattaggtccca
aaatttgtttactaaaaacacatgtggatatcttgactgatttttccatggagggcacagttaagccgctaaaggcattatccgccaagtac
aatttttactcttcgaagacagaaaatttgctgacattggtaatacagtcaaattgcagtactctgcgggtgtatacagaatagcagaatg
ggcagacattacgaatgcacacggtgtggtgggcccaggtattgttagcggtttgaagcaggcggcagaagaagtaacaaaggaac
ctagaggccttttgatgttagcagaattgtcatgcaagggctccctatctactggngaatatactaagggtactgttgacattgcgaagag
cgacaaagattttgttatcggctttattgctcaaagagacatgggtggaagagatgaaggttacgattggttgattatgacacccggtgtg
ggtttagatgacaagggagacgcattgggtcaacagtatagaaccgtggatgatgtggtctctacaggatctgacattattattgttggaa
gaggactatttgcaaagggaagggatgctaaggtagagggtgaacgttacagaaaagcaggctgggaagcatatttgagaagatgc
ggccagcaaaactaa (SEQ ID NO:13)

The TeSS repeats are unstable in the absence of selection (i.e., growth on SC-Ura), likely due to slippage during DNA replication. Clone 3 matches exactly the designed sequence, suggesting this design can indeed complement growth. Two out of four clones have a truncated CA repeat, which likely increases the stability. Other embodiments of the telomerator can incorporate this design to generate a more stable construct.

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We claim:

1. A telomerator cassette comprising a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises an intron comprising an endonuclease recognition site flanked by telomere seed sequences.

2. The telomerator cassette of claim 1, wherein the intron is the ACT1 intron.

3. The telomerator cassette of claim 1, wherein the selectable marker is an auxotrophic marker.

4. The telomerator cassette of claim 3, wherein the auxotrophic marker is the URA3 gene.

5. The telomerator cassette of claim 1, wherein the endonuclease recognition site is specific for I-SceI.

6. The telomerator cassette of claim 1, wherein the I-SceI endonuclease recognition site comprises SEQ ID NO:2.

7. The telomerator cassette of claim 1, wherein the telomere seed sequences comprise SEQ ID NO:1 and SEQ ID NO:3.

8. A circular chromosome comprising the telomerator cassette of claim 1.

9. A eukaryotic host cell comprising the circular chromosome of claim 8.

10. The eukaryotic host cell of claim 9, wherein the eukaryote is yeast.

11. The eukaryotic host cell of claim 10, wherein the yeast is *S. cerevisiae.*

12. The eukaryotic host cell of claim 9, wherein the host cell comprises a vector encoding the endonuclease under the control of an inducible promoter.

13. A telomerator cassette comprising a nucleic acid that encodes the auxotrophic marker URA3, wherein the nucleic acid comprises an intron that comprises the I-SceI endonuclease recognition site flanked by telomere seed sequences.

14. The telomerator cassette of claim 13, wherein the telomere seed sequences comprise SEQ ID NO:1 and SEQ ID NO:3.

15. The telomerator cassette of claim 13, wherein the I-SceI endonuclease recognition site comprises SEQ ID NO:2.

16. A circular chromosome comprising the telomerator cassette of claim 13.

17. A yeast host cell comprising the circular chromosome of claim 16.

18. The yeast host cell of claim 17, wherein the yeast host cell comprises a vector encoding the I-SceI endonuclease under the control of an inducible promoter.

19. The yeast host cell of claim 18, wherein the inducible promoter is the GAL1 promoter.

20. A yeast host cell comprising:

a. a circular chromosome comprising a telomerator cassette encoding a selectable marker, wherein the nucleic acid encoding the selectable marker comprises an intron that comprises an endonuclease recognition site flanked by telomere seed sequences; and

b. a vector encoding the endonuclease under the control of an inducible promoter.

21. The yeast host cell of claim 20, wherein the selectable marker is an auxotrophic marker.

22. The yeast host cell of claim 21, wherein the auxotrophic marker is the URA3 gene.

23. The yeast host cell of claim 20, wherein the endonuclease recognition site is specific for I-SceI.

24. The yeast host cell of claim 20, wherein the I-SceI endonuclease recognition site comprises SEQ ID NO:2.

25. The yeast host cell of claim 20, wherein the telomere seed sequences comprise SEQ ID NO:1 and SEQ ID NO:3.

26. A method for engineering a circular chromosome capable of being inducibly linearized comprising the step of integrating a telomerator cassette into a circular chromosome, wherein the telomerator cassette comprises a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises an intron comprising an endonuclease recognition site flanked by telomere seed sequences.

27. The method of claim 26, wherein the integrating step is accomplished by transforming a telomerator cassette into a host cell comprising the circular chromosome.

28. The method of claim 27, wherein the host cell comprises a vector encoding the endonuclease under the control of an inducible promoter.

29. A telomerator cassette comprising a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises an endonuclease recognition site flanked by telomere seed sequences.

30. A telomerator cassette comprising a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding the selectable marker comprises an endonuclease recognition site flanked by telomere seed sequences such that the selectable marker retains its function.

31. A telomerator cassette comprising a nucleic acid encoding a selectable marker, wherein the nucleic acid encoding a selectable marker comprises the original marker sequence operably linked as a protein fusion with an endonuclease recognition site flanked by telomere seed sequences.

32. The telomerator cassette of any one of claims 29-31, wherein the selectable marker is an auxotrophic marker.

33. The telomerator cassette of claim 32, wherein the auxotrophic marker is the URA3 gene.

34. The telomerator cassette of any one of claims 29-31, wherein the endonuclease recognition site is specific for I-SceI.

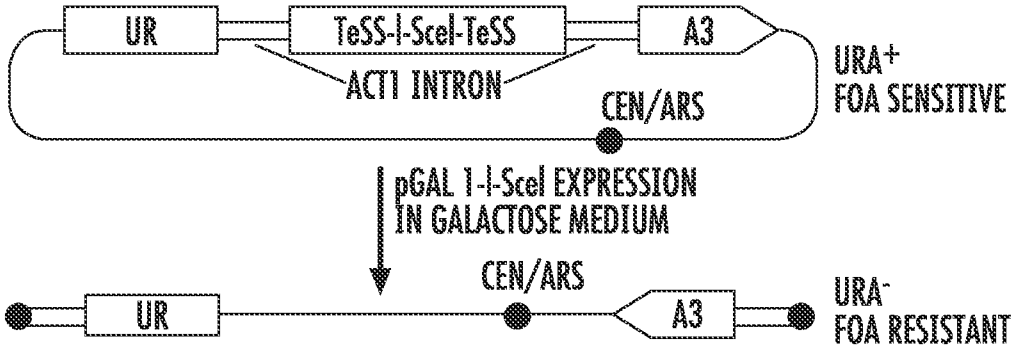
35. The telomerator cassette of claim 34, wherein the I-SceI endonuclease recognition site comprises SEQ ID NO:2.

36. The telomerator cassette of claim 29, wherein the telomere seed sequences comprise SEQ ID NO:1 and SEQ ID NO:3.

37. A circular chromosome comprising the telomerator cassette of any one of claims 29-36.

38. A eukaryotic host cell comprising the circular chromosome of claim 37.

A.



B.

THE ENTIRE SEQUENCE BELOW IS SEQ ID NO: 4.

5′ TGTGTGGTGTGTGGGTGTGTGTGGGTGTGTGGGTGTGTGGG		SEQ ID NO:1
TAGGGATACAGGGTAAT		SEQ ID NO:2
CACCCACCACACACACCCACACACACCACACACCCACCCA	3′	SEQ ID NO:3

FIG. 1

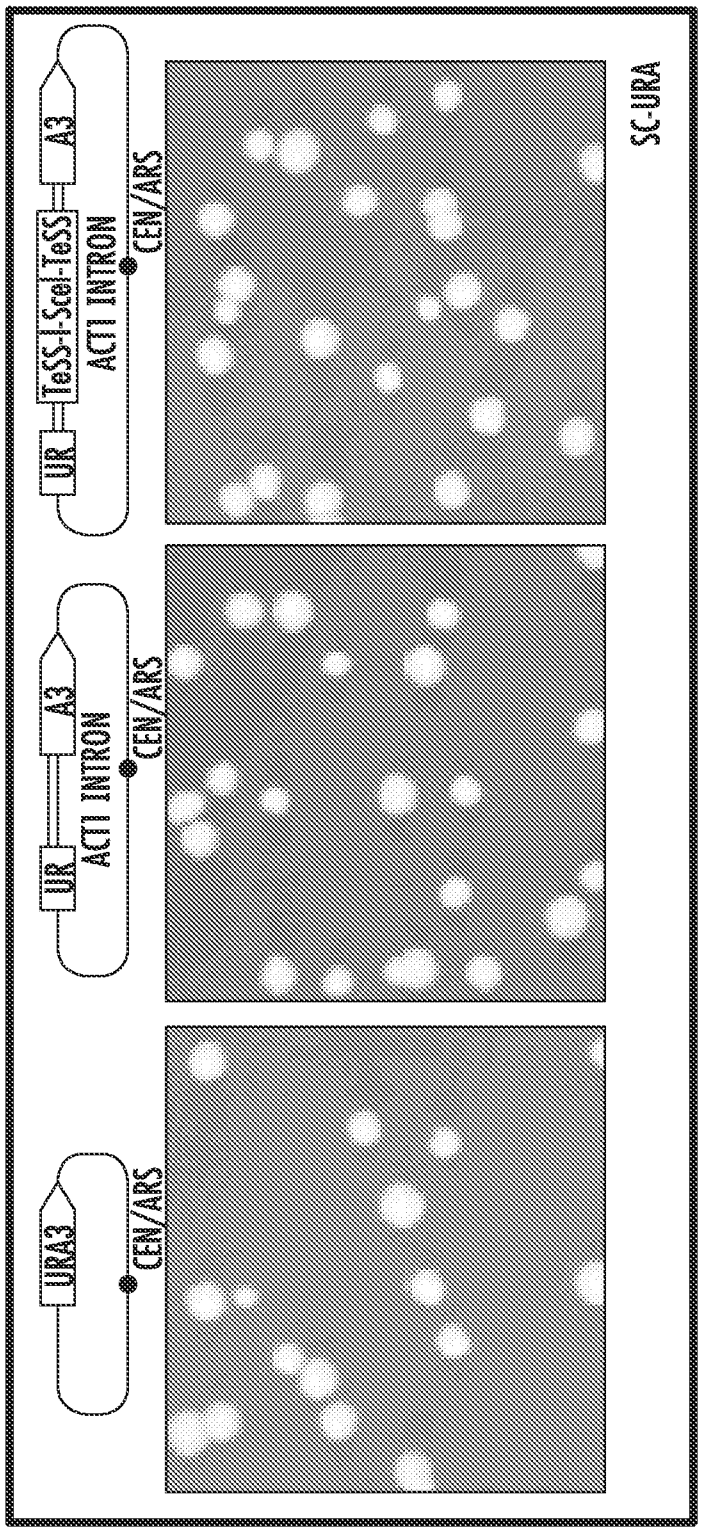

URA3
CEN/ARS
UR
ACT1 INTRON
A3
CEN/ARS
UR
TeSS-I-SceI-TeSS
ACT1 INTRON
A3
CEN/ARS
SC-URA

FIG. 2

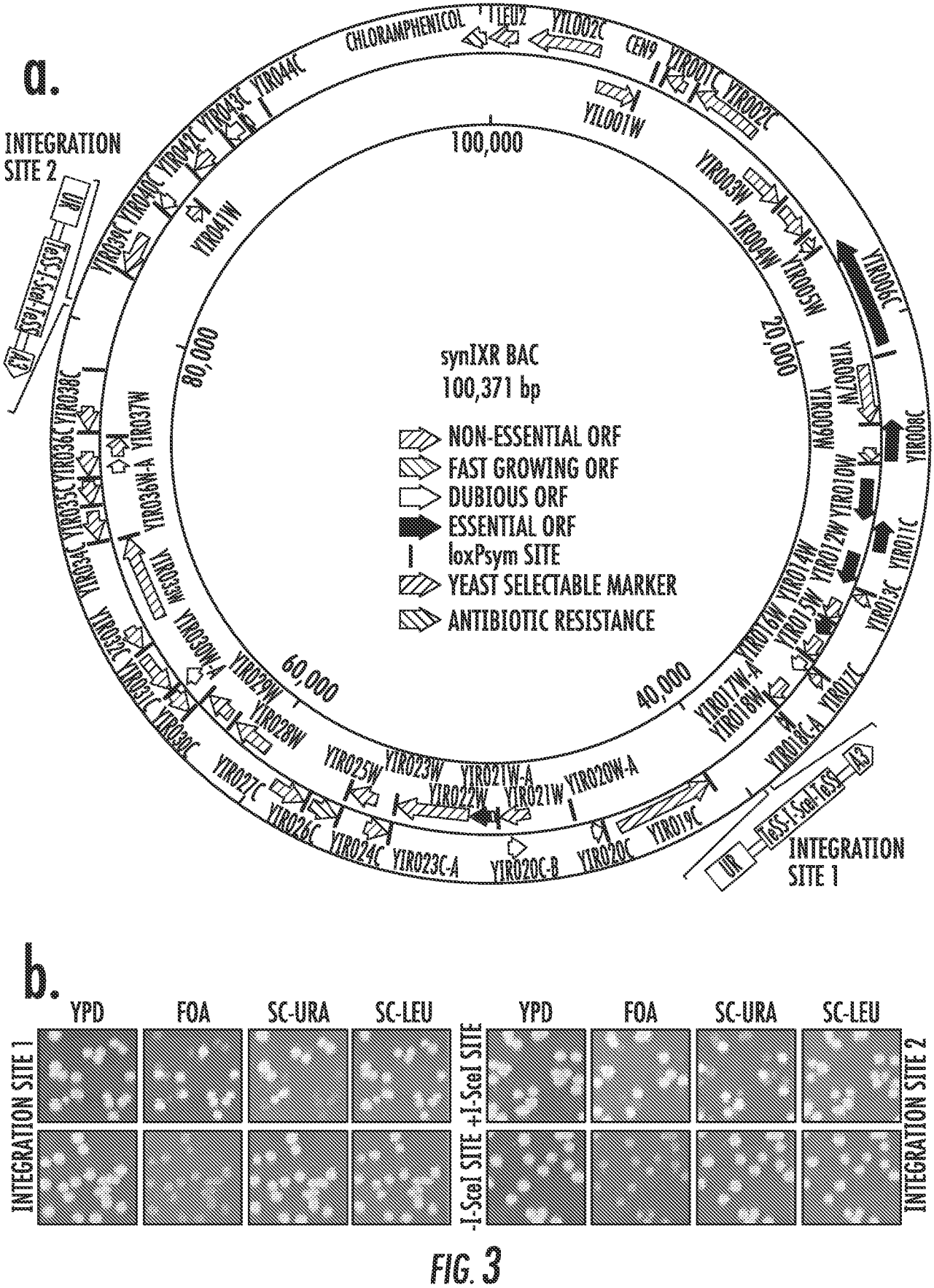

a.
INTEGRATION SITE 2
INTEGRATION SITE 1
synIXR BAC
100,371 bp
NON-ESSENTIAL ORF
FAST GROWING ORF
DUBIOUS ORF
ESSENTIAL ORF
loxPsym SITE
YEAST SELECTABLE MARKER
ANTIBIOTIC RESISTANCE
100,000
80,000
60,000
40,000
20,000
b.
YPD
FOA
SC-URA
SC-LEU
INTEGRATION SITE 1
INTEGRATION SITE 2
-I-SceI SITE +I-SceI SITE

FIG. 3

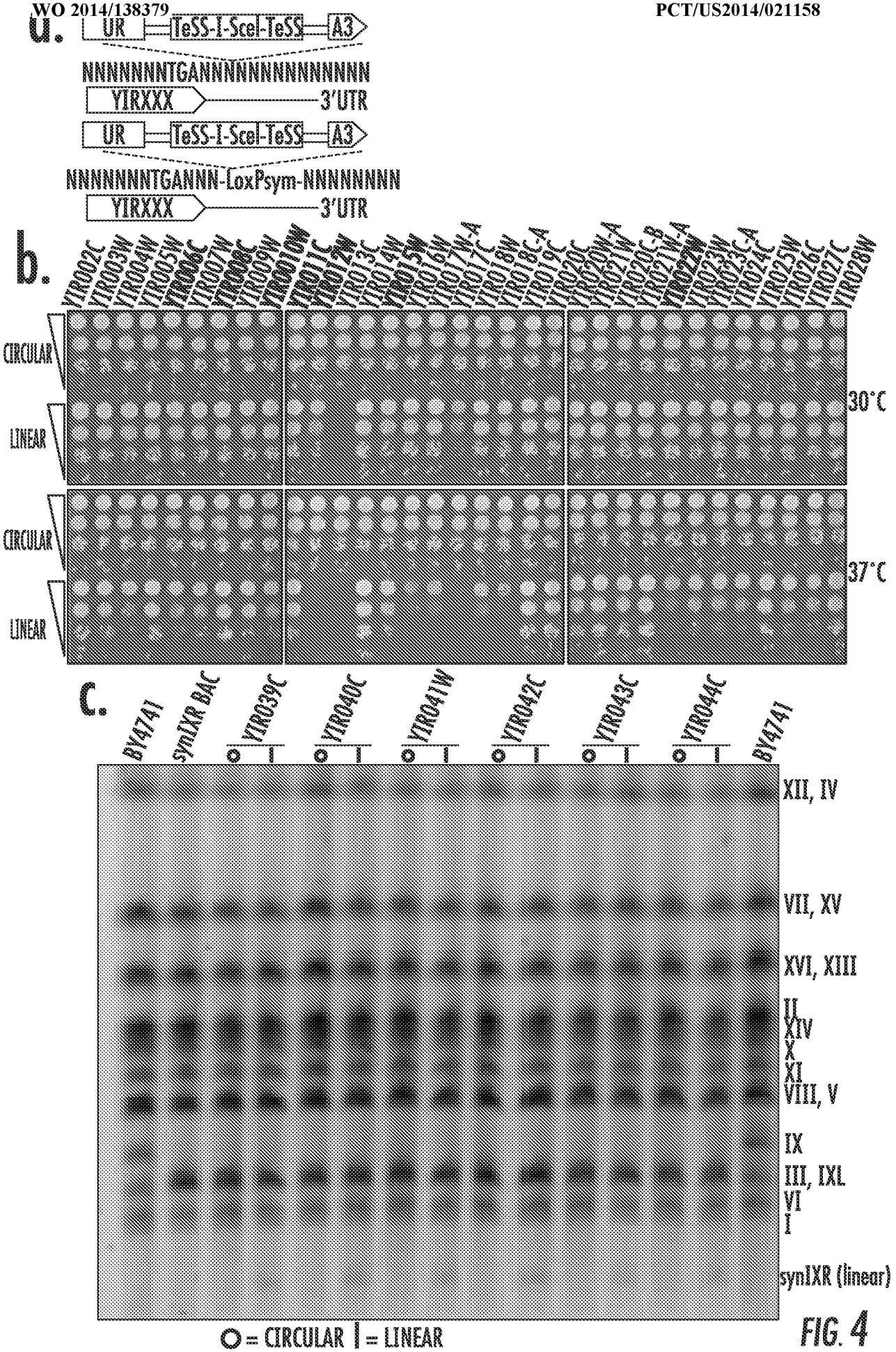

a.
UR
TeSS-I-SceI-TeSS
A3
NNNNNNNTGANNNNNNNNNNNNN
YIRXXX
3'UTR
UR
TeSS-I-SceI-TeSS
A3
NNNNNNNTGANNN-LoxPsym-NNNNNNNN
YIRXXX
3'UTR
b.
YIR002C
YIR003W
YIR004W
YIR005W
YIR006C
YIR007W
YIR008C
YIR009W
YIR010W
YIR011C
YIR012W
YIR013C
YIR014W
YIR015W
YIR016W
YIR017W-A
YIR017C
YIR018W
YIR018C-A
YIR019C
YIR020C
YIR020W-A
YIR021W
YIR020C-B
YIR021W-A
YIR022W
YIR023W
YIR023C-A
YIR024C
YIR025W
YIR026C
YIR027C
YIR028W
CIRCULAR
LINEAR
30°C
CIRCULAR
LINEAR
37°C
c.
BY4741
synIXR BAC
YIR039C
YIR040C
YIR041W
YIR042C
YIR043C
YIR044C
BY4741
XII, IV
VII, XV
XVI, XIII
II
XIV
X
XI
VIII, V
IX
III, IXL
VI
I
synIXR (linear)
○ = CIRCULAR | = LINEAR

FIG. 4

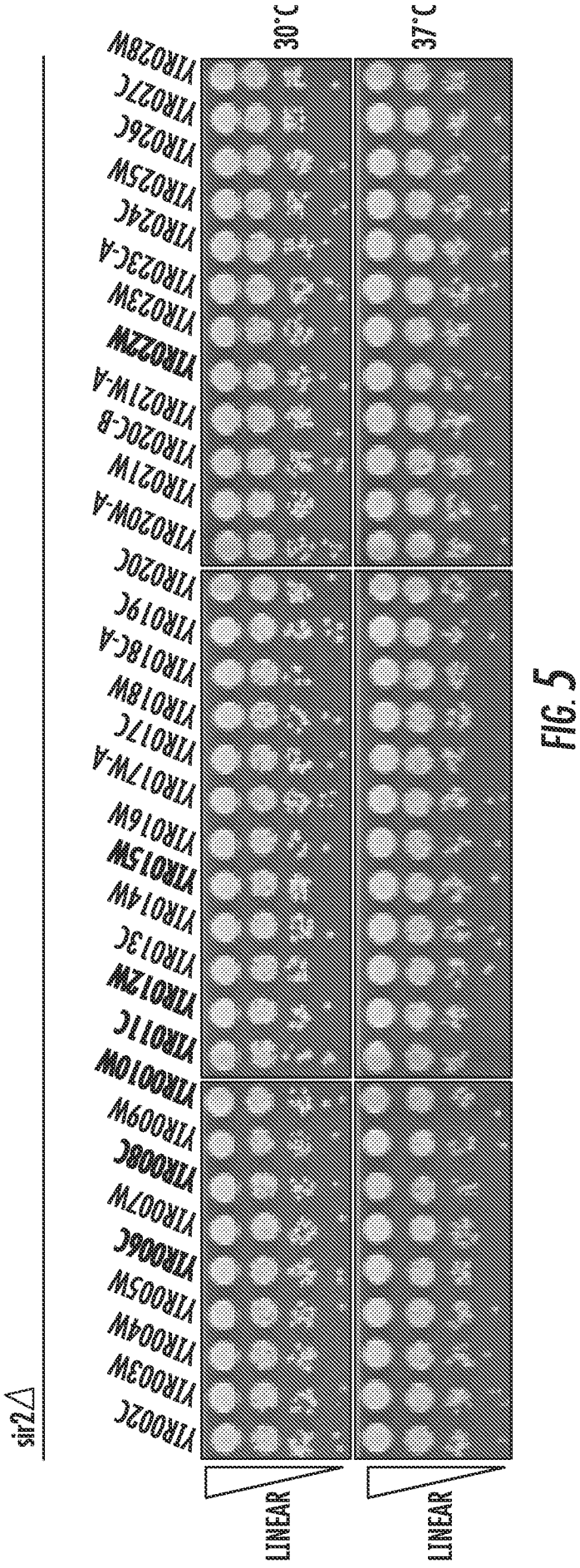

30°C
37°C
sir2Δ
YIR002C
YIR003W
YIR004W
YIR005W
YIR006C
YIR007W
YIR008C
YIR009W
YIR010W
YIR011C
YIR012W
YIR013C
YIR014W
YIR015W
YIR016W
YIR017W-A
YIR017C
YIR018W
YIR018C-A
YIR019C
YIR020C
YIR020W-A
YIR021W
YIR020C-B
YIR021W-A
YIR022W
YIR023W
YIR023C-A
YIR024C
YIR025W
YIR026C
YIR027C
YIR028W
LINEAR
LINEAR

FIG. 5

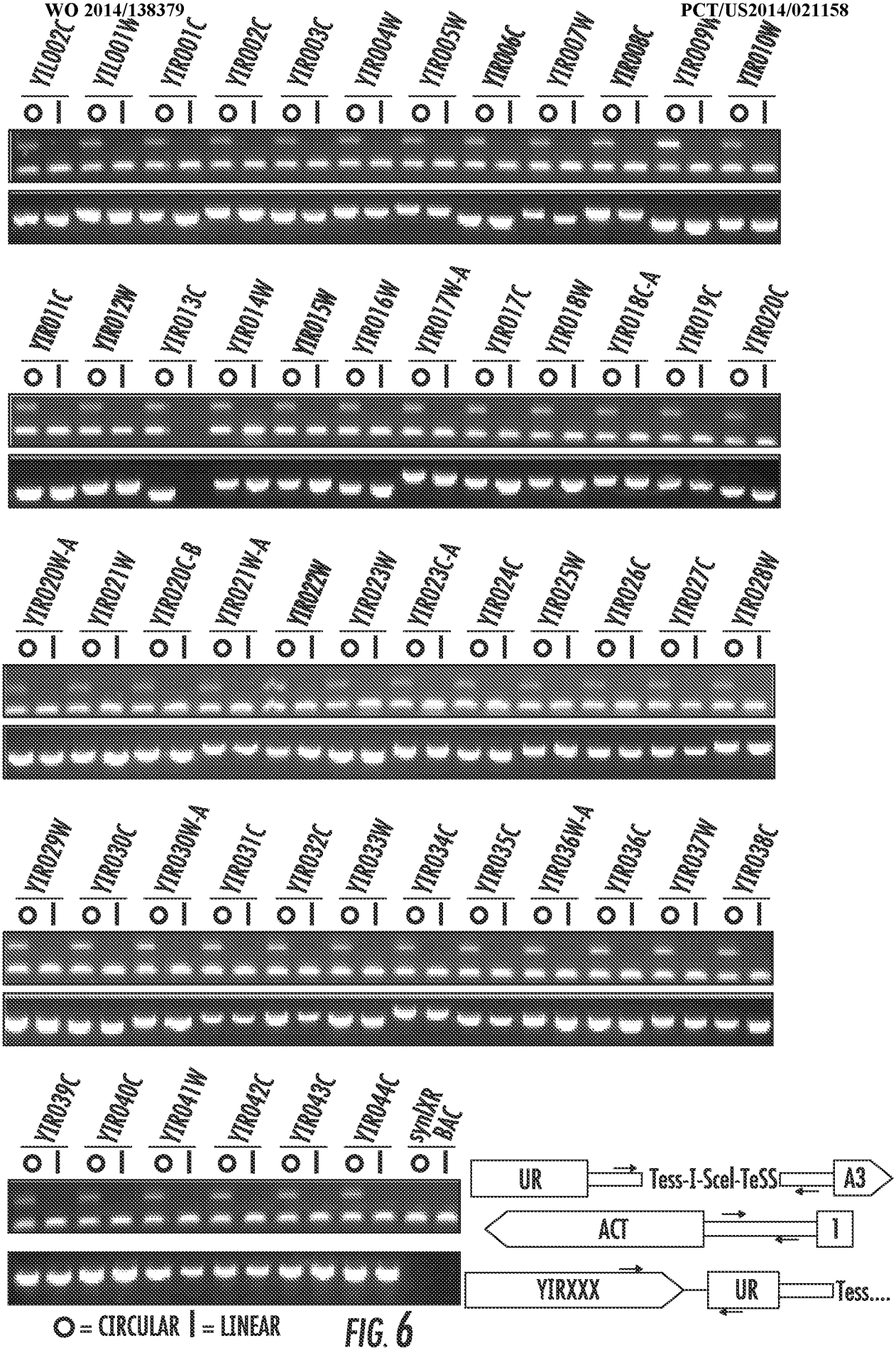

YIL002C
YIL001W
YIR001C
YIR002C
YIR003C
YIR004W
YIR005W
YIR006C
YIR007W
YIR008C
YIR009W
YIR010W
YIR011C
YIR012W
YIR013C
YIR014W
YIR015W
YIR016W
YIR017W-A
YIR017C
YIR018W
YIR018C-A
YIR019C
YIR020C
YIR020W-A
YIR021W
YIR020C-B
YIR021W-A
YIR022W
YIR023W
YIR023C-A
YIR024C
YIR025W
YIR026C
YIR027C
YIR028W
YIR029W
YIR030C
YIR030W-A
YIR031C
YIR032C
YIR033W
YIR034C
YIR035C
YIR036W-A
YIR036C
YIR037W
YIR038C
YIR039C
YIR040C
YIR041W
YIR042C
YIR043C
YIR044C
SymXR BAC
UR
Tess-I-SceI-TeSS
A3
ACT
1
YIRXXX
UR
Tess....
O = CIRCULAR | = LINEAR

FIG. 6

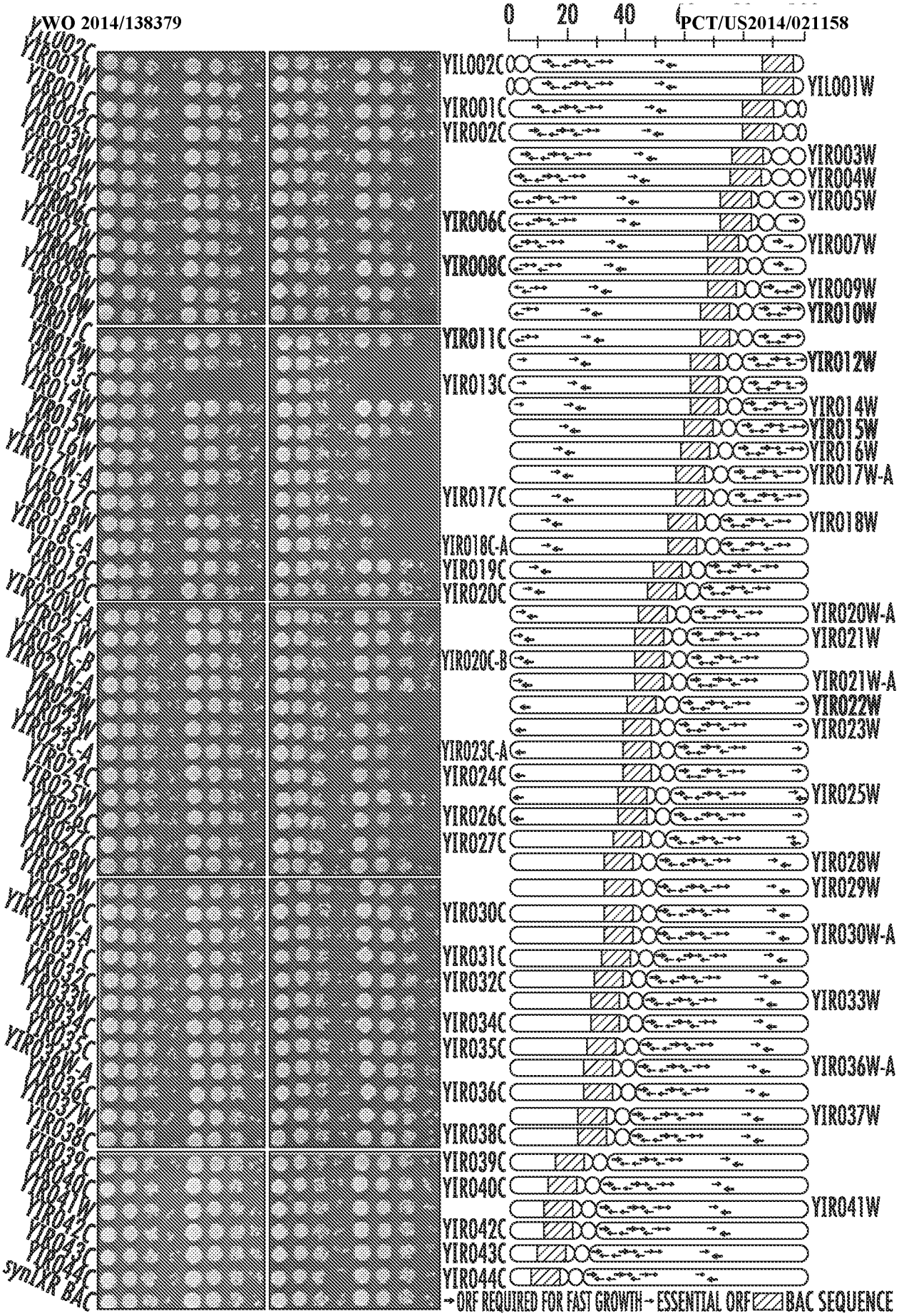

0
20
40
YIL002C
YIL001W
YIR001C
YIR002C
YIR003W
YIR004W
YIR005W
YIR006C
YIR007W
YIR008C
YIR009W
YIR010W
YIR011C
YIR012W
YIR013C
YIR014W
YIR015W
YIR016W
YIR017W-A
YIR017C
YIR018W
YIR018C-A
YIR019C
YIR020C
YIR020W-A
YIR021W
YIR020C-B
YIR021W-A
YIR022W
YIR023W
YIR023C-A
YIR024C
YIR025W
YIR026C
YIR027C
YIR028W
YIR029W
YIR030C
YIR030W-A
YIR031C
YIR032C
YIR033W
YIR034C
YIR035C
YIR036W-A
YIR036C
YIR037W
YIR038C
YIR039C
YIR040C
YIR041W
YIR042C
YIR043C
YIR044C
synLXR BAC
→ORF REQUIRED FOR FAST GROWTH ←ESSENTIAL ORF BAC SEQUENCE

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/021158

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/65(2006.01)i, C12N 15/81(2006.01)i, C12N 1/19(2006.01)i

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 15/65; C12N 15/03; C12N 15/02; C12N 15/87; C12N 15/81; C12N 1/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: telomerator cassette, selectable marker, intron, endonuclease recognition site, telomere seed sequences, ACT1, URA3, I-SceI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NOSEK, JOZEF et al., `On the origin of telomeres: a glimpse at the pre-telomerase world', Bioessays, 2006, Vol.28, pp.182-190 See abstract.	1-36
A	WOODS, JON P. et al., `Autonomous replication of foreign DNA in Histoplosma capsulatum; role of native telomeric sequences', Journal of Bacteriology, February 1993, Vol.175, No.3, pp.636-641 See abstract.	1-36
A	DOUVILLE, NICHOLAS et al., `DNA linearization through confinement in nanofluidic channels', Analytical and Bioanalytical Chemistry, 2008, Vol.391, pp.2395-2409 See abstract.	1-36
A	WO 00-06715 A1 (GENOTYPES INC.) 10 February 2000 See abstract, and claims 1-4.	1-36
A	US 2008-0160616 A1 (DUJON, BERNARD et al.) 3 July 2008 See abstract, and claims 23-28.	1-36

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"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
21 July 2014 (21.07.2014)	**22 July 2014 (22.07.2014)**

Name and mailing address of the ISA/KR
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Korean Intellectual Property Office
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HEO, Joo Hyung
Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/021158

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

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

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

2. ☒ Claims Nos.: 38
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 38 is unclear since it refers to claim which is not searchable due to not being drafted in accordance with the second and third sentence of Rule 6.4(a).

3. ☒ Claims Nos.: 37
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

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

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

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

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2014/021158

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 00-06715 A1	10/02/2000	AU 2000-17599 A	21/02/2000
		US 2005-0019924 A1	27/01/2005
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US 2008-0160616 A1	03/07/2008	AT 453711 T	15/01/2010
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		WO 96-14408 A3	29/08/1996