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A Nimble Cloning-compatible vector system for high-throughput gene functional analysis in plants

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ABSTRACT

Plant expression vectors are essential tools for gene functional analysis and molecular plant breeding. The gene of interest is transferred to the vector by molecular cloning technology. Nimble Cloning is a newly developed molecular cloning method with the advantages of simplicity, efficiency, and standardization. In this study, we developed a "pNC" vector system that contains 55 Nimble Cloning-compatible vectors for functional analysis of genes in plants. These vectors contain the NC frame flanked by unique adapters for one-step and standardized Nimble Cloning. We demonstrate that the pNC vectors are convenient and effective for the functional analysis of plant genes, including the study of gene ectopic expression, protein subcellular localization, protein–protein interaction, gene silencing (RNAi), virus-induced gene silencing, promoter activity, and CRISPR–Cas9-mediated genome editing. The "pNC" vector system represents a high-throughput toolkit that can facilitate the large-scale analysis of plant functional genomics.

Key words: nimble cloning, plant expression vector, gene function, ectopic expression, genome editing

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INTRODUCTION

With the accelerating production of plant gene and whole-genome sequences, high-throughput systems for analysis of gene function are in great demand (Huang et al., 2022). There are various methods for functional analysis of plant genes, including gain of function by ectopic expression and loss of function by gene silencing or gene knockout (Liu et al., 2016). Almost all methods require the use of plant expression vectors.

The gene of interest is transferred to the expression vector by molecular cloning technology. A rapid, efficient, and standardized molecular cloning method can greatly facilitate vector construction for high-throughput functional analysis of genes. Although many methodologies have been developed for molecular cloning, the main cloning techniques now used for routine vector construction are restriction enzyme/ligase cloning, Gateway cloning, Golden Gate cloning, and Gibson assembly (Casini et al., 2015; Wang et al., 2021; Young et al., 2021). Each of these methods has its compatible vectors.

As the conventional cloning approach, restriction enzyme/ligase cloning uses type II restriction endonucleases to cut the DNA fragment and the vector plasmid and then uses DNA ligases to reassemble them into a recombinant plasmid. Compatible vectors for restriction enzyme/ligase cloning usually contain multiple cloning sites (MCSs) to facilitate the insertion of different genes. Restriction enzyme/ligase cloning and MCS-based vectors are still used extensively (He et al., 2018). However, they are often limited by the lack of suitable restriction sites. In addition, restriction enzyme/ligase cloning requires two separate reactions with restriction enzymes and ligases, which makes cloning laborious and time consuming and limits its application in high-throughput gene functional analysis. Gateway cloning is a restriction–site-independent method that exploits the lambda phage integration and excision mechanism for site-specific recombination cloning (Hartley et al., 2000). The Gateway vector system contains donor vectors and destination vectors. Once the gene of interest has been cloned into the donor vector, it can be easily transferred into any destination vector. The Gateway system has been widely used for a variety of molecular biology applications (Curtis and Grossniklaus, 2003; Karimi et al., 2007, 2013). However, it has several disadvantages such as the high cost of commercial enzyme mixes, the unwanted 25-bp attB junk sequence (scars), and the inconvenience of assembling

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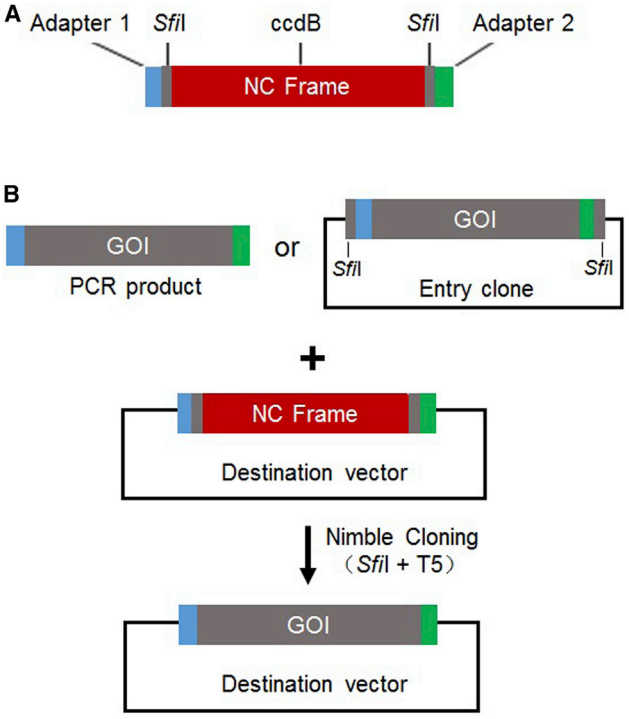

Figure 1. Schematic diagram of Nimble Cloning.

(A) The structure of the NC frame. The NC frame consisting of the cassette "adapter 1–*Sfi*I–ccdB gene–*Sfi*I–adapter 2" is used for one-step, standardized molecular cloning.

(B) Schematic diagram of the Nimble Cloning method. The PCR product flanked by the adapters or the DNA fragment in the entry clone can be cloned into the Nimble Cloning-compatible vector in a one-step Nimble Cloning reaction, replacing the NC frame of the Nimble Cloning-compatible vector.

multiple sequences. Golden Gate cloning is a restriction enzyme/ligase-based one-pot cloning method. It relies on type IIS restriction enzymes, which cut outside of their recognition sequence (Engler et al., 2008, 2009). Golden Gate-compatible vectors contain two recognition sites for type IIS restriction enzymes that are eliminated from the vector after digestion. Thus, the two steps of digestion and ligation can be replaced by a single restriction–ligation step. Golden Gate cloning is convenient for assembling a large number of fragments and for assembling modular DNA parts (Binder et al., 2014; Engler et al., 2014; Pollak et al., 2018; Vasudevan et al., 2019). The main disadvantage of Golden Gate cloning is that the gene of interest must be free of the recognition sequence of the enzymes used. Because the recognition sequences of type IIS restriction enzymes are often less than 7 bp, they are frequently present within the DNA sequences to be cloned. This disadvantage limits the application of Golden Gate cloning. Gibson assembly is a recombination-based molecular cloning method that can assemble multiple overlapping DNA fragments in a single isothermal reaction (Gibson et al., 2009; Gibson, 2011). Gibson assembly is mostly sequence independent, and it can thus use both MCS-based and Golden Gate-based vectors without the need for vector modification. Gibson assembly and its derived cloning methods (Fu et al., 2014; Xia et al., 2018) are commonly used in both routine cloning and synthetic biology (Young et al., 2021). However, Gibson assembly requires two-step cloning. The vector is first linearized by enzyme digestion or PCR and then used for recombination. Also, there is currently no vector system for standardized Gibson assembly in routine cloning.

Based on these cloning methods, a large number of plant expression vectors have been developed. Some of them were designed as vector toolboxes to facilitate large-scale vector construction. The restriction enzyme/ligase–cloning-based vector toolboxes use MCSs to enable easy exchange of the target genes between different vectors (Goderis et al., 2002; Tzfira et al., 2005; Citovsky et al., 2006; Li et al., 2014; He et al., 2018). The T-A–cloning-based vector systems were developed for directly cloning PCR fragments with a T overhang without restriction digestion of the PCR fragments (Chen et al., 2009; Wang et al., 2013). Gateway-compatible vector toolboxes enable rapid recombinational cloning of any gene of interest (sequence independent) and facilitate high-throughput gene functional analysis in plants (Curtis and Grossniklaus 2003; Earley et al., 2006; Karimi et al., 2007, 2013; Mann et al., 2012). Golden Gate-based vector toolboxes were developed for modular assembly of multiple DNA components, thus facilitating multigene expression in plants (Binder et al., 2014; Engler et al., 2014; Pollak et al., 2018; Vasudevan et al., 2019). A set of pGate vectors contain type IIS restriction enzymes and "attL1 and attL2" sequences; they can thus be used for both Gateway and Golden Gate cloning (Luo et al., 2018). Recently, a vector toolbox based on modular Gibson assembly was developed for protein localization and bimolecular fluorescence complementation (BiFC) analyses in plants (Han et al., 2022a). These vector toolboxes are useful for plant gene functional analysis. However, the disadvantages of the cloning methods on which they are based remain limitations to their application.

Nimble Cloning is a newly developed method for one-step and standardized molecular cloning (Yan et al., 2020). It uses an enzyme mix of the restriction enzyme *Sfi*I and the T5 exonuclease to simultaneously linearize the vector and generate 3′ overhangs for recombination (Figure 1). Like Gateway cloning and Golden Gate cloning, Nimble Cloning requires its own compatible vectors. To construct a Nimble Cloning-based vector, the NC frame comprising the "adapter 1–*Sfi*I–ccdB gene–*Sfi*I–adapter 2" sequence must be inserted into the cloning site (Figure 1). Although Nimble Cloning is a promising system for standardized molecular cloning in diverse applications, only a few compatible vectors have been reported to date (Yan et al., 2020; Tuo et al., 2021a, 2021b).

In this study, we developed a "pNC" vector system that contains 55 plant expression vectors based on Nimble Cloning. These vectors can be used for different applications in plants, including ectopic gene expression, gene silencing (by plant hairpin RNA expression vectors and virus vectors), protein subcellular localization, BiFC for protein–protein interaction, promotor activity analysis, and CRISPR–Cas9-mediated genome editing. All 55 plant expression vectors contain the NC frame flanked by unique adapters for one-step and standardized Nimble Cloning. The gene of interest flanked by the adapters can be transferred to any of these vectors by a single cloning reaction. We demonstrate that the pNC vector system is convenient and effective for functional analysis of plant genes. It provides a high-throughput toolkit that will accelerate large-scale functional analysis of genes in plants.

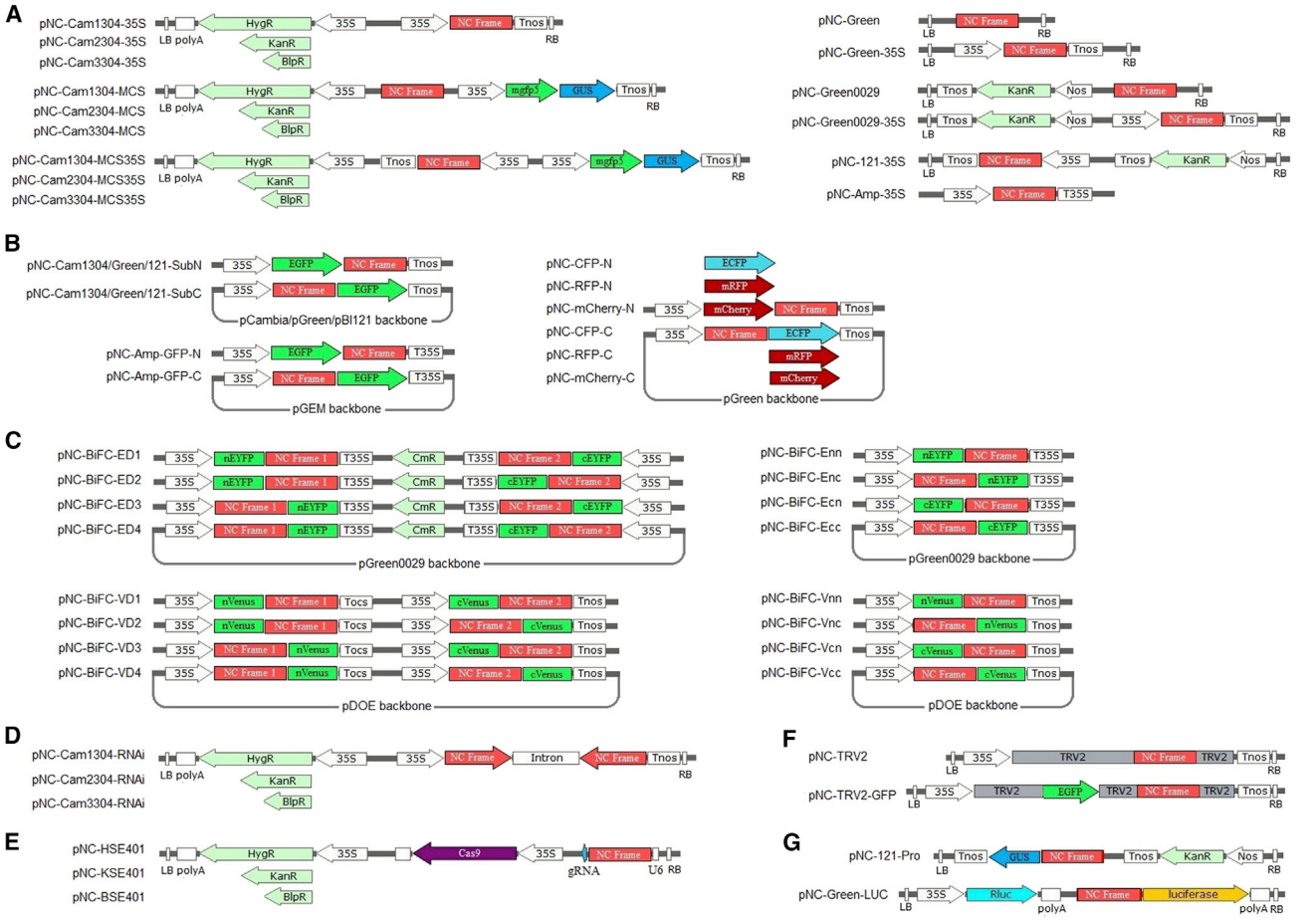

Figure 2. Schematic diagrams of pNC vectors.

pNC vectors for **(A)** ectopic gene expression, **(B) protein subcellular localization, (C) BiFC, (D) gene silencing, (E) CRISPR–Cas9-mediated genome editing, (F) virus-induced gene silencing, and (G) study of promoter activity.**

NC frame, DNA cassette for Nimble Cloning; 35S, CaMV 35S promoter; polyA, CaMV polyA signal; Tnos, Nos terminator; T35S, CaMV 35S terminator; HygR, hygromycin resistance; KanR, kanamycin resistance; BlpR, Basta resistance.

RESULTS

Construction of pNC vectors

We constructed a set of Nimble Cloning-compatible vectors for functional analysis of genes in plants. It includes 55 plant expression vectors for different applications, including 15 vectors for ectopic gene expression; three RNAi vectors for gene silencing; two vectors for virus-induced gene silencing; 14 vectors for study of protein subcellular localization; 16 BiFC vectors for protein–protein interactions; two vectors for analysis of promoter activity; and three vectors for CRISPR–Cas9-mediated genome editing (Figure 2; Supplemental Table 1; Supplemental Data 1). The vectors for ectopic gene expression and protein subcellular localization include four types of plasmid backbones: the plant binary expression vectors pCambia, pGreen, and pBI121 and the transient expression vector pGEM. The vectors for protein subcellular localization include four types of fluorescent protein genes: *GFP*, *CFP*, *RFP*, and *mCherry*. The pCambia backbone vectors for ectopic expression, RNAi, and genome editing include three resistance genes (hygromycin, kanamycin, and Basta) as selectable markers for plant transformation. The plant BiFC vectors include eight vectors (pNC-BiFC-ED1–4/Enn/Enc/Ecn/Ecc) derived from the pSAT BiFC vectors that utilize EYFP split between amino acid residues 174 and 175 and eight vectors (pNC-BiFC-VD1–4/Vnn/Vnc/Vcn/Vcc) derived from the pDOE BiFC vectors that utilize monomeric Venus split at residue 210. The two sets of pNC BiFC vectors contain four double open reading frame (ORF) expression vectors and four traditional single ORF expression vectors, respectively. The pNC BiFC vector for double ORF expression enables coexpression of two fluorescent protein fragments from a single vector. Two genes of interest can be simultaneously cloned into the vector by one-step Nimble Cloning because of the two different NC frames in the vector. The pNC RNAi vectors also contain two NC frames, one of which is inverted. A single DNA fragment can be simultaneously recombined into the vector in the sense and antisense orientations to form hairpin RNA (hpRNA) constructs using one-step Nimble Cloning.

Transient expression of pNC ectopic expression vectors

We constructed 15 pNC vectors for ectopic gene expression in plants. They were based on the widely used binary vector backbones pCambia, pGreen, and pBI121. Some of them have the

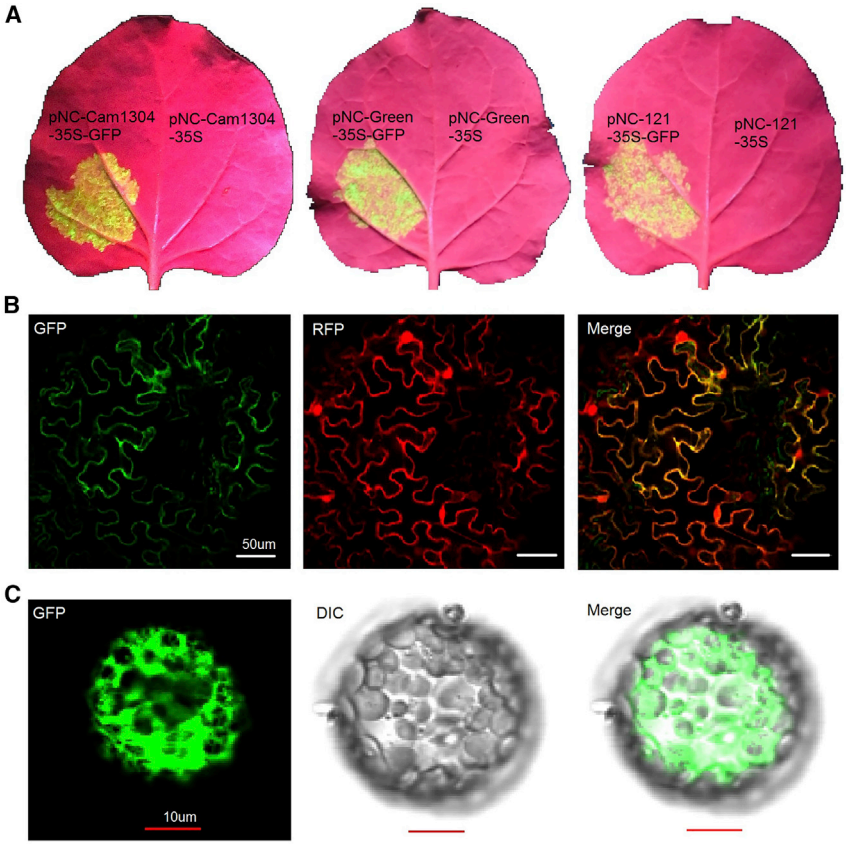

Figure 3. Transient expression of pNC ectopic expression vectors.
(A) GFP expression in *N. benthamiana* leaves using pNC-Cam1304-35S, pNC-Green-35S, and pNC-121-35S.
(B) Expression of both RFP and GFP in *N. benthamiana* leaves using pNC-Cam1304-MCS35S-RFP.
(C) Expression of pNC-Green-35S-GFP in a protoplast from an *N. benthamiana* leaf.
DIC, differential interference contrast imaging.

NC frame without the promoter and terminator (pNC-Cam1304/2304/3304-MCS, pNC-Green, and pNC-Green0029), whereas others have the NC frame between the promoter and terminator. pNC-Cam1304/2304/3304-MCS and pNC-Cam1304/2304/3304-MCS35S include gfp/gus expression cassettes as selectable markers for plant transformation. To test the expression of the vectors, the GFP gene was cloned to pNC-Cam1304-35S, pNC-Green-35S, and pNC-121-35S and the RFP gene was cloned into pNC-Cam1304-MCS35S by Nimble Cloning. *Agrobacterium* harboring these constructs was infiltrated into *Nicotiana benthamiana* leaves for transient expression. Strong GFP fluorescence was observed for leaves infiltrated with pNC-Cam1304-35S-GFP, pNC-Green-35S-GFP, and pNC121-35S-GFP at 3 days post-infiltration (Figure 3A). Both GFP fluorescence and RFP fluorescence were observed under confocal microscopy for leaves infiltrated with pNC-Cam1304-MCS35S-RFP (Figure 3B). The plasmid of pNC-Green-35S-GFP was also used for protoplast transformation. Strong GFP fluorescence was observed under confocal microscopy in protoplasts from *N. benthamiana* leaves transformed with pNC–Green-35S-GFP (Figure 3C).

Analysis of subcellular localization using pNC vectors

We constructed 14 pNC vectors for the study of protein subcellular localization in plants. They include four types of vector backbones (pCambia, pGreen, pBI121, and pGEM) and four types of fluorescent protein genes (*GFP*, *CFP*, *RFP*, and *mCherry*). The fluorescent protein genes were located at the 5′ or 3′ end of the NC frame for easy construction of N-terminal and C-terminal fusions of the fluorescent protein to the target protein. Papaya eIFiso4E, which is located in both the nucleus and cytoplasm, and papaya ringspot virus VPg (prsv-VPg), which is located in the nucleus, were used to test the utility of the pNC vectors for studying protein subcellular localization. The eIFiso4E gene was cloned into pNC-Green-SubN, and the prsv-VPg gene was cloned into pNC-Green-SubC, pNC-CFP-N, pNC-RFP-N, and pNC-mcherry-N. *Agrobacterium* harboring the sublocation constructs was infiltrated into *N. benthamiana* leaves. All sublocation constructs were expressed in the infiltrated leaves 3 days post-agroinfiltration, with the relative fluorescence at the correct subcellular locations (Figure 4A–4E). The results indicated that the Nimble Cloning-compatible vectors were effective for studying protein subcellular localization in plants. The pNC-Green-SubN-eIFiso4E and pNC-Green-SubN-VPg plasmids were also used for protoplast transformation of *N. benthamiana* to confirm the locations of the two proteins. The results of protoplast transformation showed that eIFiso4E is located in both the nucleus and cytoplasm and that prsv-VPg is located in the nucleus (Figure 4F and 4G).

Protein interaction assay with pNC BiFC vectors

Two sets of pNC BiFC vectors were constructed for studying protein–protein interactions in plants, each with four double ORF expression vectors and four traditional single ORF expression vectors. The interaction between papaya eIFiso4E and prsv-Vpg and the self-interaction of the helper component proteinase (HCPro) of prsv were tested with the pNC BiFC vectors. The two sets of single ORF expression vectors and the double ORF expression vectors pNC-BiFC-VD1–4 were used to study the interaction between papaya eIFiso4E and prsv-Vpg. *A. tumefaciens* cells containing the BiFC construct were infiltrated into *N. benthamiana* leaves. The fluorescent signal was observed exclusively in the nucleus at 3 days post-agroinfiltration, with no obvious differences among the vectors (Figure 5A–5C). The single ORF expression vectors pNC-BiFC-Vnn/Vcn/Vnc/Vcc were used to study the self-interaction of HCPro. The fluorescent signal was observed in the cytoplasm, and the strongest signal was observed with the vectors Vnn-HCPro and Vcn-HCPro (Figure 5D).

RNAi with pNC vectors

We constructed three pNC RNAi vectors (pNC-cam1304-RNAi, pNC-cam2304-RNAi, and pNC-cam3304-RNAi) for gene-silencing research in plants. They include hygromycin,

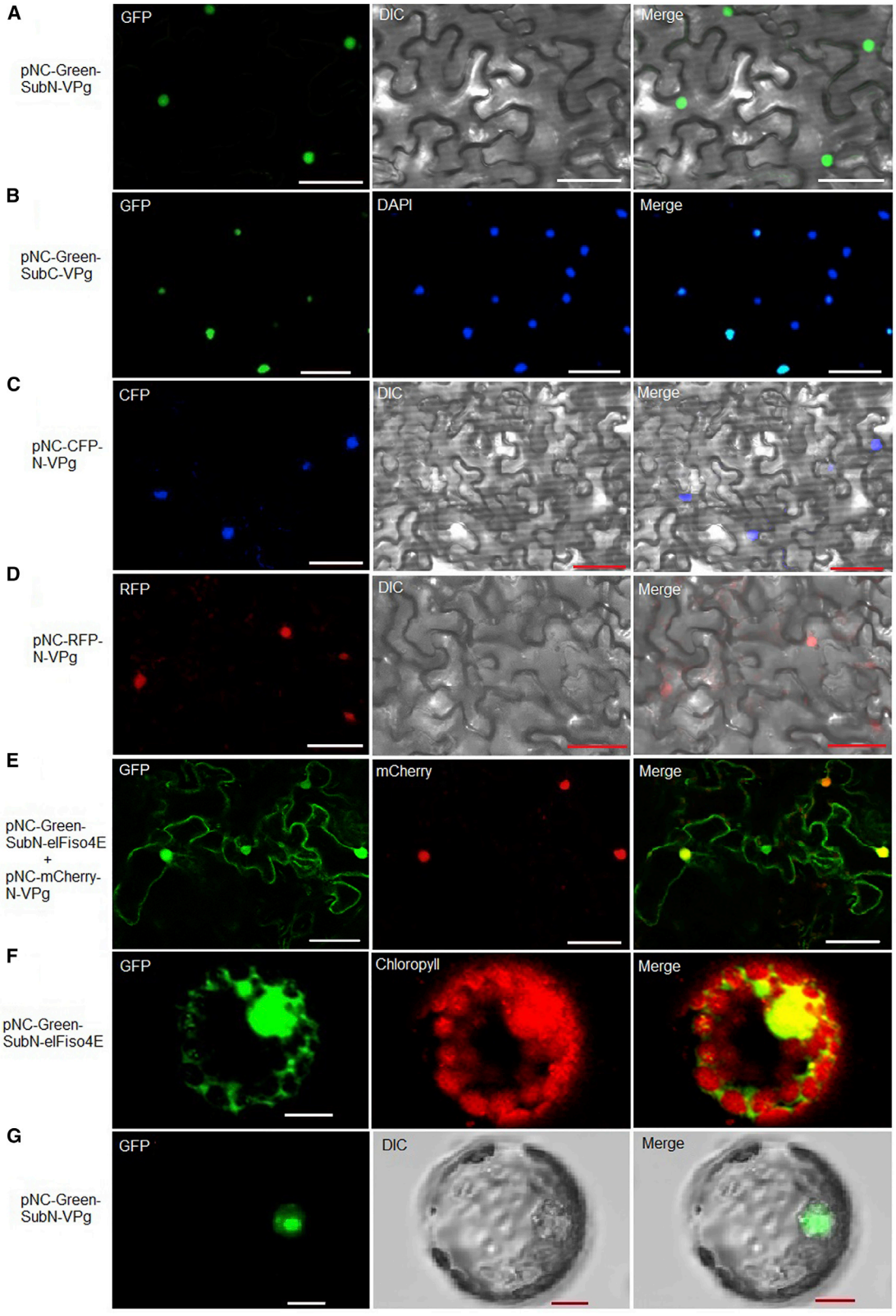

Figure 4. Analysis of subcellular localization using pNC vectors.

(A–E) Agroinfiltration of *N. benthamiana* leaves. Scale bars: 50 μm.

(B) DAPI was used as the nuclear marker.

(E) Coinfiltration with pNC-Green-SubN-eIFiso4E and pNC-mCherry-VPg.

(F and G) Protoplast transformation of *N. benthamiana* leaves. Scale bars: 10 μm.

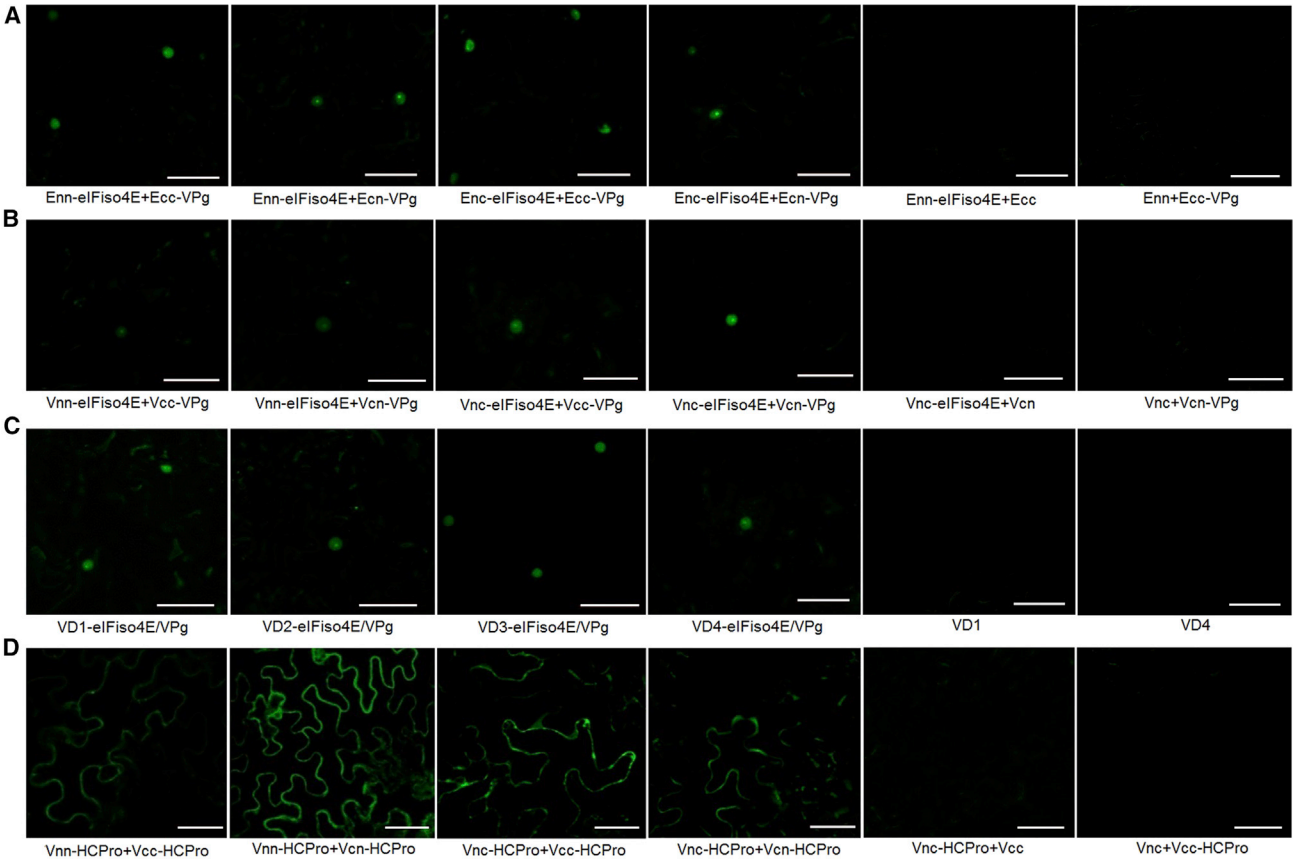

Figure 5. Protein interaction assay with pNC BiFC vectors.

(A) Interaction of eIFiso4E and prsv-VPg visualized using pNC-BiFC-Enn/Enc/Ecn/Ecc. Empty Ecc and Enn were used as the negative controls.

(B and C) Interaction of IFiso4E and prsv-VPg visualized using pNC-BiFC-Vnn/Vnc/Vcn/Vcc and pNC-BiFC-VD1–4. Empty Vcn, Vnc, VD1, and VD4 were used as the negative controls.

(D) Self-interaction of prsv-HCPro visualized using pNC-BiFC-Vnn/Vnc/Vcn/Vcc. Empty Vcc and Vnc were used as the negative controls. Scale bars: 50 μm.

kanamycin, and Basta resistance genes, respectively. All three vectors have the "NC frame–Pdk intron–inverted NC frame" cassette between the CaMV 35S promoter and the Nos terminator (Figure 6A). The marker gene *EGFP* was chosen to test the gene-silencing ability of the pNC RNAi vectors by *Agrobacterium*-mediated transient expression in *N. benthamiana*. A 299-bp fragment of *EGFP* was amplified and then cloned into the three pNC RNAi vectors to form the hpRNA constructs pNC-cam1304-RNAi-GFP, pNC-cam2304-RNAi-GFP, and pNC-cam3304-RNAi-GFP by a one-step Nimble Cloning reaction. *A. tumefaciens* cultures containing pNC-Cam1304-GFP were mixed with *A. tumefaciens* containing the hpRNA construct or the pNC RNAi empty vector as a control. The mixed *Agrobacterium* cultures were infiltrated into *N. benthamiana* leaves. Leaf areas infiltrated with the control cells had a strong GFP signal at 3 days post-agroinfiltration, whereas all leaf areas infiltrated with the three hpRNA gene-silencing constructs had much weaker GFP signals (Figure 6B). There were no obvious differences in *EGFP silencing* among the three hpRNA constructs according to the fluorescence signals. Real-time RT–PCR was performed to confirm the knockdown of *EGFP*. The results showed that GFP mRNA levels of samples infiltrated with the three hpRNA constructs were reduced to 25%–29% compared with the control samples (Figure 6C).

Virus-induced gene silencing with pNC vectors

Two pNC vectors were constructed for virus-induced gene silencing based on tobacco rattle virus (TRV). To test their gene-silencing ability, a 370-bp fragment of the *N. benthamiana phytoene desaturase* (PDS) gene was cloned into pNC-TRV2 by Nimble Cloning. *N. benthamiana* seedlings agroinfiltrated with pNC-TRV2-PDS initially developed mild photobleaching in newly developed leaves at 12 days post-inoculation (dpi) (data not shown). Large areas of photobleaching were observed at 24 dpi (Figure 6D). Real-time RT–PCR showed that the expression of *NbPDS* in the pNC-TRV2-PDS-infected leaves was reduced to 19% at 24 dpi compared with control samples infected with empty pNC-TRV2 (Figure 6E). These results indicated that the TRV-based pNC vectors were effective for virus-induced gene silencing in plants.

Analysis of promoter activity using pNC vectors

The vectors pNC-121-pro and pNC-Green-luciferase were constructed for analysis of promoter activity in plants. The pNC-121-pro vector has the NC frame in front of the GUS gene. We used the CaMV 35S promoter to test the utility of pNC-121-pro for analysis of promoter activity. The 35S promoter was amplified and inserted into pNC-121-pro to form pNC-121-pro-35S

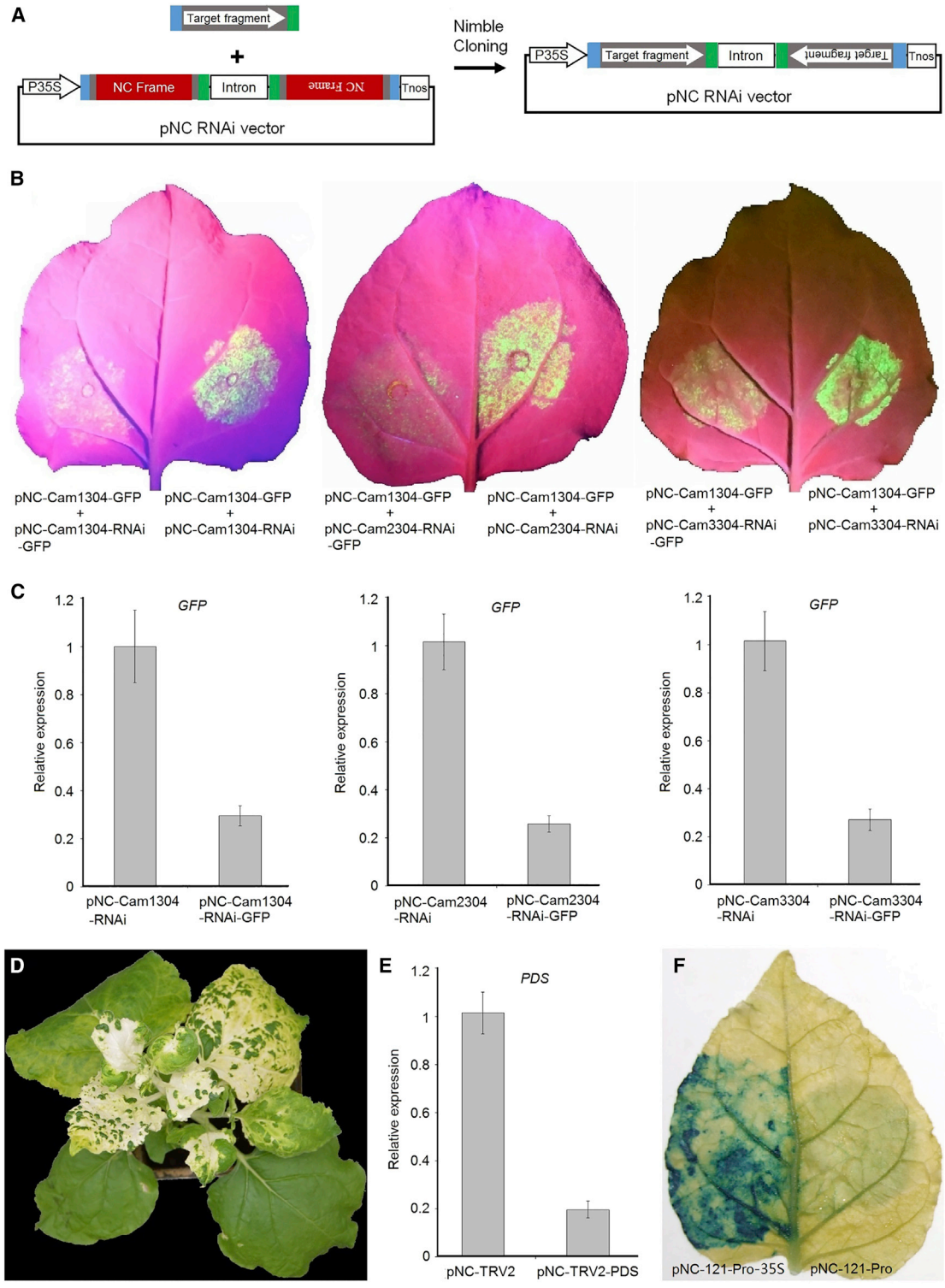

Figure 6. Gene silencing and promoter activity analysis with pNC vectors.

(A) Schematic diagram of hpRNA construction by Nimble Cloning. A single DNA fragment flanked by the adapters can be cloned into the pNC RNAi vector in the sense and antisense orientations simultaneously because of the presence of both the NC frame and the inverted NC frame in the pNC RNAi vector.

(B) Silencing *GFP* expression in *N. benthamiana* leaves by pNC-RNAi vectors. Empty pNC-RNAi vectors were used as controls.

(C) Real-time PCR for analysis of *GFP* silencing by pNC-RNAi vectors.

(D) Silencing of *PDS* by pNC-TRV2-PDS in *N. benthamiana* at 24 dpi.

(E) Real-time PCR for analysis of *PDS* silencing by pNC-TRV2-PDS in *N. benthamiana* at 24 dpi.

(F) Promoter activity analysis (GUS staining) with pNC-121-Pro-35S. Empty vector pNC-121-Pro was used as the control.

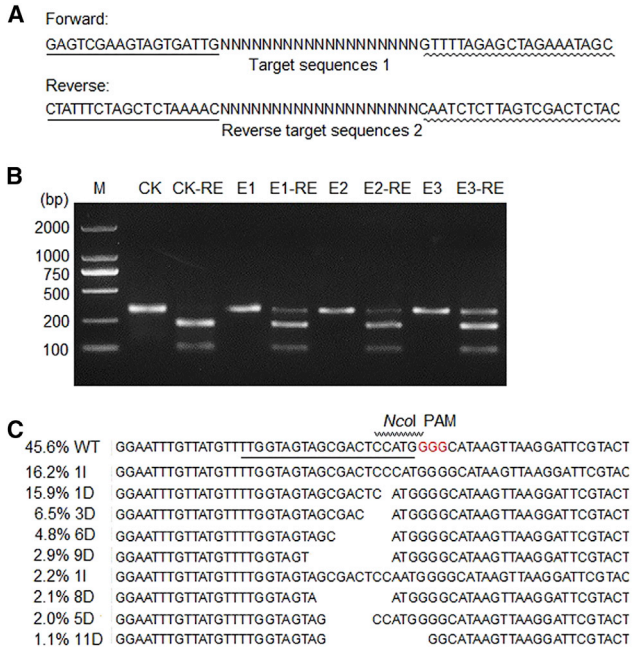

Figure 7. Genome editing with the pNC vector.

(A) Primer design for construction of pNC vectors for genome editing. The underlined sequences are homologous sequences of the pNC genome-editing vectors, which flank the NC frame for seamless cloning by Nimble Cloning. The sequences marked with wavy lines are the specific sequences of the pCBC-DT1T2 template.

(B) Restriction enzyme digestion of PCR products. CK, infiltrated with empty pNC-HSE401; E1–E3, three samples infiltrated with pNC-HSE401-gPDS; RE, restriction digested by *Nco*I.

(C) Mutations in *N. benthamiana* leaves infiltrated with pNC-HSE401-gPDS. WT, wild type; I, insertion; D, deletion.

by Nimble Cloning. pNC-121-pro-35S was infiltrated into *N. benthamiana* leaves by agroinfiltration. Empty pNC-121-pro was used as the negative control. The infiltrated leaves were harvested for the GUS assay at 3 days post-agroinfiltration. The areas infiltrated with pNC-121-pro-35S produced a blue GUS-staining signal, whereas the areas infiltrated with empty pNC-121-pro as a control produced no GUS-staining signal (Figure 6F). These results indicated that the Nimble Cloning-compatible vector pNC-121-pro was effective for analysis of promoter activity in plants.

Genome editing with pNC vectors

We constructed three pNC vectors, pNC-HSE401, pNC-KSE401, and pNC-BSE401, for genome editing in plants. They include the hygromycin, kanamycin, and Basta resistance genes, respectively. All three vectors have the NC frame between the *Arabidopsis* U6 gene promoter and the guide RNA (gRNA) scaffold and a Cas9 expression cassette under control of the CaMV 35S promoter. Two target sequences can be amplified in a single PCR fragment using pCBC vectors as templates (Figure 7A) and then assembled into pNC genome-editing vectors to form two gRNA expression cassettes by one-step Nimble Cloning. If only a single target sequence is needed, the two gRNA expression cassettes can be designed to contain the same target sequence. The PDS gene of *N. benthamiana* was chosen to test the pNC vectors in genome editing. A 20-nt sequence containing a *Nco*I restriction site in front of the PAM sequence NGG was used as the target sequence and assembled into pNC-HSE401 to form two gRNA expression cassettes with the same target sequence. *Agrobacterium* harboring the construct pNC-HSE401-gPDS or empty pNC-HSE401 was infiltrated into *N. benthamiana* leaves. Genomic DNA was extracted from the infiltrated leaf tissues 5 days post-agroinfiltration. The DNA fragment with the edited area was amplified and digested by *Nco*I. The results showed that the DNA fragment from the pNC-HSE401-gPDS-infiltrated leaf was partly resistant to *Nco*I digestion, whereas the DNA fragment from the pNC-HSE401-infiltrated leaf was completely digested by *Nco*I (Figure 7B). To further examine the mutations, the target sites were analyzed by a Hi-TOM high-throughput sequencing assay with a 1% threshold. The sequencing results confirmed that 54% of the reads from the pNC-HSE401-gPDS-infiltrated leaf sample included insertions and deletions (Figure 7C). The insertions and deletions were grouped into nine different types, seven of which (35.5% reads) abolished the *Nco*I recognition site within the target region. These results indicated that pNC vectors were effective for genome editing in plants.

DISCUSSION

Nimble Cloning is a promising system for standardized molecular cloning in diverse applications. We have reported a few Nimble Cloning-compatible vectors previously (Yan et al., 2020; Tuo et al., 2021a, 2021b). These vectors have been provided to dozens of labs and successfully used for determining gene function (Li et al., 2021; Tan et al., 2021; Zhu et al., 2021, 2022; An et al., 2022). However, if Nimble Cloning is to be a widely used system for standardized molecular cloning, more compatible vectors will be needed. In this study, we constructed a set of 55 Nimble Cloning-compatible plant expression vectors. This vector system is very convenient, as the gene of interest flanked by the adapters can be transferred to any of the system vectors by a one-step Nimble Cloning reaction. We demonstrate that this vector system is effective for gene functional analysis in plants, including the study of gene ectopic expression, gene silencing, protein subcellular localization, protein–protein interaction, promoter activity, and CRISPR–Cas9-mediated gene editing. Some of the vectors constructed in this study have been successfully used in several laboratories (Jiang et al., 2022a, 2022b; Liu et al., 2022a, 2022b, 2022c; Han et al., 2022b). These vectors will further facilitate application of the Nimble Cloning system in plant science and provide a high-throughput toolkit for large-scale analysis of plant functional genomics.

Nimble Cloning uses the relatively rare-cutting restriction enzyme *Sfi*I to linearize the vector (Ghareeb et al., 2016). Thus, the vector backbone must be free of an *Sfi*I recognition site. The 55 pNC vectors include four types of plasmid backbone: the plant binary expression vectors pCambia, pGreen, and pBI121 and the transient expression vector pGEM. The pBI121backbone has one *Sfi*I recognition site (Frisch et al., 1995), and the pCambia backbone has five *Sfi*I recognition sites. The others are free of an *Sfi*I recognition site. The *Sfi*I recognition sites in pBI121 and pCambia have been eliminated by site-specific mutation in this study. These *Sfi*I-free plasmid backbones were

used to construct the pNC vectors. Thus, the backbones of all 55 pNC vectors in this study are *Sfi*I free and are suitable for use as backbones for construction of other Nimble Cloning-compatible vectors.

Two sets of pNC BiFC vectors were constructed in this study. The pNC-BiFC-ED1–4/Enn/Enc/Ecn/Ecc set was derived from the pSAT BiFC vectors that utilize EYFP split between amino acid residues 174 and 175 (Citovsky et al., 2006). The pSAT BiFC vectors contain plant expression cassettes that use the tandem CaMV35S promoter and the tobacco etch virus translation leader to promote high expression levels in a wide range of plant species and tissues. They have been widely used for studying protein–protein interactions in plants. One of their disadvantages is that the pSAT BiFC vectors are not plant binary vectors, so they cannot be used for *Agrobacterium*-mediated transformation. The BiFC expression cassettes must be transferred into the T-DNA region of binary plasmids and can then be used for *Agrobacterium*-mediated transformation (Tzfira et al., 2005). To avoid this inconvenience, the pNC BiFC vectors pNC-BiFC-ED1–4/Enn/Enc/Ecn/Ecc use the pGreen plasmid as a backbone. pGreen is a kind of plant binary vector with a relatively small size. It can be used directly for both transient and stable transformation by biolistic bombardment, protoplast transformation, and *Agrobacterium*-mediated transformation. The pGreen backbone vectors require a helper plasmid to provide a replication protein (RepA) in *Agrobacterium*. The *Agrobacterium*-containing pSoup helper plasmid can be used as a host for pGreen backbone vectors. The other set of pNC BiFC vectors (pNC-BiFC-VD1–4/Vnn/Vnc/Vcn/Vcc) are derived from pDOE BiFC vectors that utilize monomeric Venus split at residue 210. The BiFC system using Venus split at residue 210 can significantly reduce the background signal and increase confidence in both true positives and true negatives (Gookin and Assmann, 2014). Both sets of pNC BiFC vectors include four double ORF expression vectors that enable the expression of two test proteins with a single vector (Grefen and Blatt, 2012; Gookin and Assmann, 2014). Two genes of interest flanked by the adapters can be simultaneously cloned into the double ORF expression vector by one-step Nimble Cloning. The BiFC vectors with double ORF expression cassettes can enhance transformation efficiency compared with the single ORF expression BiFC vectors (Han et al., 2022a). We also observed similar results with the pNC BiFC vectors (data not shown).

CRISPR–Cas9-mediated genome editing is being rapidly adopted for gene functional analysis and molecular breeding in plants (Mao et al., 2019; Gao, 2021; Liu et al., 2022d). A convenient and effective vector will facilitate the study of genome editing (Xing et al., 2014; Ma et al., 2015). We constructed three pNC vectors for genome editing in plants. One or multiple target sequences can be assembled into the pNC genome-editing vectors by Nimble Cloning using procedures similar to those described previously for Gibson assembly. The main difference between Nimble Cloning and Gibson assembly for construction of genome-editing vectors is that Nimble Cloning uses circular plasmids, whereas Gibson assembly requires linearized plasmids. Furthermore, the Nimble Cloning-compatible vectors for genome editing are a part of a standardized cloning system that will facilitate the high-throughput construction of vectors for large-scale functional gene analysis in plants.

In summary, we developed a set of plant expression vectors based on Nimble Cloning. They are convenient and effective for gene functional analysis in plants and are freely available to the plant research community.

METHODS

Strains, plant material, and reagents

Escherichia coli strains DB3.1 and DH5α (Weidi Biotech, Shanghai, China) were used for cloning the pNC plasmids and the loaded pNC plasmids, respectively. *Rhizobium radiobacter* strain GV3101 was used for plant expression. Wild-type *N. benthamiana* was used for the analysis of transient expression. Nimble Mix was obtained from NC Biotech (Haikou, China). Primers were ordered from Sangon Biotech (Shanghai, China).

Plasmid construction

The Nimble Cloning-compatible vectors were generated by inserting the cloning cassette (NC frame) into the desired vector via Gibson assembly. The NC frame comprises the "adapter 1–*Sfi*I–ccdB gene–*Sfi*I–adapter 2" sequence. To generate pNC vectors with the pCambia backbone (Hajdukiewicz et al., 1994), *Sfi*I recognition sites in pCambia vectors were eliminated by site-specific mutation. The plant ectopic expression vectors pNC-Cam1304/2304/3304-35S were constructed by inserting the NC frame into pCambia1304/2304/3304 between the *Nco*I and *Pml*I sites, replacing the gfp/gus sequences; pNC-Cam1304/2304/3304-MCS were constructed by inserting the NC frame into pCambia1304/2304/3304 between the *Sac*I and *Pst*I sites; pNC-Cam1304/2304/3304-MCS35S were constructed by inserting the 35S promoter–NC frame–Nos terminator into pCambia1304/2304/3304 between the *Sal*I and *EcoR*I sites; pNC-Green/Green0029 were constructed by inserting the NC frame into pGreenII 0000 and pGreen0029 between the *Hind*III and *EcoR*I sites (Hellens et al., 2000); pNC-Green/Green0029-35S were constructed by inserting the 35S promotor–NC frame–Nos terminator into pGreenII 0000 and pGreen0029 between the *Hind*III and *EcoR*I sites; pNC-121-35S was constructed by inserting the NC frame into pBI121 between the *BamH*I and *Sac*I sites, replacing the gus sequence (Jefferson et al., 1987); and pNC-Amp-35S was constructed by inserting the 35S promotor–NC frame–35S terminator into pGEM-T between the *Sal*I and *Sph*I sites. The plant sublocation vectors pNC-Green/Cam1304/121-SubN/SubC were constructed by inserting gfp/NC-frame or NC-frame/gfp into pNC-Green-35S/Cam1304-35S/121-35S, replacing the NC frame; pNC-CFP/mCherry/RFP-N/C were constructed by inserting fluorescent protein genes and the NC frame into pNC-Green-35S, replacing the NC frame; pNC-Amp-GFP-N/C were constructed by inserting gfp/NC-frame or NC-frame/gfp into pNC-Amp-35S, replacing the NC frame. The plant RNAi vectors pNC-Cam1304/2304/3304-RNAi were constructed by inserting the NC frame–Pdk intron–inverted NC frame into pCambia1304/2304/3304 between the *Nco*I and *Pml*I sites, replacing the gfp/gus sequences. The plant BiFC vectors pNC-BiFC-ED1–4/Enn/Enc/Ecn/Ecc and pNC-BiFC-VD1–4/Vnn/Vnc/Vcn/Vcc were derived from the pSAT BiFC (Citovsky et al., 2006) and pDOE BiFC vectors (Gookin and Assmann, 2014) by inserting the NC frame into the MCSs, respectively. The plant genome-editing vectors pNC-HSE401/KSE401/BSE401 were constructed by inserting the NC frame into pHSE401/KSE401/BSE401 between the two *Bsa*I sites (Xing et al., 2014). The vector pNC-121-Pro for analysis of plant promoter activity was constructed by inserting the NC frame into pBI121 between the *Hind*III and *BamH*I sites, replacing the 35S promotor sequence. The primer sequences used are listed in Supplemental Table 2.

Nimble cloning reaction

The following components were added to a PCR microtube: 50–100 ng pNC plasmids (1–2 μl), 30–50 ng PCR fragment (1–3 μl), and 5 μl 2× Nimble Mix. Distilled water was added to the tube for a final volume of 10 μl. Tubes were mixed well by pipette and then incubated in a water bath or a thermocycler at 50°C for 0.5–1 h. The reaction mixture was

used for *E. coli* transformation or stored at −20°C if not used immediately (Yan et al., 2020).

N. benthamiana transient expression

Agroinfiltration was performed for *N. benthamiana* transient expression following previously reported procedures with a slight modification (Sparkes et al., 2006). *N. benthamiana* plants were grown in a growth chamber at 25°C under a 16-h-light/8-h-dark cycle. Plasmids were transformed into *Agrobacterium* strain GV3101. A single colony of each transformed *Agrobacterium* was used to inoculate 5 ml LB medium supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin. Bacteria were grown overnight to obtain an OD_{600} of 1.0–1.5 at 28°C with shaking at 200 RPM. The cultures were pelleted by centrifugation at 2000 × *g* for 5 min. The cells were resuspended with 1 ml infiltration buffer (50 mM MES [pH 5.6], 10 mM $MgCl_2$, and 100 mM acetosyringone) and then centrifuged again. The pelleted cells were diluted with infiltration buffer to a final OD_{600} of 0.1–0.3 and then incubated for 1–2 h at 25°C in the dark before agroinfiltration of *N. benthamiana* leaves using a 1-ml needleless syringe.

Fluorescence and GUS assays

The expression of fluorescent protein was observed under a confocal microscope (Olympus FluoView FV1000, Tokyo, Japan) or UV lamp (UVP, Upland, CA, USA). GUS assays were performed as described previously (Yan et al., 2012).

RNA isolation and real-time PCR

Total RNA was isolated with an RNAprep Pure Plant kit (Tiangen, China) and treated with DNase I to remove DNA contamination. Real-time PCR was performed as previously described (Yan et al., 2012).

Genome editing

N. benthamiana leaves were infiltrated by *A. tumefaciens* carrying the plasmid pNC-HSE401-gPDS. Genomic DNA was extracted from the infiltrated leaf at 5 dpi using a TransDirect Plant Tissue PCR Kit (Transgene, Beijing, China). The target regions were amplified with site-specific primers. Mutants were evaluated by Hi-TOM sequencing with a 1% threshold (Liu et al., 2019).

Availability of data and materials

The 55 Nimble Cloning-compatible vectors and their sequences and maps are available in Addgene with Addgene ID numbers 193384–193438 (Supplemental Table 1). They are also available from the corresponding authors upon request.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

P.Y., P.Z., and X.G. conceived and designed the project. P.Y. and H.D. performed the experiments. P.Y., D.T., and W.S. analyzed the data. P.Y. wrote the manuscript. All authors commented on the manuscript.

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