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Method for simply constructing two-component viral vector and related applications thereof

Abstract

A method for simply constructing a two-component virus vector and related applications. The two-component plant viral vector is optimized, so that a two-component virus genome is placed in a single plasmid. Due to the use of the single plasmid, the activation, resuspension and mixing processes of auxiliary bacteria are avoided, the workload of an *Agrobacterium* experimental stage is simplified by half, the potential cross-contamination risk in the bacteria mixing process is avoided, and the virus infectivity is improved, so that the use of the two-component viral vector is greatly simplified.

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Background/Summary

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

(1) The content of the electronic sequence listing (SEQUENCE-0213-0319PUS1. xml; Size: 33,468 bytes; and Date of Creation: Jun. 24, 2024) is herein incorporated by reference in its entirety.

TECHNICAL FIELD

(2) This application belongs to the technical field of bioengineering, and specifically relates to a method for simply constructing a two-component viral vector and related applications thereof.

BACKGROUND

(3) Plant viral vectors are a special type of plant expression vectors that are often used in virus-induced gene silencing (VIGS) experiments. Compared with ordinary plant expression vectors, the viral vectors have unique advantages in studying plant gene functions. The principle is as follows: according to characteristics that plants have RNAi immune response to viruses and the plant viruses may replicate in the plants and systematically infect the plants, endogenous gene fragments or full lengths can be expressed through plant viral vectors, thereby achieving virus-mediated gene silencing (VIGS), virus-mediated gene overexpression (VOX), virus-induced flowering (VIF), and virus-mediated gene editing (VIGE), etc.

(4) At present, relatively few viral vectors have been modified and used to study plant gene functions, mainly some RNA viruses and a few DNA viruses. Among them, a single-stranded linear RNA virus TRV has become the most widely used viral vector due to its wide host range. The TRV virus contains two genomic components, which are placed into two plasmids pTRV1 and pTRV2. When a TRV system is used, a target fragment needs to be constructed at the multiple cloning site of pTRV2, and then the two plasmids are mixed and delivered to plant cells. The TRV virus on the plasmid is transcribed by the 35S expression cassette, so the viral activity can be restored after entering plant cell transcription. The two-component circular DNA virus CLCrV is a widely used viral vector in cotton. Its vector system includes two genomes, pCLCrVA and pCLCrVB (hereinafter referred to as pVA and pVB). When a CLCrV system is used, the target fragment needs to be constructed at the multiple cloning site of VA, and then the two plasmids are mixed and delivered to the plant cells. CLCrV viral coding components on the plasmid are flanked by their respective CR regions (conservative regions containing splicing sites). When viral replicase is expressed, the linear CLCrV viral components will be spliced to re-form circular VA and VB genomes, so as to restore the viral activity. It is worth noting that the VA multiple cloning site contains a coding frame of viral proteins, so gene overexpression can be achieved by placing exogenous genes in the coding frame. In the past, the optimization of TRV viral vectors mainly focused on optimizing the multiple cloning site region of pTRV2 plasmid to improve the vector construction mode, while the optimization of CLCrV viral vectors mainly changed from gene gun to *Agrobacterium* infection. In addition, there are still optimizations on the conditions of *Agrobacterium* infection such as temperature and a formula of a resuspension. At present, there are no related reports in constructing the viral vectors with multiple genomic components into a single plasmid system.

(5) The current mainstream method of using plant viral vectors is to deliver them into plant cells across cell barriers through *Agrobacterium*. Therefore, the use of the plant viral vectors is mainly divided into three steps: plasmid construction, *Agrobacterium* transformation and infection. Since the above two types of viral vectors are two-component systems, the infection steps require additional activation, shaking, and resuspension of the auxiliary bacteria (carrying the plasmid pTRV1 or pCLCrVB) and a mixing step of main bacteria (carrying the plasmid pTRV2 or pCLCrVA). This process greatly increases the workload of the infection step and increases the risk of cross-contamination of a bacterial solution. When high-throughput experiments are required, the complexity of experimental operations and the risk of contamination increase significantly. On the other hand, there is currently little development of new viral vectors in the field of botany. Due to their host specificity and effectiveness, two-component viral vectors are often not easily replaced by new viral vectors in scientific research applications. Therefore, their simplified transformation has extremely high application value.

SUMMARY

(6) In view of this, the present disclosure provides a method for simply preparing a two-component plant viral vector and related applications thereof. In view of the replication characteristics of two-component DNA and RNA viruses, according to the present disclosure, the two-component virus genomes are concatenated and transformed into a single plasmid system, which greatly simplifies experimental operations and effectively avoids potential experimental contamination.

(7) In order to achieve the above objects, the present disclosure provides the following technical solutions.

(8) The present disclosure provides a pair of adapter sequences, the adapter sequences including sequences shown in SEQ ID NO.31 and SEQ ID NO.32. As a result, the VIGS vectors for multiple viruses of one gene through a single PCR product can be simply and rapidly constructed. In an actual operation, the present disclosure reserves the adapter sequences in advance to facilitate subsequent replacement of a target gene.

(9) The present disclosure also provides a method for simply constructing a two-component viral vector, including the following steps: modifying a viral vector skeleton and constructing plasmid series of a two-component virus; the modifying a viral vector skeleton includes the following steps: analyzing and obtaining an enzyme cutting site at a multiple cloning site, and modifying the enzyme cutting site at the multiple cloning site in the viral vector skeleton into a restriction endonuclease or exonuclease enzyme cutting site in a multiple cloning site region; a flanking sequence of the enzyme cutting site is modified into the above-mentioned adapter sequence; the constructing plasmid series of the two-component virus includes the following steps: concatenating the adapter sequences through PCR and homologous recombination to obtain a single plasmid of the two-component viral vector.

(10) The present disclosure also provides an application of the above-mentioned adapter sequences or the above-mentioned method of simplified construction in completing the construction of multiple viral vectors through one PCR product.

(11) The present disclosure provides two VIGS plasmids: pVS and pVS2. Usually, DNA virus and RNA viral vectors have different multiple cloning sites, and the construction of multiple viral vectors cannot be completed through one PCR product. In the present disclosure, the multiple cloning site adapter sequences as same as the pVS plasmid based on CLCrV virus are introduced into the pVS2 plasmid based on TRV virus, and thus can be used to construct gene silencing plasmids of the two viruses at the same time.

(12) The present disclosure provides a two-component DNA viral vector, and the method for preparing the two-component DNA viral vector is as shown above; when the two-component DNA viral vector is a circular DNA virus, the two components in the two-component DNA viral vector are flanked by respective CR regions, the CR regions may be linked by any enzyme cutting site sequence, and the other end of the CR region is linked to

a T-DNA border of the plasmid or other prokaryotic expression components; and the CR region comprises a conserved splicing site during viral replication.
(13) The present disclosure provides a two-component RNA viral vector, and the method for preparing the two-component RNA viral vector is as shown above; when the two-component RNA viral vector is a linear RNA virus, the two components of the two-component RNA viral vector are placed in two expression cassettes for independent expression; each of the expression cassettes is a 35S expression cassette, a promoter is 35S, and a terminator is NOS. The application preferably includes denovo development of vectors for multi-component viruses that have not been vectorized.
(14) In view of the replication characteristics of the two-component circular DNA virus, the two genomes of the virus only need to be placed into the T-DNA border in any direction and order; the two genomes of a two-component RNA virus need to be placed into two different eukaryotic gene expression cassettes for transcription. Preferably, the DNA virus is CLCrV and the RNA virus is TRV.
(15) The present disclosure provides an application of the above-mentioned two-component DNA viral vector and/or the above-mentioned two-component RNA viral vector in *Agrobacterium* infection.
(16) In the present disclosure, the *Agrobacterium* infection further includes downstream applications of viral vectors; and the downstream applications include VIGS, VOX, VIGE, and VIF.
(17) Based on the technical solution provided above, the present disclosure provides a method for concatenating multiple viral genome modules into a single plasmid, which can more easily use viral vectors to perform complex genetic manipulation. The usual method for combining viral vectors for gene manipulation is to inject a mixture of *Agrobacterium* containing different gene-manipulating viral plasmids. The present disclosure provides a more modular way to achieve complex genetic manipulation by concatenating multiple viral genomes in the single plasmid to achieve specific application purposes.
(18) The beneficial effects of the present disclosure are as follows: when the genomes of the two-component virus are concatenated and modified into a single plasmid system, it cannot only simplify experimental operations and avoid experimental contamination, but also improve the infectivity of the virus and increase the success rate of the experiment. Single-plasmid viral vectors containing two components or multiple components are also suitable for high-throughput experiments, such as VIGS mutant libraries and VIF-based large-scale material rapid seed reproduction and the like. A flow chart for the use of simplified DNA and RNA viral vectors is shown in FIG. **1**.

Description

BRIEF DESCRIPTION OF DRAWINGS
(1) FIG. **1** is a flow chart for use of simplified DNA and RNA viral vectors.
(2) FIG. **2** shows an albino phenotype of a plant with PDS gene silencing after successfully silencing a cotton PDS gene by using a VS1 system;
(3) FIG. **3** shows an albino phenotype of a plant with PDS gene silencing after successfully silencing a cotton CLA gene by using a VS2 system;
(4) FIG. **4** shows simultaneously overexpression of an SFT gene and silencing of an SP gene by using the VS1 system concatenated by a plurality of VA genome;
(5) FIG. **5** shows a PCR identification results of a bacterial solution in Example 4.
DETAILED DESCRIPTION
(6) The present application will be further explained below in conjunction with Examples.
Example 1
(7) A method for simplifying a two-component DNA virus—CLCrV vector
(8) In this example, a simplifying process of the two-component DNA virus is illustrated by taking the two-component virus-CLCrV as an example. Whether the simplified plasmid works normally is determined through a VIGS phenotype result of a cotton PDS gene after *Agrobacterium* infection.
(9) A specific implementation process is briefly introduced below.
(10) (1) Modification of a Viral Vector Skeleton
(11) Through two rounds of plasmid point mutation experiments, a Bsa I enzyme cutting site on a pVA plasmid was mutated sequentially and a 35S expression cassette was deleted.
(12) Point mutation primers used are as follows:
(13) TABLE-US-00001 clcrv M-BSA F: 5′-GGAAAGACACCTTTTCGACCTTTTTCCCCT-3′, SEQ ID NO. 1; clcrv M-Bsa R: 5′-AAAAGTGTCTTTCCTGTGGATAGCACGTACAT-3′, SEQ ID NO. 2; clcrv-D-HygR F: 5′-TAATTCGGGGATAGCCCTTTGGTCTTCTGAGACTGT-3′, SEQ ID NO. 3; clcrv-D-HygR R: 5′-CAAAGGGCTATCCCGAATTAATTCGGCGTTAATTCA-3′, SEQ ID NO. 4.
(14) A PCR reaction system is shown in Table 1, and a PCR amplification program is shown in Table 2.
(15) TABLE-US-00002 TABLE 1 PCR reaction system Component Volume Phanta Max Super-Fidelity DNA Polymerase 1 μL 2 × Phanta Max Buffer 10 μL dNTP Mix 0.5 μL Upstream primer 0.8 μL Downstream primer 0.8 μL Template DNA 1 μL Supplementing double distilled water to 20 μL
(16) TABLE-US-00003 TABLE 2 PCR Amplification program Step Temperature Time Cycle number Predenaturation 95° C. 5 min 1 Denaturation 95° C. 15 sec 33 Annealing 58° C. 15 sec Extension 72° C. 8 min Extension 72° C. 5 min 1
(17) Further, after the amplified PCR product was purified, 1 μL of Dpn I was added and placed at 37° C. to digest the template plasmid for 2 hours.
(18) Further, a linear PCR product was recombined by using a ClonExpress® II One Step Cloning Kit.
(19) Further, a recombinant product was transformed and entered into *E. coli* strain DH5α. After overnight growth on a plate, monoclones were selected for colony PCR identification and positive transformants were screened.
(20) Further, whether the point mutation was successful was confirmed by a method of a Sanger sequencing. The plasmid that was finally sequenced was named pMDA.
(21) (2) Construction of Plasmid Series of the Two-Component Virus
(22) Through two rounds of vector construction, the plasmid series for constructing the two-component virus was obtained. First, a silencing fragment of a cotton PDS gene was constructed into a multiple cloning site of pMDA to obtain a pMDA-PDS plasmid, and then VB was constructed into the pMDA-PDS plasmid to obtain pMDAB-PDS (hereinafter referred to as pVS-PDS).
(23) The PCR amplification primers used are as follows:
(24) TABLE-US-00004 clcrv-PDS F: 5′-AACGCTAGCGAATTCACTAGTGCCTGAAGACTGGAG-3′, SEQ ID NO. 5; clcrv-PDS R: 5′-GGCATGCCTGCAGACTAGTGCTTTACTCTGATCC-3′, SEQ ID NO. 6; Clone VB F: 5′-AACCTATCCCAAGTGGAGCTCCGGGGGATCCACTAGTAAAC-3′, SEQ ID NO. 7; Clone VB R: 5′-CATGATTACGAATTCGAGCTCATTCGAGCTCCAGAACGATC-3′, SEQ ID NO. 8.
(25) The PCR reaction system is shown in Table 3, and the PCR amplification program is shown in Table 4.
(26) TABLE-US-00005 TABLE 3 PCR reaction system Component Volume Phanta Max Super-Fidelity DNA Polymerase 1 μL 2 × Phanta Max Buffer 10 μL dNTP Mix 0.5 μL Upstream primer 0.8 μL Downstream primer 0.8 μL Template DNA 1 μL Supplementing double distilled water to 20 μL
(27) TABLE-US-00006 TABLE 4 PCR amplification program Step Temperature Time Cycle number Predenaturation 95° C. 5 min 1 Denaturation 95° C. 15 sec 33 Annealing 58° C. 15 sec Extension 72° C. 2 min Extension 72° C. 5 min 1

(28) The amplified PCR products were recovered by using an agarose gel DNA recovery kit (enhanced type).
(29) The vectors used in the two rounds of experiments were linearized by using restriction enzymes SpeI and SacI.
(30) The enzyme cutting system is shown in Table 5.
(31) TABLE-US-00007 TABLE 5 Enzyme cutting system Component Volume 10 × Cut smart buffer 10 μL SpeI/Sac I 1 μL Vector 2000 ng Supplementing double 100 μL distilled water to
(32) The reaction system was placed in a 37° C. incubator for enzyme cutting overnight.
(33) Further, an ultra-thin DNA product purification kit was used to recover the linearized vector.
(34) Further, the PCR product and linearized vector were recombined by using a ClonExpress® II One Step Cloning Kit.
(35) Further, a recombinant product was transformed and entered into *E. coli* strain DH5α. After overnight growth on a plate, monoclones were selected for colony PCR identification and positive transformants were screened.
(36) Further, whether the PCR fragment was successfully constructed was confirmed by a method of a Sanger sequencing. The final plasmid is pVS-PDS.
(37) (3) *Agrobacterium* Infection
(38) Since the VA and VB genomes of the CLCrV virus were placed in a single plasmid, and the plasmid contained the silencing fragment of cotton PDS, whether the simplified method was successful could be determined by way of whether cotton cotyledons infected by the pVS-PDS *Agrobacterium* were whitened.
(39) Further, pVS-PDS was transformed and entered into *Agrobacterium* GV3101 through heat shock transformation.
(40) Further, after the plate grew on kana and rifampicin double-resistant plates for two days, monoclones were selected for colony PCR identification and positive transformants were screened.
(41) Further, positive monoclones were selected and shook overnight until the OD value was 0.5-1.0.
(42) Further, the bacteria solution was collected at the bottom of a centrifuge tube by centrifugalization at 6000 rpm for 10 minutes, a supernatant was discarded, and the mixture was adjusted with a resuspending solution till OD=0.5-1.0.
(43) A formula of the resuspending solution is shown in Table 6.
(44) TABLE-US-00008 TABLE 6 Formula of the resuspending solution Resuspending solution 100 mL $MgCl_2$ (1M) 1 mL MES(0.5M) 2 mL AS (100 mM) 200 μL
(45) Further, after standing for 3 hours in the dark, pores were punched to inject the resuspending solution on the back of the flat cotyledons of cotton at a cotyledonary stage.
(46) Further, after being kept in the dark for 12 h, the mixture was cultured in a 25-28° C. culture room.
(47) As shown in FIG. **2**, the silencing phenotype of pVS-PDS-injected plants can last until the later stages of cotton growth and development, and obvious albino phenotypes can be seen in leaves, stems, and bracts. The above results show that the simplification of the two-component DNA virus-CLCrV vector is successful.
Example 2
(48) Method for Simplifying Two-Component RNA Virus-TRV Vector
(49) In this example, a two-component virus-TRV was used as an example to illustrate the simplification process of the two-component RNA virus. Whether the simplified single plasmid can work normally is determined by the VIGS phenotype results of the CLA gene of new cotton leaves after *Agrobacterium* infection.
(50) A specific implementation process is briefly introduced below.
(51) (1) Modification of a Viral Vector Skeleton
(52) Since the viral component on the pTRV1 plasmid contained five dispersed Bsa I enzyme cutting sites, all Bsa I enzyme cutting sites on the pTRV1 plasmid were synonymously mutated through staged gene synthesis and vector construction by a gene synthesis company. Then, the resistance selection gene BlpR in the 35S expression cassette was deleted by BamHI enzyme cutting, and two concatenated Bsa Is were introduced by gene synthesis for subsequent concatenated pTRV2 plasmids. The gene fragments synthesized in stages were shown in SEQ ID NO. 9. SEQ ID NO. 10 and SEQ ID NO. 11. Synthetic fragments used for intermediate plasmid construction was shown in SEQ ID NO. 12. The final intermediate plasmid was named 2_3_2_1_VIGS pYL192 (TRV1).
(53) TABLE-US-00009 SEQ ID NO. 9: GTTATTGCTTTTAGATAGAGTTCCTGCTCTGCAAGAGGTGGATGACATCG
GTGGTCAATGGTCGTTTTGGGTAACTAGAGGTGAGAAAAGGATTCATTCC
TGTTGTCCAAATCTAGATATTCGGGATGATCAGAGAGAAATTTCTCGACA
GATATTTCTTACTGCTATTGGTGATCAAGCTAGAAGTGGTAAGAGACAGA
TGTCGGAGAATGAGCTGTGGATGTATGACCAATTTCGTGAAAATATTGCT
GCGCCTAACGCGGTTAGGTGCAATAATACATATCAGGGTTGTACATGTAG
GGGTTTTTCTGATGGTAAGAAGAAAGGCGCGCAGTATGCGATAGCTCTTC
ACAGCCTGTATGACTTCAAGTTGAAAGACTTGATGGCTACTATGGTTGAG
AAGAAAACTAAAGTGGTTCATGCTGCTATGCTTTTTGCTCCTGAAAGTAT
GTTAGTGGACGAAGGTCCATTACCTTCTGTTGACGGTTACTACATGAAGA
AGAACGGGAAGATCTATTTCGGTTTTGAGAAAGATCCTTCCTTTTCTTAC
ATTCATGACTGGGAAGAGTACAAGAAGTATCTACTGGGGAAGCCAGTGAG
TTACCAAGGGAATGTGTTCTACTTCGAACCGTGGCAGGTGAGAGGAGACA
CAATGCTTTTTTCGATCTACAGGATAGCTGGAGTTCCGAGGAGGTCGCTA
TCATCGCAAGAGTACTACCGAAGAATATATATCAGTAGATGGGAAAACAT
GGTTGTTGTCCCAATTTTCGATCTGGTCGAATCAACGCGAGAGTTGGTCA
AGAAAGACCTGTTTGTAGAAACAATTCATGGACAAGTGTTTGGATTACAT
AGCTAGGTTTATCTGACCAGCAGCTGACCATAAGCAATGTTAAATCATAC
TTGAGTTCAAATAATTGGGTCTTATTCATAAACGGGGCGGCCGTGAAGAA
CAAGCAAAGTGTAGATTCTCGAGATTTACAGTTGTTGGCTCAAACTTTGC
TAGTGAAGGAACAAGTGGCGCGACCTGTCATGAGGGAGTTGCGTGAAGCA
ATTCTGACTGAGACGAAACCTATCACGTCATTGACTGATGTGCTGGGTTT
AATATCAAGAAAACTGTGGAAGCAGTTTGCTAACAAGATCGCAGTCGGCG
GATTCGTTGGCATGGTTGGTACTCTAATTGGATTCTATCCAAAGAAGGTA
CTAACCTGGGCGAAGGACACACCAAATGGTCCAGAACTATGTTACGAGAA
CTCGCACAAAACCAAGGTGATAGTATTTCTGAGTGTTGTGTATGCCATTG
GAGGAATCACGCTTATGCGTCGAGACATCCGAGATGGACTGGTGAAAAAA
ACTATGTGATATGTTTGATATCAAACGGGGGGCCCATGTCTTAGACGTTG
AGAATCCGTGCCGCTATTATGAAATCAACGATTTCTTTAGCAGTCTGTAT TCGGCATCTGAGTCCCGGTGAGACG; SEQ ID NO. 10:
TGCCGCGCTTACGAAGGCGGCTTTGGCAAGATTTTTTGTTACTGAGACGG
TCTTATGACGGTTTCGGTCTAGGTTTGATGTCTTTAGACATCATGAAGGG CCTTGCG; SEQ ID NO. 11:

GCCGAAGTATTTTCACAGAAGAAGAGAAACTGTCCTAAATCATGTTGGTG
GGAAGAAGAGTGAACACAAGTTAGACGTTTTTGACCAAAGGGATTACAAA
ATGATTAAATCTTACGCGTTTCTAAAGATAGTAGGTGTACAATTG; SEQ ID NO. 12:
GGATCCCAGGAAACAGCTATGACCAATTCCCGATCTAGTAACATAGATGA
CACCGCGCGCGATAATTTATCCTAGTGAGACCGTAGGTCTCATTCTACTG
CGATCACTGACATACCCCAGCCAGGGCAACACCATAGGTGCAATGTTTTT
ATCCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGTCTTGCGA
AGGATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGAGATG.

(54) On the other hand, in order to use the same PCR product to construct TRV and CLCrV vectors at the same time, the adapter sequence on the pTRV2 plasmid was replaced to be exactly the same as CLCrV, and the same Bsa I enzyme cutting strategy as CLCrV was used at the multiple cloning site.

(55) The adapter and enzyme cutting site sequences were introduced into both ends of the CLA gene through overlap extension PCR amplification.

(56) The primers used in overlap extension PCR are as follows:

(57) TABLE-US-00010 V2 F1: 5′-CTTTGGAAGAAGACTTGTACACTTATTACAAATTCGAT-3′, SEQ ID NO. 13; V2 R1: 5′-TCCTTAAATCCCTAAAGCTTGGGATTAGGACGTATCGGACCTC-3′, SEQ ID NO. 14; V2 F2: 5′-AAGCTTAGGGATTTAAGGACGTGAACTCTGTTGA-3′, SEQ ID NO. 15; V2 R2: 5′-ATTCGCTAGCGTTAACTGGCCAATTCGGTAACCTTACTCACAGAATC TAA GTC-3′, SEQ ID NO. 16; V2 F3: 5′-GCCAGTTAACGCTAGCGAATCGAGACCGCCCTTTGTGCATCTTCATT TCC T-3′, SEQ ID NO. 17; V2 R3: 5′-GGGACATGCCCGGGCCTCGAATGGCATGCCTGCAGACTAGTTGAGAC CATTAACACCGTTGCGGCTAAGC-3′, SEQ ID NO. 18.

(58) The PCR reaction system is shown in Table 7, and the PCR amplification program is shown in Table 8.

(59) TABLE-US-00011 TABLE 7 PCR reaction system Component Volume Phanta Max Super-Fidelity DNA Polymerase 1 μL 2 × Phanta Max Buffer 10 μL dNTP Mix 0.5 μL Upstream primer 0.8 μL Downstream primer 0.8 μL Template DNA 1 μL Supplementing double distilled water to 20 μL

(60) TABLE-US-00012 TABLE 8 PCR amplification program Step Temperature Time Cycle number Predenaturation 95° C. 5 min 1 Denaturation 95° C. 15 sec 33 Annealing 58° C. 15 sec Extension 72° C. 1 min Extension 72° C. 5 min 1

(61) Further, the amplified overlap extension PCR product was purified.

(62) Further, the pTRV2 plasmid was linearized by BsrG I and Xho I.

(63) The enzyme cutting system is shown in Table 9.

(64) TABLE-US-00013 TABLE 9 Enzyme cutting system Component Volume 10 × Cut smart buffer 10 μL SpeI/Sac I 1 μL Vector 2000 ng Supplementing double 100 μL distilled water to

(65) The reaction system was placed in a 37° C. incubator for enzyme cutting overnight.

(66) Further, the PCR product and linearize the pTRV2 plasmid were recombined by using a ClonExpress® II One Step Cloning Kit.

(67) Further, a recombinant product was transformed and entered into *E. coli* strain EPI300. After overnight growth on a plate, monoclones were selected for colony PCR identification and positive transformants were screened.

(68) Further, whether the modification of pTRV2 was successful was confirmed by a method of a Sanger sequencing. The finally modified pTRV1 and pTRV2 plasmids were named 2_3_2_1_VIGS pYL192 (TRV1) and V2-CLA-Bsa I respectively.

(69) (2) Construction of Plasmid Series of Two-Component Virus

(70) The viral component on the V2-CLA-Bsa I plasmid was amplified by PCR.

(71) The PCR amplification primers used are as follows:

(72) TABLE-US-00014 Clone V2 F: 5′-TGTCAGTGATCGCAGTAGAATGTACTAATT-3′, SEQ ID NO. 19; Clone V2 R:5′-CGCGCGATAATTTATCCTAGTTTGCG-3′, SEQ ID NO. 20.

(73) The PCR reaction system is shown in Table 10, and the PCR amplification program is shown in Table 11.

(74) TABLE-US-00015 TABLE 10 PCR reaction system Component Volume Phanta Max Super-Fidelity DNA Polymerase 1 μL 2 × Phanta Max Buffer 10 μL dNTP Mix 0.5 μL Upstream primer 0.8 μL Downstream primer 0.8 μL Template DNA 1 μL Supplementing double distilled water to 20 μL

(75) TABLE-US-00016 TABLE 11 PCR amplification program Step Temperature Time Cycle number Predenaturation 95° C. 5 min 1 Denaturation 95° C. 15 sec 33 Annealing 58° C. 15 sec Extension 72° C. 2 min Extension 72° C. 5 min 1

(76) The amplified PCR products were recovered by using an agarose gel DNA recovery kit (enhanced type).

(77) An intermediate plasmid 2_3_2_1_VIGS pYL192 (TRV1) was linearized by using a restriction enzyme Bsa I.

(78) The enzyme cutting system is shown in Table 12.

(79) TABLE-US-00017 TABLE 12 Enzyme cutting system Component Volume 10 × Cut smart buffer 10 μL Bsa I 1 μL Vector 2000 ng Supplementing double 100 μL distilled water to

(80) The reaction system was placed in a 37° C. incubator for enzyme cutting overnight.

(81) Further, the linearized vector was recovered by an ultra-thin DNA product purification kit.

(82) Further, the PCR product and linearized vector were recombined by using a ClonExpress® II One Step Cloning Kit.

(83) Further, a recombinant product was transformed and entered into *E. coli* strain EPI300. After overnight growth on a plate, monoclones were selected for colony PCR identification and positive transformants were screened.

(84) Further, a Sanger sequencing method was performed by using bacterial liquid PCR products to confirm positive clones. Whether the concatenated plasmid was successfully constructed was finally confirmed by next-generation sequencing of the entire plasmid. The final plasmid was named pVS2-CLA.

(85) (3) *Agrobacterium* Infection

(86) Since the RNA1 and RNA2 genomes of the TRV virus were placed in a single plasmid, and the plasmid contained the silencing fragment of cotton CLA, whether the simplified method was successful could be determined by way of whether cotton cotyledons infected by the pVS2-CLA *Agrobacterium* were whitened.

(87) Further, pVS2-CLA was transformed and entered into *Agrobacterium* GV3101 through heat shock transformation.

(88) Further, after the plate grew on kana and rifampicin double-resistant plates for two days, monoclones were selected for colony PCR identification and positive transformants were screened.

(89) Further, positive monoclones were selected and shook overnight until the OD value was 0.5-1.0.

(90) Further, a bacteria solution was collected at the bottom of a centrifuge tube by centrifugalization at 6000 rpm for 10 minutes, a supernatant was discarded, and the mixture was adjusted with the resuspending solution till OD=0.1-0.3.

(91) A formula of the resuspending solution is shown in Table 13.

(92) TABLE-US-00018 TABLE 13 Formula of the resuspending solution Resuspending solution 100 mL MgCl2 (1M) 1 mL MES(0.5M) 2 mL AS (100 mM) 200 μL

(93) Further, after standing for 3 hours in the dark, pores were punched to inject the resuspending solution to the back of the flat cotyledon of cotton at a cotyledonary stage.

(94) Further, after being kept in the dark for 12 h, the mixture was cultured in a culture room at 25° C. or below.
(95) As shown in FIG. **3**, 2 weeks after injection, the plants injected with pVS2-CLA turned completely white above the cotyledon node, indicating that the CLA gene is successfully silenced. Therefore, the simplification of the two-component RNA virus-TRV vector was successful. When the two-component RNA viral vector was used in cotton, the concentration of the bacteria solution was OD=0.3.

Example 3

(96) Simultaneous Implementation of VIGS, VOX, and VIF by Using a Simplified Two-Component Viral Vector.
(97) In this example, two plasmids, pVF-SFT and pVS-SP, were first constructed to overexpress an SFT gene and silence the SP gene respectively. Using these two plasmids as templates, a plasmid pVF-SFT-SP was obtained by way of overlap extension PCR. The two VA genomes in the pVF-SFT-SP plasmid shared the same CR region, thereby ensuring that both VA genomes could restore circularity. In addition, an empty pVSe containing no endogenous gene fragments was constructed as a negative control, and whether the three-component single plasmid system could successfully overexpress the SFT gene and silence the SP gene was verified by using the qRT-PCR results and flowering time phenotypes, and thus VIGS, VOX, and VIF were implemented simultaneously. The schematic diagram is shown in FIG. **4**.
(98) A specific implementation process is briefly introduced below.
(99) (1) Construction of Two Plasmids, pVF-SFT and pVS-SP
(100) The primers used were as follows:
(101) TABLE-US-00019 VF-SFT F: 5′-ATGGCATGCCTGCAGACTAGTATGCCTAGAGATAGAGATCCTTTGG TT G-3′, SEQID NO. 21; VF-SFT R: 5′-GGCCAGTTAACGCTAGCGAATTCATGTCCTACGGCCACCGG-3′, SEQ ID NO. 22; VS GHSP F: 5′-ATGGCATGCCTGCAGACTAGTGCGTCTTCTAGCAGCTGTTTCC C-3′, SEQ ID NO. 23; VS GHSP R: 5′-GGCCAGTTAACGCTAGCGAATGAGTGATTGGGGATGTTATTGATG CCC-3′, SEQ ID NO. 24.
(102) The PCR reaction system is shown in Table 14, and the PCR amplification program is shown in Table 15.
(103) TABLE-US-00020 TABLE 14 PCR reaction system Component Volume Phanta Max Super-Fidelity DNA Polymerase 1 μL 2 × Phanta Max Buffer 10 μL dNTP Mix 0.5 μL Upstream primer 0.8 μL Downstream primer 0.8 μL Template DNA 1 μL Supplementing double distilled water to 20 μL
(104) TABLE-US-00021 TABLE 15 PCR amplification program Step Temperature Time Cycle number Predenaturation 95° C. 5 min 1 Denaturation 95° C. 15 sec 33 Annealing 58° C. 15 sec Extension 72° C. 1 min Extension 72° C. 5 min 1
(105) The amplified PCR products were recovered by using an agarose gel DNA recovery kit (enhanced type).
(106) The pVS vector was linearized by using a restriction enzyme BsaI.
(107) An enzyme cutting system is shown in Table 16. In Table 16, the volume of the component 10× Cut smart buffer is 10 μL, the volume of the component BsaI is 1 μL, and the volume of the component carrier is 2000 ng. Finally, double distilled water is supplemented to 100 μL.
(108) TABLE-US-00022 TABLE 16 Enzyme cutting system Component Volume 10 × Cut smart buffer 10 μL BsaI 1 μL Vector 2000 ng Supplementing double distilled 100 μL water to
(109) The reaction system was placed in a 37° C. incubator for enzyme cutting overnight.
(110) Further, the linearized vector was recovered by an ultra-thin DNA product purification kit.
(111) Further, the PCR product and linearized vector were recombined by using a ClonExpress® II One Step Cloning Kit.
(112) Further, a recombinant product was transformed and entered into *E. coli* strain DH5α. After overnight growth on a plate, monoclones were selected for colony PCR identification and positive transformants were screened.
(113) Further, whether the PCR fragment was successfully constructed was confirmed by a method of a Sanger sequencing. The final plasmids obtained were pVF-SFT and pVS-SP.
(114) (2) Construction of pVF-SFT-SP Plasmid
(115) The primers used are as follows:
(116) TABLE-US-00023 VS GHSP F: 5′-ATGGCATGCCTGCAGACTAGTGCGTCTTTCTAGCAGCTGTTTCC C-3′, SEQ ID NO. 25; VF R1: 5′-CTAGGCTAGTCAGGCGCAAAATGAT-3′, SEQ ID NO. 26; VF F1: 5′-ATCATTTTGCGCCTGACTAGCCTAG-3′, SEQ ID NO. 27; VF-SFT R: 5′-GGCCAGTTAACGCTAGCGAATTCATGTCCTACGGCCACCG G-3′, SEQ ID NO. 28.
(117) The first two primers were used to amplify the SP silent fragment and CR region, and the last two primers were used to amplify the full-length cDNA of SFT.
(118) The PCR reaction system is shown in Table 17, and the PCR amplification program is shown in Table 18.
(119) TABLE-US-00024 TABLE 17 PCR reaction system Component Volume Phanta Max Super-Fidelity DNA Polymerase 1 μL 2 × Phanta Max Buffer 10 μL dNTP Mix 0.5 μL Upstream primer 0.8 μL Downstream primer 0.8 μL Template DNA 1 μL Supplementing double distilled water to 20 μL
(120) TABLE-US-00025 TABLE 18 PCR amplification program Step Temperature Time Cycle number Predenaturation 95° C. 5 min 1 Denaturation 95° C. 15 sec 33 Annealing 58° C. 15 sec Extension 72° C. 2 min Extension 72° C. 5 min 1
(121) The amplified PCR products were recovered by using an agarose gel DNA recovery kit (enhanced type).
(122) A pVS vector was linearized by using a restriction enzyme BsaI.
(123) An enzyme cutting system is shown in Table 19.
(124) TABLE-US-00026 TABLE 19 Enzyme cutting system Component Volume 10 × Cut smart buffer 10 μL BsaI 1 μL Vector 2000 ng Supplementing double 100 μL distilled water to
(125) The reaction system was placed in a 37° C. incubator for enzyme cutting overnight.
(126) Further, the linearized vector was recovered by an ultra-thin DNA product purification kit.
(127) Further, the PCR product and linearized vector were recombined by using a ClonExpress® II One Step Cloning Kit.
(128) Further, a recombinant product was transformed and entered into *E. coli* strain DH5α. After overnight growth on a plate, monoclones were selected for colony PCR identification and positive transformants were screened.
(129) Further, whether the PCR fragment was successfully constructed was confirmed by a method of a Sanger sequencing. The final plasmid was pVF-SFT-SP.
(130) (3) Construction of a Negative Control Plasmid pVSe
(131) The primers used are as follows:
(132) TABLE-US-00027 VSE F: 5′-ATGGCATGCCTGCAGACTAGTGAGACCGTGTAAGAGGTCTCG-3′, SEQ ID NO. 29; VSE R: 5′-GGCCAGTTAACGCTAGCGAATCGAGACCTCTTACACGGTCTC-3′, SEQ ID NO. 30.
(133) Referring to the authorized patent CN113215145A, an annealed product is prepared through fragment annealing, and then recombined and transformed.
(134) (4) *Agrobacterium* Infection
(135) The pVF-SFT and pVF-SFT-SP plasmids were transformed into *Agrobacterium* GV3101 through heat shock transformation.
(136) Further, after the plate grew on kana and rifampicin double-resistant plates for two days, monoclones were selected for colony PCR identification and positive transformants were screened.
(137) Further, positive monoclones were selected and shook overnight until the OD value is 0.5-1.0.
(138) Further, the bacteria solution was collected at the bottom of the centrifuge tube by centrifugalization at 6000 rpm for 10 minutes, a supernatant

was discarded, and the mixture was adjusted with the resuspending solution till OD=0.5-1.0.
(139) A formula of the resuspending solution is shown in Table 20.
(140) TABLE-US-00028 TABLE 20 Formula of resuspending solution Resuspending solution 100 mL MgCl2 (1M) 1 mL MES(0.5M) 2 mL AS (100 mM) 200 μL
(141) Further, after standing for 3 hours in the dark, pores were punched to inject the resuspending solution to the back of the flat cotyledon of cotton at a cotyledonary stage.
(142) Further, after being kept in the dark for 12 h, the mixture was cultured in a 28° C. culture room.
(143) Further, phenotypic observation was conducted one month after injection, and RNA was extracted from new leaves, and the expression levels of SFT and SP were detected by fluorescence quantitative PCR. It could be seen from the test results that flower buds preferentially appeared in the infected plants in the VF-SFT group. Five weeks after injection, statistics showed that no flower buds appeared in the VSe control group, 42% of the plants in the VF-SFT group had flower buds, and 14% of the plants in the VF-SFT-SP group had flower buds.
(144) It can be seen from the above that compared with the control group, SP was successfully silenced and SFT was successfully overexpressed. In general, the flowering effect induced by viruses in the VF-SFT group is better than that in the VF-SFT-SP group. The above results show that by using the simplified two-component viral vector to concatenate multiple A genome copies can achieve VIGS, VOX, and VIF simultaneously. The experimental results in one month after injection are shown in Table 21.
(145) TABLE-US-00029 TABLE 21 Experimental results of fluorescence quantitative PCR Standard mean SP expression level deviation VSe group 0.977 0.091 VF-SFT-SP group 0.382 0.155 Standard mean SFT gene expression (Log2) deviation VSe group 0.986 0.399 VF-SFT-SP group 2374.835 57.413
Example 4
(146) Simultaneous construction of two VIGS plasmids of simplified two-component viral vector from a single PCR product
(147) In this example, primers containing adapter sequences on both sides of the vector's multiple cloning site were designed, and partial fragments of a target gene GhOMT1 to be silenced were amplified by PCR. This PCR product could be used to construct the VIGS plasmids of two viruses at the same time.
(148) A specific implementation process is briefly introduced below.
(149) (1) Construction of Two Plasmids, pVS GhOMT1 and pVS2 GhOMT1
(150) The primers used are as follows:
(151) TABLE-US-00030 VS GhOMT1 F2: 5′-GGCCAGTTAACGCTAGCGAATTGTTGCACCATGACCAAGTCTT CA-3′, SEQ ID NO. 33; VS GhOMT1 R2: 5′-ATGGCATGCCTGCAGACTAGTTGATGCCCTTGATTTGAGGATAC TTTG-3′, SEQ ID NO. 34;
(152) The PCR reaction system is shown in Table 22, and the PCR amplification program is shown in Table 23.
(153) TABLE-US-00031 TABLE 22 PCR reaction system Component Volume Phanta Max Super-Fidelity DNA Polymerase 1 μL 2 × Phanta Max Buffer 10 μL dNTP Mix 0.5 μL Upstream primer 0.8 μL Downstream primer 0.8 μL Template DNA 1 μL Supplementing double distilled water to 20 μL
(154) TABLE-US-00032 TABLE 23 PCR amplification program Step Temperature Time Cycle number Predenaturation 95° C. 5 min 1 Denaturation 95° C. 15 sec 33 Annealing 58° C. 15 sec Extension 72° C. 1 min Extension 72° C. 5 min 1
(155) The amplified PCR products were recovered by using an agarose gel DNA recovery kit (enhanced type).
(156) The pVS and pVS2 vectors were linearized by using a restriction enzyme BsaI.
(157) An enzyme cutting system is shown in Table 24.
(158) TABLE-US-00033 TABLE 24 Enzyme cutting system Component Volume 10 × Cut smart buffer 10 μL BsaI 1 μL Vector 2000 ng Supplementing double 100 μL distilled water to
(159) The reaction system was placed in a 37° C. incubator for enzyme cutting overnight.
(160) Further, the linearized vector was recovered by an ultra-thin DNA product purification kit.
(161) Further, the PCR product and linearized vector were recombined by using a ClonExpress® II One Step Cloning Kit.
(162) Further, recombinant products of pVS-GhOMT1 and pVS2-GhOMT1 were transformed and entered into *E. coli* strains DH5α and EPI300 respectively. After overnight growth on a plate, four monoclones were selected for colony PCR identification and positive transformants were screened.
(163) Further, whether the PCR fragment was successfully constructed was confirmed by a method of a Sanger sequencing.
(164) As shown in FIG. **5**, PCR identification results of a bacteria solution show that all eight single clone bands are correct. Sanger sequencing results also confirm that GhOMT1 is successfully constructed into two viral plasmids, pVS and pVS2. This shows that through the use of adapter sequences, the present disclosure can simply and quickly construct VIGS vectors for multiple viruses of one gene through the single PCR product. In the future, through the simplified construction method and the use of the adapters in the present disclosure, more different viral vectors can be expanded for the rapid construction of multiple viral plasmids of single genes.
(165) The above is merely preferred embodiments of the present disclosure. It should be noted that those skilled in the art can further make several improvements and modifications without departing from the principle of the present disclosure. The improvements and modifications shall also be regarded as the scope of protection of the present disclosure.
(166) TABLE-US-00034 Sequence table <110> Cotton Research Institute, Chinese Academy of Agricultural Sciences Sanya National Institute of Southern Propaganda, Chinese Academy of Agricultural Sciences <120> A method for simply constructing two-component viral vector and related applications thereof <141> 2023-11-14 <160> 34 <170> SIPOSequenceListing 1.0 <210> 2 <211> 30 <212> DNA <213> Artificial Sequence <400> 2 ggaaagacac cttttcgacc tttttcccct 30 <210> 3 <211> 33 <212> DNA <213> Artificial Sequence <400> 3 aaaaggtgtc tttcctgtgg atagcacgta cat 33 <210> 4 <211> 36 <212> DNA <213> Artificial Sequence <400> 4 taattcgggg atagccctttt ggtcttctga gactgt 36 <210> 5 <211> 37 <212> DNA <213> Artificial Sequence <400> 5 caaagggcta tccccgaatt aattcggcgt taattca 37 <210> 6 <211> 36 <212> DNA <213> Artificial Sequence <400> 6 aacgctagcg aattcactag tgcctgaaga ctggag 36 <210> 7 <211> 34 <212> DNA <213> Artificial Sequence <400> 7 ggcatgcctg cagactagtg ctttactctg atcc 34 <210> 8 <211> 41 <212> DNA <213> Artificial Sequence <400> 8 aacctatccc aagtggagct ccgggggatc cactagtaaa c 41 <210> 9 <211> 41 <212> DNA <213> Artificial Sequence <400> 9 catgattacg aattcgagct cattcgagct ccagaacgat c 41 <210> 9 <211> 1473 <212> DNA <213> Artificial Sequence <400> 9 gttattgctt ttagatagag ttcctgctct gcaagaggtg gatgacatcg gtggtcaatg 60 gtcgttttgg gtaactagag gtgagaaaag gattcattcc tgttgtccaa atctagatat 120 tcggggatgat cagagagaaa tttctcgaca gatatttctt actgctattg gtgatcaagc 180 tagaagtggt aagagacaga tgtcggagaa tgagctgtgg atgtatgacc aatttcgtga 240 aaatattgct gcgcctaacg cggttaggtg caataataca tatcagggtt gtacatgtag 300 gggtttttct gatggtaaga agaaaggcgc gcagtatgcg atagctcttc acagcctgta 360 tgacttcaag ttgaaagact tgatggctac tatggttgag aagaaaacta aagtggttca 420 tgctgctatg ctttttgctc ctgaaagtat gttagtggac gaaggtccat taccttctgt 480 tgacggttac tacatgaaga agaacgggaa gatctatttc ggttttgaga aagatccttc 540 cttttcttac attcatgact gggaagagta caagaagtat ctactgggga agccagtgag 600 ttaccaaggg aatgtgttct acttcgaacc gtggcaggtg agaggagaca caatgctttt 660 ttcgatctac aggatagctg gagttccgag gaggtcgcta tcatcgcaag agtactaccg 720 aagaatat atcagtagat gggaaaacat ggttgttgtc ccaattttcg atctggtcga 780 atcaacgcga gagttggtca agaaagacct gtttgtagag aaacaattca tggacaagtg 840 tttggattac atagctaggt tatctgacca gcagctgacc ataagcaatg ttaaatcata 900 cttgagttca aataattggg tcttattcat aaacggggcg gccgtgaaga acaagcaaag 960 tgtagattct cgagatttac agttgttggc tcaaactttg ctagtgaagg

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Claims

1. A method for simply constructing a two-component viral vector comprising a first genomic component and a second genomic component, the method comprising: modifying a restriction site within the multiple cloning site of the first genomic component into a new restriction site, modifying the flanking sites of the new restriction site into the adapter sequences set forth by SEQ ID NO: 31 and SEQ ID NO: 32, and concatenating the first genomic component via the adapter sequences through PCR and homologous recombination; ligating the concatenated first genomic component and the second genomic component into a plasmid to obtain a single plasmid encoding the two-component viral vector; wherein the two-component viral vector is a two-component DNA or a two-component RNA viral vector; when the two-component DNA viral vector is the cotton leaf crumple virus (CLCrV), the two genomic components are flanked by respective common regions (CRs), the plasmid is the *Agrobacterium* Ti plasmid, the CRs can be linked via a restriction site sequence, and the two genomic components are inserted between the T-DNA borders of the *Agrobacterium* Ti plasmid; when the two-component RNA viral vector is the tobacco rattle virus (TRV), the two genomic components are placed in two separate expression cassettes for independent expression from the 35S promoter, wherein each expression cassette comprises the NOS terminator.