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Efficient assembly of long DNA fragments and multiple genes with improved nickase-based cloning and Cre/*loxP* recombination

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Summary

Functional genomics, synthetic biology and metabolic engineering require efficient tools to deliver long DNA fragments or multiple gene constructs. Although numerous DNA assembly methods exist, most are complicated, time-consuming and expensive. Here, we developed a simple and flexible strategy, unique nucleotide sequence-guided nicking endonuclease (UNiE)-mediated DNA assembly (UNiEDA), for efficient cloning of long DNAs and multigene stacking. In this system, a set of unique 15-nt 3′ single-strand overhangs were designed and produced by nicking endonucleases (nickases) in vectors and insert sequences. We introduced UNiEDA into our modified Cre/*loxP* recombination-mediated TransGene Stacking II (TGSII) system to generate an improved multigene stacking system we call TGSII-UNiE. Using TGSII-UNiE, we achieved efficient cloning of long DNA fragments of different sizes and assembly of multiple gene cassettes. Finally, we engineered and validated the biosynthesis of betanin in wild tobacco (*Nicotiana benthamiana*) leaves and transgenic rice (*Oryza sativa*) using multigene stacking constructs based on TGSII-UNiE. In conclusion, UNiEDA is an efficient, convenient and low-cost method for DNA cloning and multigene stacking, and the TGSII-UNiE system has important application prospects for plant functional genomics, genetic engineering and synthetic biology research.

Introduction

Molecular cloning and DNA assembly are crucial techniques widely used in genetic engineering. In conjunction with the development of multi-omics technology and functional genomics, more and more genomes, biosynthetic pathways and functional genes have been elucidated, increasing the demand for cloning and stacking of larger or more complex DNA molecules in various fields. To date, various DNA assembly strategies have been designed and developed. These include restriction fragment ligation-based methods using rare-cutting homing endonucleases and zinc-finger nuclease (Zeevi *et al*., 2012), BioBrick (Knight, 2003), Golden Gate cloning (Engler *et al*., 2008), DNA polymerase-dependent circular polymerase extension cloning (CPEC) (Quan and Tian, 2009), overlapping extension (OE) PCR cloning (Bryksin and Matsumura, 2010) and Ω-PCR (Chen *et al*., 2013), exonucleases-based sequence and ligation-independent cloning (SLIC) (Li and Elledge, 2007), In-Fusion (Benoit *et al*., 2006) and Gibson assembly (GA) (Gibson *et al*., 2009), site-specific recombination-based Gateway cloning (Walhout *et al*., 2000) and Cre/*loxP*-based assembly (Guo *et al*., 1999; Lin *et al*., 2003; Zhu *et al*., 2017; Zhu and Liu, 2021). These methods have facilitated the development of molecular cloning techniques.

Plant functional genomics, synthetic biology, molecular farming and genetic engineering of complex multiple traits or metabolic pathways often require cloning of long DNA (LDNA) fragments and stacking of multigenic genes (Zhu *et al*., 2020; Zhu *et al*., 2022). However, it is still difficult to meet all these needs using the above-mentioned methods. For example, DNA polymerase-dependent PCR cloning approaches are not suitable for LDNA cloning and may introduce undesired mutation during multi-round PCR. Improved traditional restriction fragment ligation methods with rare-cutting restriction enzyme sites are limited by the sparseness of such cloning sites (Zeevi *et al*., 2012). The isocaudomer-dependent BioBrick system and type IIS restriction endonucleases-based Golden Gate cloning enable parallel assembly of multiple short DNA fragments in a single-tube reaction (Anderson *et al*., 2010; Engler *et al*., 2009; Lampropoulos *et al*., 2013). As a widely used method, Golden Gate cloning for assembly of multiple fragments is usually effective, except when the assembled fragments contain multiple restriction sites. Several site-specific recombinase systems can effectively assemble large DNA fragments. Multisite Gateway cloning, using several sets of the λ-integrase recombination sites, allows assembly of several genes on the same vectors *in vitro* using the expensive commercial Gateway kit (Buntru *et al*., 2013; Karimi *et al*., 2007). The A118/TP901-1 recombinase-mediated

GA*A*NTRY (Gene Assembly in *Agrobacterium* by Nucleic acid Transfer using Recombinase technologY) system enables the stacking of multiple genes in *Agrobacterium tumefaciens* through multi-round recognition of specific attB and attP sites (Collier *et al*., 2018; Mccue *et al*., 2019; Hathwaik *et al*., 2021). The TransGene Stacking II (TGSII) system enables the assembly of multiple genes involved in different complex biosynthetic pathways by Cre/*loxP* and irreversible *loxP* site-mediated multi-round gene stacking cycles (Wang *et al*., 2020; Zhu *et al*., 2017; Zhu *et al*., 2018). These site-specific recombination systems have assembled large and complex multigene constructs, but their operation process is still relatively time-consuming and laborious for assembly of multiple genes due to the need for multiple rounds of recombination.

Compared with the restriction enzyme-based methods and site-specific recombination systems, exonuclease-mediated overlapping sequences-based methods for assembly of multiple DNA fragments are simpler. In SLIC and the In-Fusion reaction, 3′ homologous DNA ends are digested by T4 DNA polymerase in the absence of dNTPs to generate 5′ overhangs, while the GA utilizes T5 exonuclease to digest the 5′ ends to create 3′ overhangs (Gibson, 2011; Li and Elledge, 2007; Zhu *et al*., 2007). Then, the DNA fragments with complementary overhangs are annealed and joined together by RecA-mediated recombination or DNA polymerase/DNA ligase-mediated ligation *in vitro* (Benoit *et al*., 2006; Gibson, 2009; Gibson, 2011; Zhu *et al*., 2007). However, the commercial mixture kits for In-Fusion and GA are relatively expensive and are inefficient for stacking multiple fragments containing repetitive sequences.

Nicking endonucleases (NiEs) recognize specific short DNA sites and nick a strand of double-stranded DNA; therefore, they can be used to generate single-strand complementary overhangs (Abrosimova *et al*., 2019; Too *et al*., 2010). NiE-mediated ligation-independent cloning (NiE-LIC) can clone short DNA fragments (Gong *et al*., 2020; Wang *et al*., 2013; Yang *et al*., 2010). However, the cloning or assembly efficiency is low for multiple long DNA fragments due to the designed short overhanging sequences generated by NiEs, which limits the application of this method.

In this study, we designed a set of unique nucleotide sequences (UNSs) and used them for NiE-mediated (UNiE) DNA assembly (UNiEDA). Based on our TGSII system (Zhu *et al*., 2017; Zhu and Liu, 2021), we developed a new UNiE-compatible vector system, TGSII-UNiE, which can achieve quick and flexible multigene stacking. Using this system, we demonstrated betanin biosynthesis in wild tobacco (*Nicotiana benthamiana*) leaves and transgenic rice (*Oryza sativa*). This system has great potential for applications in plant functional genomics, synthetic biology and genetic improvement of agronomic traits.

Results

Development of the TGSII-UNiE vector system

Although NiEs were used for DNA assembly (Oster and Phillips, 2011; Yang *et al*., 2010; Wang *et al*., 2013; Gong *et al*., 2020), the assembly efficiency is low partially because the single-stranded ends generated by NiEs are relatively short (less than 10 nucleotides [nt]). However, single-stranded overhangs of 10 or 15 nt generated by NiEs have relatively higher assembly efficiency (Wang *et al*., 2013). In this study, we designed a set of (21 in total) 15-nt UNSs with a melting temperature (T_m) of 46 °C as the NiE-generated single-strand ends; they did not contain palindrome structure, and there was no partial pairing between the UNSs (Table S1). A set of UNS-containing PCR chimeric primer pairs that carry an NiE (such as Nb.BtsI) site (Table S2) were used to generate UNiE-containing LDNAs or cassettes.

As an efficient multigene stacking system, TGSII has been used to engineer anthocyanin and astaxanthin biosynthesis and to improve photosynthetic efficiency in rice (Wang *et al*., 2020; Zhu *et al*., 2017; Zhu *et al*., 2018). To develop UNiE-compatible vectors for LDNA cloning and multigene stacking, we first designed a core UNiE cloning cassette containing a *lacZ* expression cassette flanked by two UNSs and six Nb.BtsI sites (Figure 1a). We then used the UNiE cloning cassette in various vectors to generate single-stranded 15-nt overhangs at the 3′ end and selected positive clones by blue/white screening. After subcloning the UNiE cloning cassette into the binary acceptor vectors and donor vectors of TGSII, we developed an improved stacking system that we called TGSII-UNiE (Figure 1).

The TGSII-UNiE system consists of six binary vectors (pYL1300H/N/B-UNiE and pYLTAC380H/N/B-UNiE) with different selectable marker genes and two donor vectors (pYL322d1-UNiE and pYL322d2-UNiE; Figures 1b-e and S1). Because the pYL1300H/N/B-UNiE vectors with the pCAMBIA vector backbone have only moderate loading capacity for inserted DNA length (generally <15 kb), this set of vectors contained the UNiE cloning cassette for direct cloning of several target genes but not the Cre/*loxP* recombination elements (*loxP*/I-*Sce* I/*loxP1R*) originally used in TGSII for multigene assembly. However, the pYLTAC380H/N/B-UNiE vectors based on the transformation-competent artificial chromosome (TAC) (Liu *et al*., 1999) that has long (>100 kb) DNA cloning and transferring capabilities contain both the UNiE cloning cassette and the core *loxP*/I-*Sce* I/*loxP1R* elements. Therefore, pYLTAC380H/N/B-UNiE vectors allow cloning of multiple DNA fragments through the UNiEDA strategy and Cre/*loxP* recombination. The pYL322d1-UNiE and pYL322d2-UNiE donor vectors are capable of cloning long (up to ~20 kb) DNA fragments and deliver target genes into the acceptor vectors by rotational Cre/*loxP* recombination as described by Zhu *et al*. (2017). Additionally, we constructed a modified binary acceptor, pYLTAC380GW-UNiE, for transgenic marker-free purposes (Figures S1 and S2) in combination with the previous marker/marker excision plasmids (Zhu *et al*., 2017).

UNiEDA enables efficient cloning of target DNA

UNiEDA-mediated cloning was performed as shown in Figure 2a. Before carrying out UNiEDA, the distribution of the used NiE sites in the vectors and target sequences (in both strands) should be analysed to confirm that there are not two or more nicking sites located closely along the same strand or opposite strands of the target sequences, for example, <50 bp with <45% GC content and T_m < 75 °C between the sites. This is because the generation of closely spaced NiE nicks along the strands will break the sequence or melt out a short strand after the heat treatment at 70 °C. However, the presence of NiE sites with longer distances (with $T_m \geq 75$ °C) enables the base-pairing of the doubled strands flanked by the nicks to be stable enough at 70 °C (not denaturing), thus do not affect the use of UNiEDA. The 70 °C heat treatment is performed to melt out the nicked strands (with a T_m of 48 °C, see below), generating single-stranded sticky ends of the vectors and target sequences; this temperature does not

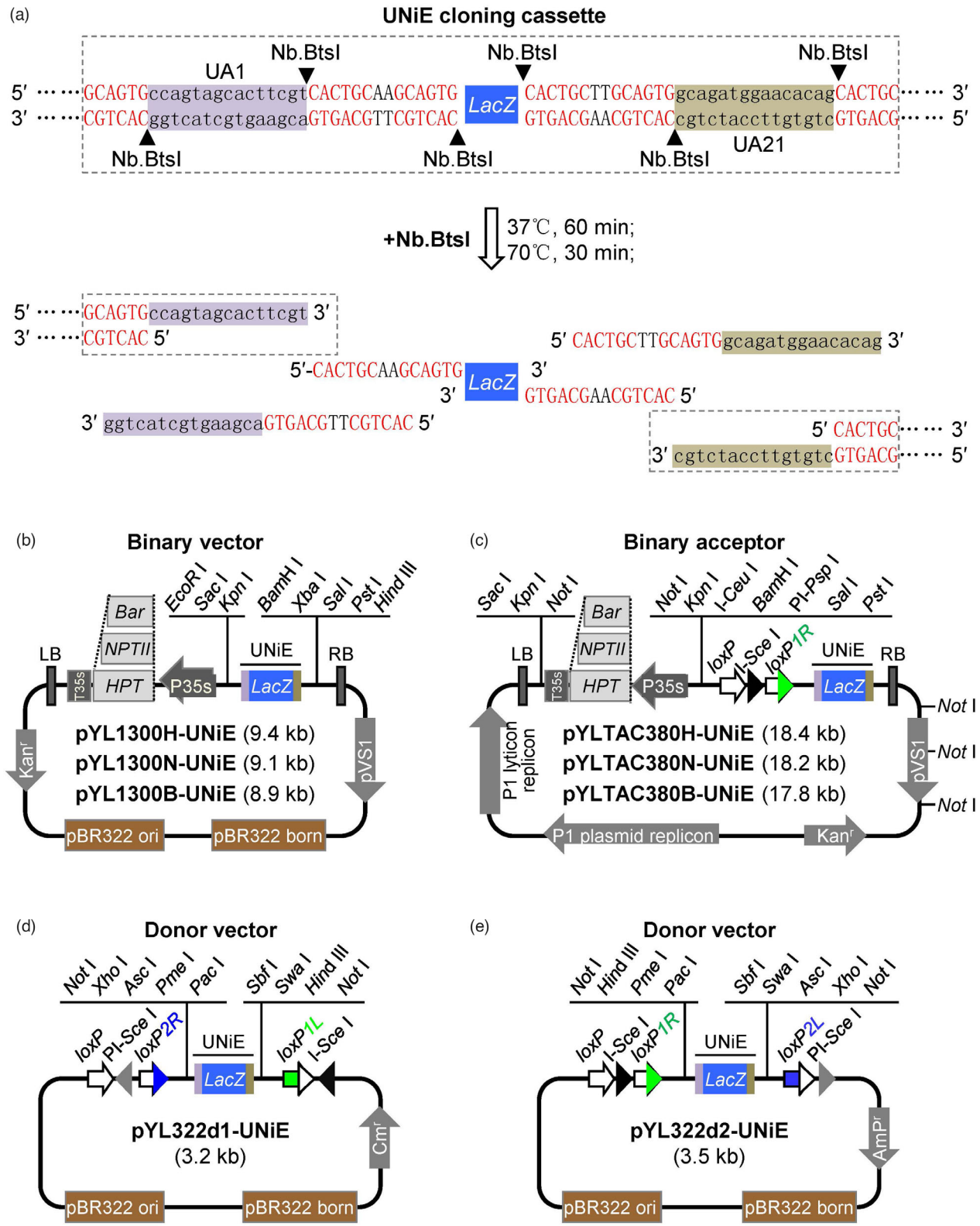

Figure 1 TGSII-UNiE vector system. (a) DNA sequence features of the UNiE cloning cassette. Red letters represent recognition sequences of Nb.BtsI, and arrows indicate its cleavage sites. The letters highlighted purple and brown are UNSs. After digestion of the vectors with Nb.BtsI, vector fragments with 3′ 15-nt UNS overhangs are produced. (b-e) Structures of UNiEDA-compatible TGSII-UNiE vectors: pYL1300H/N/B-UNiE (b) and pYLTAC380H/N/B-UNiE (c) binary vectors; TGSII-based pYL322d1-UNiE (d) and pYL322d2-UNiE (e) donor vectors. *lacZ* was used as the selectable marker of negative clones.

denature double-stranded DNAs without nicks or with nicks having longer distances between them. Briefly, a target DNA fragment is amplified using a pair of chimeric primers (each consisting of a 15-nt UNS 5′-end, a NiE site [Nb.BtsI or Nb.BsrDI] and a 3′-end target-binding sequence) and purified. The target fragment and vector are digested by the NiE (Nb.BtsI) at 37 °C, and then heated at 70 °C to melt out the nicked 15-nt strands, generating 15-nt overhangs at 3′ ends and inactivating the enzyme. The fragment and vector are purified using a purification kit or dialysis on Millipore filters with 0.2× TE buffer. Then, they are ligated using a heat-stable *Taq* DNA ligase and annealing-ligation (between 46 °C and 60 °C) thermal-cycling (see Methods), since *Taq* DNA ligase has higher activities at 55–60 °C.

To examine the cloning efficiency of LDNAs, we used UNiEDA to clone three DNA fragments (10.3, 14.8 and 22.9 kb) into the pYL1300H-UNiE and pYLTAC380H-UNiE binary vectors, respectively. We determined that the optimal initial annealing temperature for thermal-cycle ligation was 46 °C by examining the cloning efficiency of a 10.3-kb LDNA into pYL1300H-UNiE using different annealing temperatures (Figure S3). We compared positive cloning rates between UNiEDA and GA by cloning a 10.3-kb LDNA into the pYL1300H-UNiE vector and a 14.8-kb LDNA into the pYLTAC380H-UNiE vector, and the results showed that the cloning efficiencies of these two methods were similar (Figure 2b). Additionally, we found that a 1 : 3 molar ratio of vector to LDNA was most efficient for UNiEDA. Due to its larger cloning capability, pYLTAC380H-UNiE had a higher cloning efficiency than pYL1300H-UNiE for the same target fragments (Figures 2b and c). We confirmed the integrity of the resulting constructs using restriction enzyme digestion (Figures 2d and e). These results indicate that UNiEDA is an efficient approach for LDNA cloning.

Simultaneous assembly of several genes directly into binary vectors using UNiEDA

One noticeable feature of the TGSII-UNiE system is that direct assembly of multiple genes into the T-DNA region of the binary vectors can be done in a one-tube reaction. The metabolite red betanin is used as a reporter for gene expression and transformation; its biosynthesis involves the following genes: *BvCYP76AD1S* (abbr. C), *BvDODA1S* (abbr. D), *cDOPA5GT* (abbr. G) and *ADH* (abbr. A) (Chen *et al.*, 2017; Grützner *et al.*, 2021). We used these four genes, along with another fluorescent marker gene, *eGFP* (abbr. eG), in a gene stacking assay. We prepared each gene expression cassette (containing a CaMV35S promoter [P35s], a target gene and a nopaline synthase terminator [Tnos]) by overlapping PCR. Then, we amplified the five gene cassettes by sets of chimeric primers and digested them with Nb.BtsI (Figure 3a). We then assembled the five gene cassettes with 3′ UNS overhangs into Nb.BtsI-digested pYL1300H-UNiE or pYLTAC380H-UNiE binary vectors in a one-step reaction (Figure 3a).

Since the multiple gene cassettes for betanin biosynthesis had repetitive sequences (due to the use of the same promoter and terminator) and were not suitable for GA reactions (GA is not compatible for repeat-containing sequences because of the interaction among T5 exonuclease-generated single-stranded repetitive sequences), we divided the aforementioned 10.3-kb LDNA into four fragments (each ~2.5 kb) to compare the assembly efficiency of UNiEDA and GA. For the pYL1300H-UNiE vector, the UNiEDA strategy yielded a slightly higher proportion of positive clones (12.85%) than GA (9.03%); for the pYLTAC380H-UNiE vector, the UNiEDA and GA methods produced similar proportions of positive clones (17.02% and 18.75%, respectively; Figure 3b). These results show that the two methods have similar cloning efficiency.

We then investigated the efficiency of the methods for the assembly of three, four and five genes (*CDeG*, *CDGeG* and *CDGAeG*) for betanin biosynthetic pathway using pYL1300H-UNiE and pYLTAC380H-UNiE vectors. Assembly efficiency decreased with increasing number of fragments for both binary vectors (Figure 3c). For a given number of fragments, pYLTAC380H-UNiE cloning was more efficient than pYL1300H-UNiE cloning (Figure 3c). We verified the structural stability of three pYL1300H-based constructs (pYL1300H-*CDeG*, pYL1300H-*CDGeG* and pYL1300H-*CDGAeG*) in *E. coli* and *A. tumefaciens* by *Kpn* I/*BamH* I digestion (Figure 3d), and that of three pYLTAC380H-based plasmids (pYLTAC380H-*CDeG*, pYLTAC380H-*CDGeG* and pYLTAC380H-*CDGAeG*) by *Not* I digestion (Figure 3e). In addition, we confirmed the gene expression cassettes (*C*, *D*, *G*, *A* and *eG*) and the *HPT* gene of all UNiEDA-based multigene vectors in both *E. coli* and *A. tumefaciens* by PCR analyses (Figure S4). These results indicate that the TGSII-UNiE system enables simultaneous assembly of several genes in a one-tube reaction by UNiEDA.

TGSII-UNiE is easier and more efficient than TGSII for multigene stacking

Our previous TGSII uses repeated rounds of Cre/*loxP* recombination to assemble multiple gene cassettes, which is relatively time-consuming and complex. The improved TGSII-UNiE system utilizes the UNiEDA strategy to assist Cre/*loxP* recombination, simplifying the operation and improving the efficiency and capability of multigene assembly. To test its assembly capability for more genes involved in different synthetic pathways, we further stacked the hydroxycinnamoylputrescine (*HP*) gene cluster and three glutamine synthetase (*GS*) genes into the binary vector pYLTAC380H-CDGAeG constructed for betanin biosynthesis (Figure 4a).

The *HP* gene cluster, consisting of a decarboxylase gene (*OsODC*) and two putrescine hydroxycinnamoyl acyltransferases genes (*OsPHT3* and *OsPHT4*), regulates disease resistance in rice by contributing to immunity and cell death (Fang *et al.*, 2021). Three *GS* genes (*OsGS1;1*, *OsGS1;2* and *OsGS1;3*) are involved in nitrogen assimilation and recycling in plants; they catalyse condensation of glutamate and ammonium to glutamine (Kusano *et al.*, 2011; Tabuchi *et al.*, 2007). Through UNiEDA, we simultaneously cloned three *HP* genes (total size ~9.7 kb) and three *GS* genes (total size ~16.7 kb) into the donor vectors pYL322d1-UNiE and pYL322d2-UNiE, respectively, and verified the resulting constructs by *Pme* I/*Sbf* I digestion (Figure 4b). After only two rounds of Cre/*loxP* recombination, all six genes (~26.4 kb in total) in the two donor vectors were sequentially inserted into pYLTAC380H-CDGAeG to produce a multigene binary vector pYLTAC380H-GSs/HPs/CDGAeG, which contained a total of 12 genes (including *HPT*) in the T-DNA region with about 40 kb (Figure 4a). We confirmed the structures of these constructs (pYLTAC380H-CDGAeG, pYLTAC380H-HPs/CDGAeG and pYLTAC380H-GSs/HPs/CDGAeG) and their stability in both *E. coli* and *A. tumefaciens* by colony PCR and *Not* I digestion (Figures 4c and S5). These results demonstrate that the TGSII-UNiE system is easy to manipulate for rapid assembly of multiple genes, which is valuable as a means to combine diverse biosynthetic pathways and for genetic improvement of complex traits.

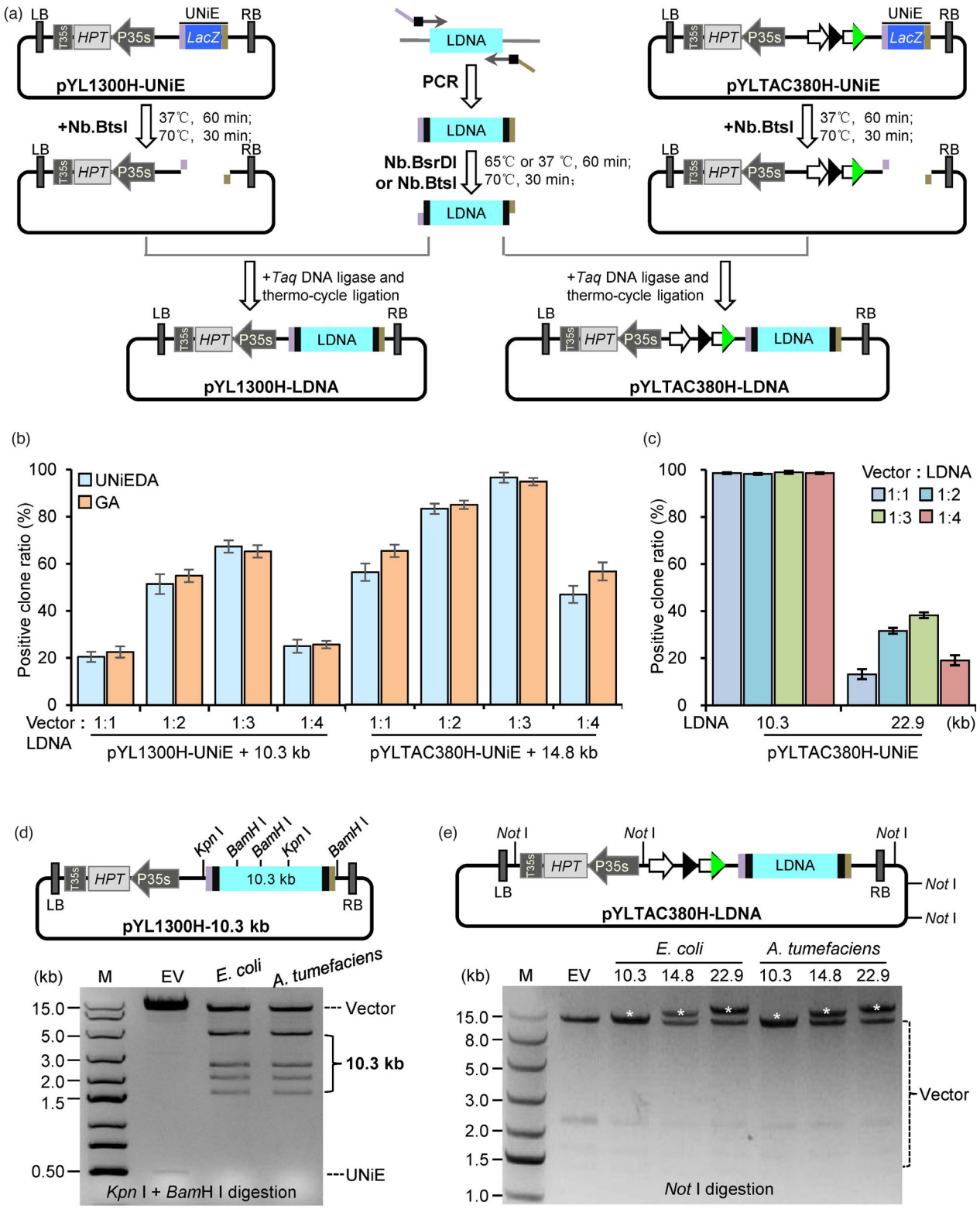

Figure 2 Efficient cloning of long DNA fragments (LDNA) using the UNiEDA method. (a) Target DNA is amplified with chimeric primers and digested with used Nb.BsrDI or Nb.BtsI. The resultant fragment with 3′ 15-nt UNS overhangs is then cloned into Nb.BtsI-digested pYL1300H-UNiE or pYLTAC380H-UNiE. (b) Cloning efficiencies of a 10.3-kb long DNA (LDNA) in pYL1300H-UNiE and a 14.8-kb LDNA in pYLTAC380H-UNiE using the UNiEDA and GA (Gibson assembly) methods with different vector:LDNA molar ratios. (c) Cloning capacities and efficiencies of UNiEDA in pYLTAC380H-UNiE determined with 10.3-kb and 22.9-kb LDNAs with different vector:LDNA molar ratios. (d) *Kpn* I- and *Bam*H I-digestion analysis of the pYL1300H-10.3 kb constructs in *E. coli* and *A. tumefaciens*. (e) *Not* I-digestion analysis of the pYLTAC380H-based constructs carrying the inserts of different sizes in *E. coli* and *A. tumefaciens*. White stars indicate the insert DNA fragments. M, DNA ladder marker, EV, empty vector.

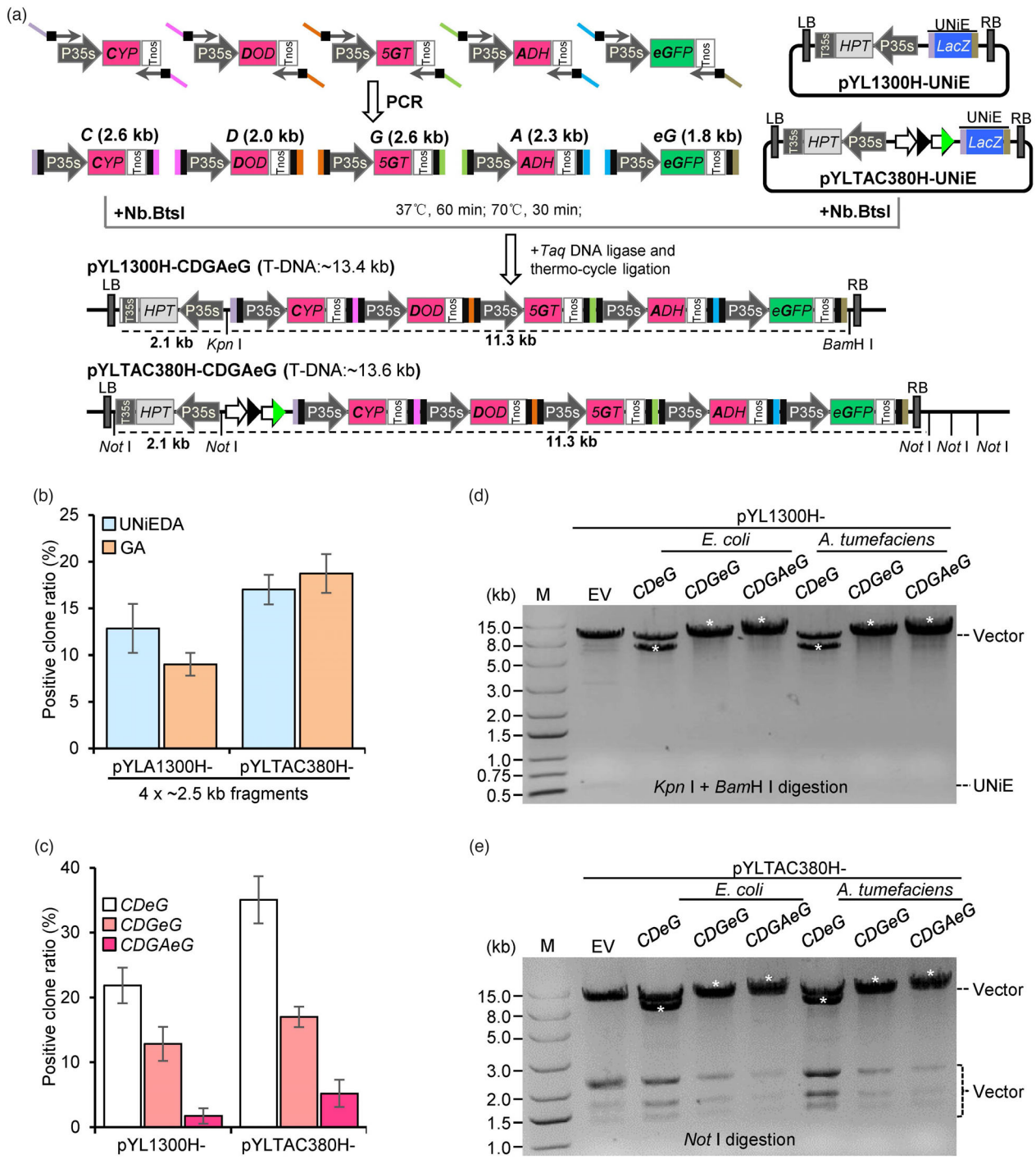

Figure 3 Simultaneous assembly of multiple genes using the UNiEDA strategy. (a) Expression cassettes carrying *BvCYP76AD1S* (*CYP*), *BvDODA1S* (*DOD*), *cDOPA5GT* (*5GT*), *ADH* and *eGFP* were amplified with chimeric primers and digested with Nb.BtsI. Then, the five fragments were simultaneously assembled into Nb.BtsI-digested pYL1300H-UNiE or pYLTAC380H-UNiE. (b) Cloning efficiencies of four fragments (each ~2.5 kb) in pYL1300H-UNiE and pYLTAC380H-UNiE using the UNiEDA and GA methods. (c) Cloning efficiencies of various expression units (CDeG, CDGeG and CDGAeG) stacked in pYL1300H-UNiE and pYLTAC380H-UNiE using UNiEDA. (d) *Kpn* I- and *Bam*H I-digestion analysis of pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG constructs in *E. coli* and *A. tumefaciens*. (e) *Not* I-digestion analysis of pYLTAC380H-CDeG, pYLTAC380H-CDGeG and pYLTAC380H-CDGAeG constructs in *E. coli* and *A. tumefaciens*. White stars indicate the insert DNA fragments. M: DNA ladder marker, EV: empty vector.

Engineering betanin biosynthesis in *N. benthamiana* leaves and rice

To confirm the reliability and functionality of constructs generated by TGSII-UNiE, we transformed six plasmids containing different gene combinations for betanin biosynthesis (pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG; pYLTAC380H-CDeG, pYLTAC380H-CDGeG and pYLTAC380H-CDGAeG) and two eGFP plasmids (pYL1300H-eG and pYLTAC380H-eG) into *A. tumefaciens* GV3101 for transient expression in *N. benthamiana*

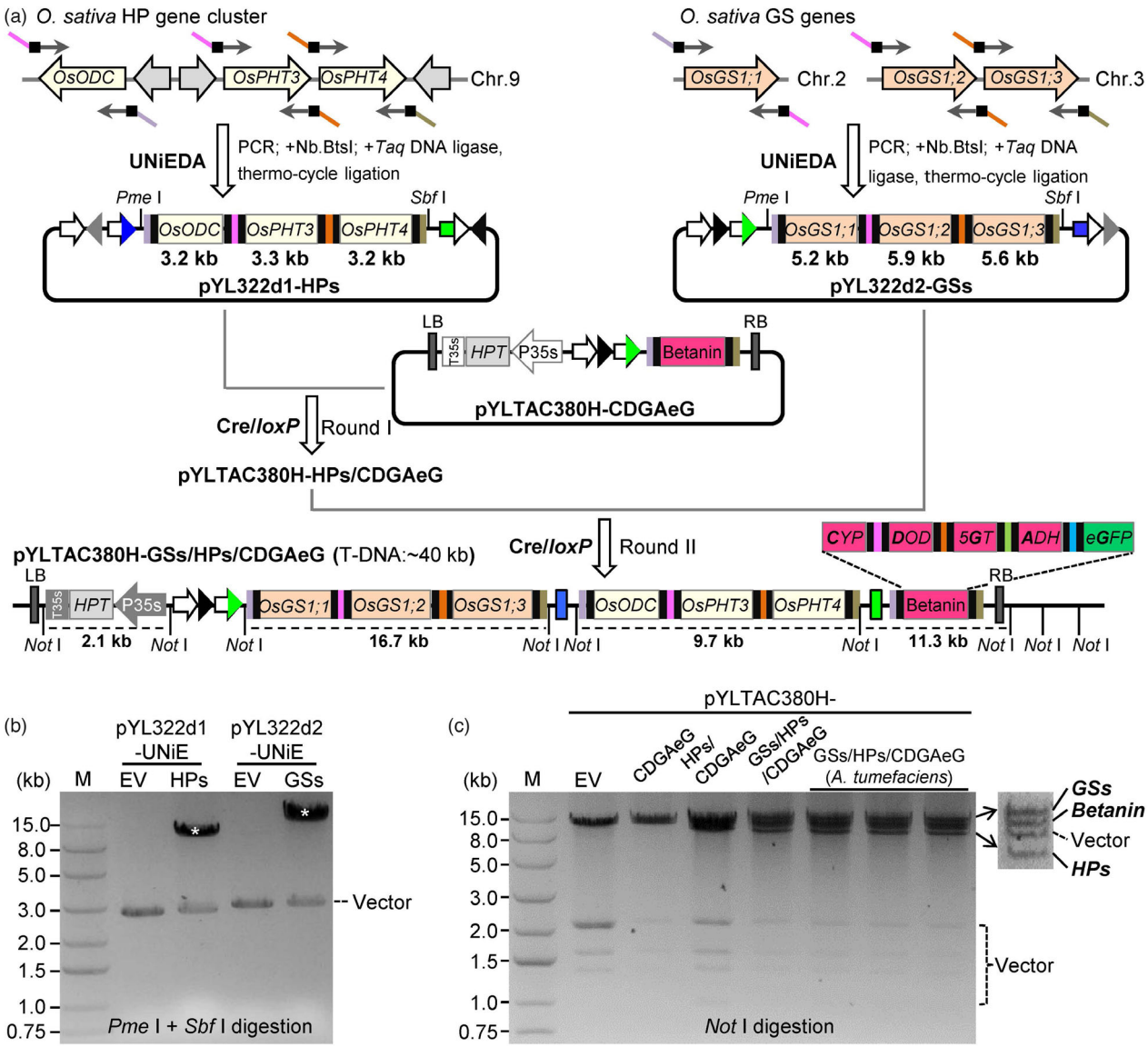

Figure 4 Efficient assembly of multigene stacking by the TGSII-UNiE system. (a) Using the UNiEDA method, three *HP* genes were assembled into pYL322d1-UNiE and three *GS* genes were assembled into pYL322d2-UNiE. Then, the *HP* and *GS* genes were recombined into a pYLTAC380H-betanin binary plasmid by two rounds of Cre/*loxP* recombination. (b) *Pme* I- and *Sbf* I-digestion analysis of the pYL322d1-*HP* and pYL322d2-*GS* plasmids. White stars indicate the insert DNA fragments. (c) *Not* I-digestion analysis of pYLTAC380H-CDGAeG, pYLTAC380H-*HPs*/CDGAeG and pYLTAC380H-*GSs*/*HPs*/CDGAeG constructs in *E. coli* and *A. tumefaciens*. M: DNA ladder marker, EV: empty vector.

leaves. The qRT-PCR results showed that the four betanin biosynthetic genes (*BvCYP76AD1S*, *BvDODA1S*, *cDOPA5GT* and *ADH*) and *eGFP* were highly expressed in infiltrated leaves of each construct (Figure 5a-e). Red betanin was successfully synthesized by pYL1300H-based or pYLTAC380H-based CDGeG and CDGAeG vectors in *N. benthamiana* leaves (Figure 5f). The constructs pYL1300H-CDeG and pYLTAC380H-CDeG produced a yellow pigment, possibly derived from production of betalains other than betanin, as previously reported (Grützner *et al.*, 2021). The two CDGAeG constructs produced a dark red colour, indicative of a higher synthetic capacity than the two CDGeG constructs possessed. However, due to the reduction of *eGFP* expression and interference from betanin pigment, the fluorescence signal of eGFP decreased in infiltrated leaves with the CDGeG and CDGAeG vectors (Figure 5f). Furthermore, the observed colour of the pigment extracts and the relative content of betanin also indicated that the three genes (*BvCYP76AD1S*, *BvDODA1S* and *cDOPA5GT*) in the CDGeG vectors were necessary for betanin biosynthesis; adding the *ADH* gene in the CDGAeG constructs further promoted betanin biosynthesis (Figure 5g and h). Additionally, our data suggest that the pCAMBIA-based pYL1300H/N/B-UNiE vectors are better than the TAC-based pYLTAC380H/N/B-UNiE constructs for the transient expression of multiple genes in plants.

Moreover, we introduced the pYL1300H-eG, pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG constructs into rice calli, respectively, by *Agrobacterium*-mediated transformation. Same as the results in *N. benthamiana* leaves, red betanin was successfully obtained in transgenic rice calli with pYL1300H-CDGeG and pYL1300H-CDGAeG, and the transgenic yellow calli

were generated by pYL1300H-CDeG vector (Figure 6a). Furthermore, one type of pale-yellow transgenic resistant calli was also produced by pYL1300H-CDGeG and pYL1300H-CDGAeG. Genotyping of transgenic hygromycin-resistance calli showed that all the corresponding gene expression units were positively detected in the pYL1300H-CDeG-transformed red calli, but only parts of the transgenes were presented in the pale-yellow calli (Figures 6a and S6a-c). Moreover, green fluorescence produced by eGFP was observed in all red calli but only in some pale-yellow calli. By differentiating and regenerating hundreds of pYL1300H-CDGAeG transformed calli, only several transgenic rice plants with red leaves and roots that synthesized betanin were obtained (Figure 6b), while most of the red calli turn brown during differentiation. Genotyping of the transgenic plants showed that four betanin synthesis genes and eGFP were presented in the red plants, while only parts of the genes were detected in the green plants (Figure S6d). Moreover, the qRT-PCR results showed that the four betanin biosynthetic genes (*BvCYP76AD1S*, *BvDODA1S*, *cDOPA5GT* and *ADH*) and *eGFP* were all highly expressed in transgenic red plants, and partial genes were expressed in transgenic green plants (Figure 6c–g). These results indicated that repeated use of the P35s promoters might affect in some cases the transgene stability or their expression. The multigene construct pYL1300H-CDeG, containing four P35s promoters, remained stable in *E. coli*, *Agrobacterium*, and in transgenic plants. Thus, use of more than four times of the same promoter in a construct to drive different target genes is not recommended in multigene transformation. In addition, over accumulation of betanin in red calli may produce negative effect on plant regeneration.

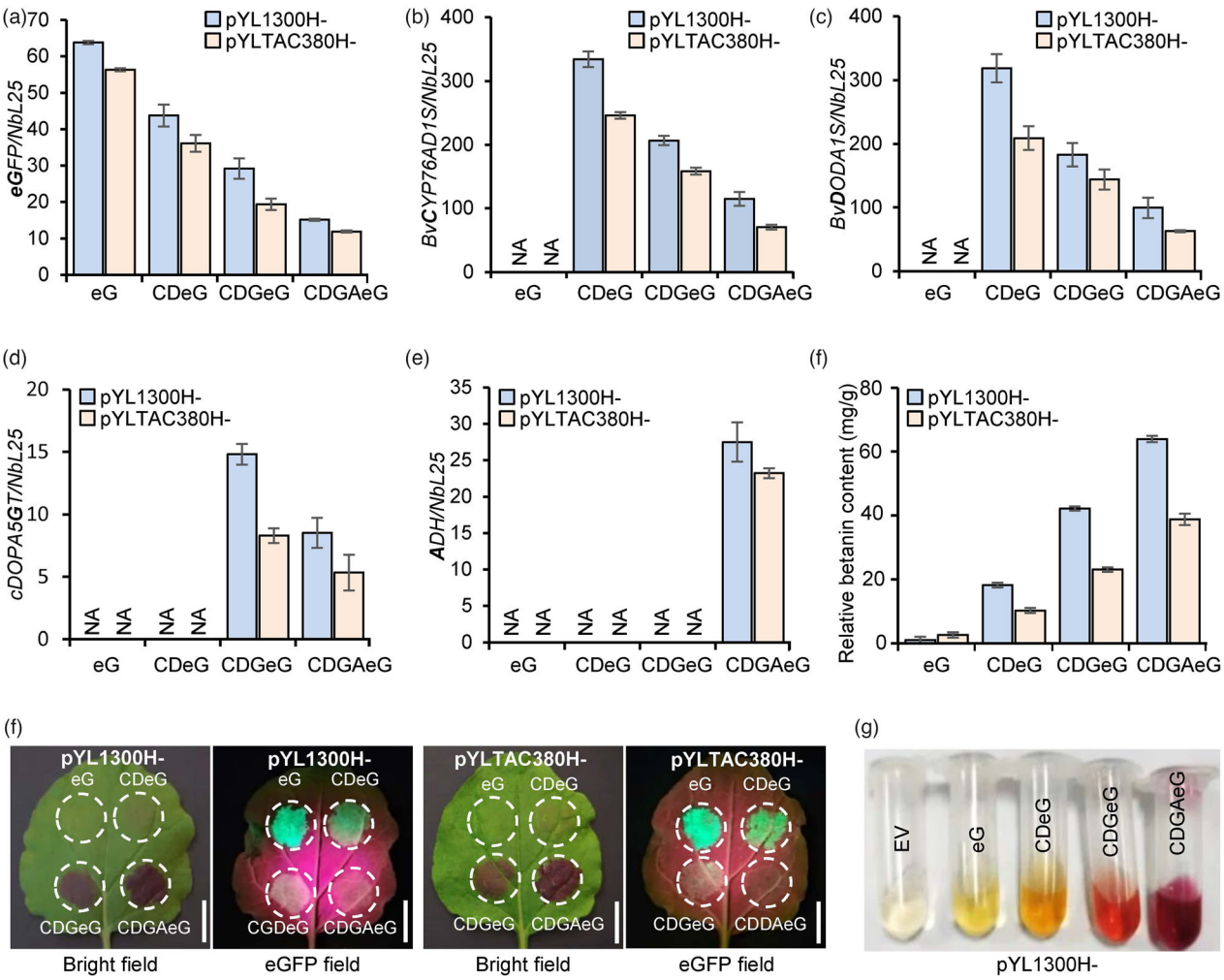

Figure 5 Engineering of betanin biosynthesis in *N. benthamiana* leaves. (a-e) Expression levels of *eGFP* (a), *BvCYP76AD1S* (b), *BvDODA1S* (c), *cDOPA5GT* (d) and *ADH* (e) in *N. benthamiana* leaves were measured by qRT-PCR. The *N. benthamiana* gene *NbL25* was used as an internal control. NA: not available. Error bars represent standard deviations (SD) of three biological replicas. (f) Phenotypes of *N. benthamiana* leaves infiltrated with pYL1300H-eG (negative control), pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG; and pYLTAC380H-eG (negative control), pYLTAC380H-CDeG, pYLTAC380H-CDGeG and pYLTAC380H-CDGAeG constructs. Bright-field images were photographed under LED excitation. eGFP field images were taken under 440–460 nm excitation with a portable fluorescence detector. Scale bar: 2 cm. (g) Colorization of betanin extract solution from infiltrated *N. benthamiana* leaves with pYL1300H-eG, pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG constructs. EV, empty vector as negative control. (h) Relative betanin contents in *N. benthamiana* leaves transfected with pYL1300H-eG, pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG; and pYLTAC380H-eG, pYLTAC380H-CDeG, pYLTAC380H-CDGeG and pYLTAC380H-CDGAeG constructs, respectively. Data are shown as mean ± standard deviation of biological replicas.

Taken together, these results confirm that the multigene vectors constructed by the UNiEDA strategy were functional and effective.

Discussion

Although nicking of DNA strands with NiEs, such as Nb.BtsI, Nb.BsrDI and Nt.BbvCI, has been utilized for DNA engineering and cloning (Bitinaite *et al*., 2007; Gong *et al*., 2020; Oster and Phillips, 2011; Yang *et al*., 2010), its application in LDNA cloning and multigene assembly is still limited. In this study, we designed a set of optimized 15-nt UNSs (Table S1) to improve the cloning efficiency of UNiEDA. Furthermore, to facilitate vector construction, we designed a UNiE cloning cassette containing the assembly elements and introduced it into our previous multigene stacking vector system, TGSII, to develop TGSII-UNiE for cloning of large fragments and stacking of multiple genes (Figures 1 and S1). The UNiE cloning cassette of TGSII-UNiE vectors exploits NiEs to generate optimized single-stranded overhangs on the vectors. We used the *lacZ* marker to select positive clones based on their white colour. Additionally, we further adopted the thermostable *Taq* DNA ligase and thermal cycling to promote the efficiencies of annealing (at 46 °C) and ligation (at 60 °C). These improvements make the UNiEDA strategy superior to previously reported NiE-mediated cloning methods and more beneficial for cloning or stacking large DNA fragments and multiple genes. Using TGSII-UNiE, we successfully cloned LDNA fragments of different sizes (the largest had 22.9 kb; Figure 2) and directly stacked five genes (total length ~11.3 kb; Figure 3) into the binary vectors. The largest construct with multiple gene cassettes had 12 genes and was ~40 kb (Figure 4). Additionally, multiple genes can be connected into an LDNA by overlapping/STI (Suppression Thermo-Interlaced) PCR (Zhao *et al*., 2022), and then, the LDNA can be cloned into a binary vector by UNiEDA. GA has been widely used in molecular cloning due to its easy operation; however, the commercial kits for this method are relatively expensive (e.g. NEB #E5520S, $18 per reaction). Comparison of the efficiency of large fragment cloning and multigene assembly showed that our UNiEDA strategy and GA have similar cloning efficiencies (Figures 2b and 3b); however, UNiEDA is much less costly (~$3 per reaction). Nonetheless, the UNiEDA method also has some limitations. For example, the UNiEDA leaves the nickase recognition sites and adapter sequences in the assembled constructs, but GA can be seamless. In addition, for some improved GA methods, such as Hot Fusion (Fu *et al*., 2014), the use of home-made reaction mixture does not require the purchase of commercial kit and reduces the price. Overall, UNiEDA strategy is a good choice for assembly of multiple fragments for its efficiency and low-cost.

With the rapid development of synthetic biology, genetic engineering of multiple traits and biosynthesis of bioactive substances, the delivery of more genes or entire sets of genes for biosynthetic pathways is required. Use of multigene vector systems for simultaneously stacking target genes in single T-DNA region is the most efficient method (Dafny-Yelin *et al*., 2007; Zhu *et al*., 2020). Site-specific recombination systems such as Multisite Gateway (Buntru *et al*., 2013), our previous Cre/*loxP*-based TGSII (Zhu *et al*., 2017) or GA*A*NTRY (Collier *et al*., 2018) enable stacking of multiple genes; however, these methods are quite time-consuming. Compared to these systems, our newly developed TGSII-UNiE system has more beneficial for transgene stacking, which can assemble more genes in parallel into the binary acceptors and/or the donor vectors at the same time, and then, the stacking of many genes from the same or different synthetic pathways is rapidly completed by a few rounds of Cre/*loxP* recombination (Figure 4). For the delivery of a few genes (at least up to the five inserts we tested), they could be assembled directly *in vitro* into a binary vector without the need for cumbersome separate steps, as shown by our test in which we stacked four betanin synthetic genes and one *eGFP* marker (Figure 3). If more genes need to be assembled, several genes can be assembled into the UNiE-donor vectors at one time and then delivered into the TGSII binary acceptor through a single recombination cycle. Using this method, we successfully assembled 11 genes (with a T-DNA region of ~40 kb) for three different synthetic pathways (Figure 4). Moreover, based on the large cloning capacity of the TAC-based binary acceptor vectors (>100 kb; Liu *et al*., 1999), and the unrestricted Cre/*loxP*-recombination cycles (Lin *et al*., 2003; Zhu *et al*., 2017), we believe that stacking of more genes is feasible. Additionally, the UNiEDA method allows the splicing of multiple DNA fragments (e.g. assembly of promoter–CDS–terminator cassettes, gene–gene fusion or modification of plasmid fragments) in one reaction. By simply inserting the UNiE cloning cassette into suitable plasmids, new types of acceptor and donor vectors can be developed for DNA assembly in other organisms. Nevertheless, for the UNiEDA method, the use of high-fidelity DNA polymerases is highly recommended for large fragment amplification or multi-DNA fragment assembly to minimize undesired mutations caused by PCR amplification.

In addition, multiple promoters are required for multigene transformation. Due to limitation of the number of useful promoters, in some cases a promoter may be used repeatedly in the constructs. However, how many times of a promoter can be effectively used to drive different genes in a construct is not clear. In this study, red betanin as a reporter was successful synthesized in leaves of *N. benthamiana* and transgenic red rice calli and plants using TGSII-UNiE based multigene vectors (Figures 5 and 6), but loss of some transgenes occurred in the transgenic pale-yellow calli and green plants (Figures 6 and S6). This may be due to the excessive reuse of the P35s promoter leading to the occurrence of recombination deletions. Therefore, multigene vectors should reduce the times of repeated use of the same promoters, and test structure stability of the constructs in *E. coli* and *A. tumefaciens*. Reuse of the same promoter is recommended no more than 4 times in the same multigene constructs.

In summary, the novel TGSII-UNiE system based on UNiEDA and Cre/*loxP* recombination developed in this study has the advantages of simple design, easy operation, efficiency, time saving and low cost, which can effectively clone long DNA fragments and rapidly assemble multiple genes, and will have broad application prospects in plant functional genomics, genetic engineering, synthetic biology and genetic improvement of agronomic traits.

Materials and methods

Development of the TGSII-UNiE vector system

To develop a set of UNiEDA-compatible vectors, a UNiE cloning cassette containing *lacZ*, two UNSs and six Nb.BtsI sites was generated by overlapping PCR, using *lacZ* of the pYL322d1 plasmid (Zhu *et al*., 2017) as a template. The resulting UNiE fragment was then cloned into the pYL1300H/N/B (digested with *Kpn* I and *BamH* I), pYLTAC380H/N/B and pYLTAC380GW (amplified by 380HV-F/380HV-R; where H, N and B represent

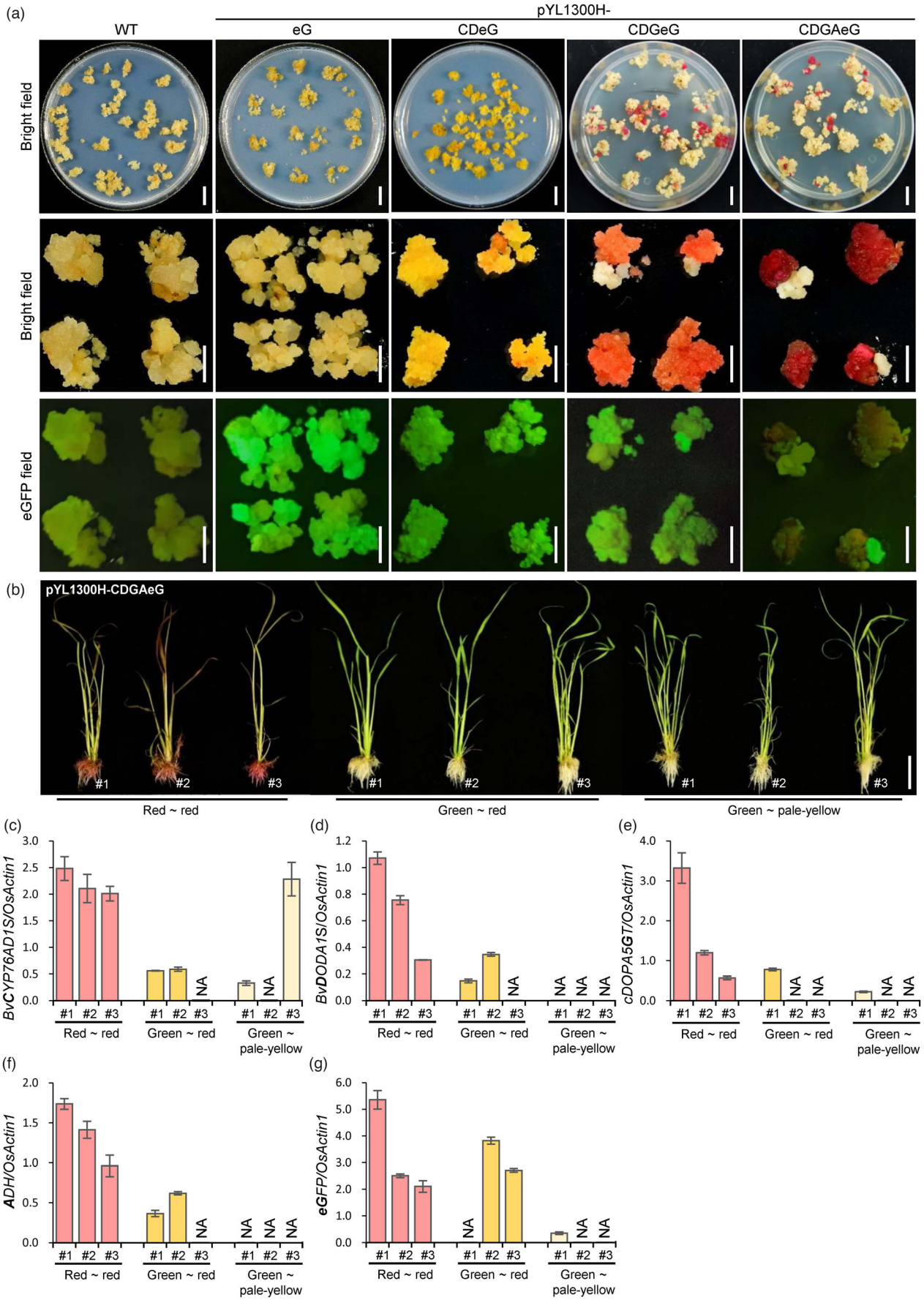
(a)
pYL1300H-
WT
eG
CDeG
CDGeG
CDGAeG
Bright field
Bright field
eGFP field
(b)
pYL1300H-CDGAeG
#1
#2
#3
Red ~ red
Green ~ red
Green ~ pale-yellow
(c)
BvCYP76AD1S/OsActin1
(d)
BvDODA1S/OsActin1
(e)
cDOPA5GT/OsActin1
(f)
ADH/OsActin1
(g)
eGFP/OsActin1
NA
Red ~ red
Green ~ red
Green ~ pale-yellow

Figure 6 Genetic engineering of betanin biosynthesis in transgenic rice. (a) Four constructs (pYL1300H-eG, pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG) were, respectively, transformed into rice calli to obtain stable transgenic calli under hygromycin selection. Bright-field images were photographed under LED excitation. eGFP field images were taken under 440–460 nm excitation with a portable fluorescence detector. Scale bar: 1 cm. (b) Phenotypes of transgenic plants transformed with pYL1300H-CDGAeG construct. Red ~ red: Red plants derived from red calli; Green ~ red: Green plants derived from red calli; Green ~ pale-yellow: Green plants derived from pale yellow calli. Scale bar: 5 cm. (c-g) Transcripts of *BvCYP76AD1S* (c), *BvDODA1S* (d), *cDOPA5GT* (e), *ADH* (f) and *eGFP* (g), in transgenic rice plants were measured by qRT-PCR. The *OsActin1* was used as an internal control. Error bars represent standard deviations (SD) of three biological replicas. NA, not available.

HPT, *NTP II* and *Bar* marker genes, respectively), pYL322d1 (digested with *Asc* I and *Hind* III) and pYL322d2 (digested with *Asc* I and *Hind* III) vectors (Zhu *et al*., 2017) by the modified Gibson cloning method (Zhu *et al*., 2014) to produce the modified binary and donor vectors for TGSII-UNiE (pYL1300H/N/B-UNiE, pYLTAC380H/N/B-UNiE, pYLTAC380GW-UNiE, pYL322d1-UNiE and pYL322d2-UNiE). All primers used in this study are listed in Table S3.

DNA assembly using the UNiEDA strategy

The UNiEDA protocol is as follows:

1. The target fragments were analysed to choose the appropriate NiEs. An absence of the selected NiE on the fragments or the presence of a few sites (the greater the distance between adjacent sites, the better, for example, distances with $T_m \geq 75$ °C) are sufficient for the use of UNiEDA. T_m values were calculated according to the formula $T_m = 69.3 + 41 \times GC\% - 650/L$ (L = primer length) (Mazars *et al*., 1991). Then, the fragments flanking NiE sites and UNSs were generated by PCR amplification using chimeric primers (5′-UNS-Nb.BsrDI/Nb.BtsI -target sequence-3′) and purified using a DNA purification kit (to remove the primers).
2. Each PCR product (insert) of ~200–300 ng was digested in a 10-μL reaction with 5 U Nb.BsrDI (NEB catalogue no. R0648S, USA) at 65 °C for 1 h, or 5 U Nb.BtsI (NEB catalogue no. R0707S, USA) at 37 °C for 1 h, and then heat inactivated at 70 °C for 30 min (also melted out the nicked short strands). Meanwhile, the TGSII-UNiE vector plasmid of ~500 ng was digested with 5 U of Nb.BtsI at 37 °C for 1 h and then heat inactivated at 79 °C for 30 min. The digested PCR products and TGSII-UNiE vector were dialyzed against 0.2× TE using a Minipore dialysis membrane (Merck catalogue no. VSWPO4700, Germany) for 30 min.
3. The digested TGSII-UNiE vector (~60 ng of 9-kb pYL1300H/N/B-UNiE; ~100 ng of 18-kb pYLTAC380H/N/B-UNiE), or 30 ng of donor vector (3.2–3.5 kb), was mixed with a suitable amount of (one or multiple) insert DNA fragment(s) with a vector: insert molar ratio of 1:3 in 10 μL 1× *Taq* DNA ligase reaction buffer. The mixture was incubated at 70 °C for 1 min and 60 °C for 1 min. Then, 0.3–0.5 μL HiFi *Taq* DNA ligase (NEB catalogue no. M0647S, USA) was added. Ligation was conducted using the following thermo-cycling conditions: 18 cycles of 46 °C for 5 min, 55 °C for 2 min, 60 °C for 2 min and 65 °C for 2 min.
4. The reaction product was dialyzed against 0.2× TE using a Minipore dialysis membrane for 30 min. Then, 2 μL of the dialysis product was transformed into *E. coli* strain DH10B by electroporation. The cells in 1 mL Super Optimal Catabolite (SOC) medium were shaken at 37 °C for 1 h. Half of the suspended cells were plated on Luria-Bertani (LB) solid medium (25 mL) with antibiotic (25 μg/mL kanamycin or 17 μg/mL chloramphenicol), 10 μL of 1 M IPTG and 40 μL of 20 mM X-gal per plate. The plate(s) were incubated at 37 °C overnight. After transformation, white colonies were selected to identify the positive recombinant plasmid. Data were calculated from three biological replicates and are shown as mean ± SD (n = 3).

LDNA cloning and multigene stacking with TGSII-UNiE vectors using UNiEDA

For LDNA cloning, different sizes of DNA fragments (10.3 kb, GeneBank accession number KX495644; 14.8 kb and 22.9 kb, GeneBank accession number KX495643) were amplified from a bacterial artificial chromosome (BAC) plasmid (Shen *et al*., 2017). After digested with Nb.BsrDI, the LDNA fragments were cloned into the pYL1300H-UNiE or pYLTAC380H-UNiE binary vectors. The resulting plasmids were verified by colony PCR and endonuclease digestion. To test structural stability, recombinant plasmids were transformed into *A. tumefaciens* strain EHA105 then isolated for endonuclease digestion.

For multigene stacking, four betanin biosynthetic genes [*BvCYP76AD1S* (*C*), *BvDODA1S* (*D*), *cDOPA5GT* (*G*) and *ADH* (*A*), sequence information was shown in Note S1] and an *eGFP* (*eG*, GeneBank accession number NC_049202) were generated by overlapping PCR and fused with Nb.BtsI-generated UNSs at the 5′ and 3′ ends. After being digested with Nb.BtsI, these expression cassettes were assembled into pYL1300H-UNiE and pYLTAC380H-UNiE binary vectors under the control of the P35s promoter, generating pYL1300H- and pYLTAC380H-CDeG/CDGeG/CDGAeG, respectively. The construct stability of these recombinant plasmids was verified by *Kpn I*/*Bam*H I or *Not* I digestion.

Similarly, three hydroxycinnamoylputrescine biosynthesis genes (*HP*s: *OsODC*, *OsPHT3* and *OsPHT4*; GeneBank accession number NC_029264) and three glutamine synthetase biosynthesis genes (*GS* genes: *OsGS1;1*, GeneBank accession number NC_029257; *OsGS1;2*, GeneBank accession number NC_029258; *OsGS1;3*, GeneBank accession number NC_029258), each with its own native promoter and terminator, were cloned into the pYL322d1-UNiE and pYL322d2-UNiE donor vectors. The positive constructs were determined by colony PCR and *Pme* I/*Sbf* I digestion.

The multigene constructs in pYL322d1-HPs and pYL322d2-GSs were sequentially delivered into pYLTAC380H-CDGAeG by Cre/*loxP* recombination as previously described (Zhu *et al*., 2017), resulting in pYLTAC380H-HPs/CDGAeG and pYLTAC380H-GSs/HPs/CDGAeG. These recombinant plasmids were further verified in *E. coli* and *A. tumefaciens* by *Not* I digestion.

Transient expression analysis in *N. benthamiana*

To engineer betanin in *N. benthamiana*, *A. tumefaciens*-mediated transformation was used to deliver the constructs according to a previously reported method with slight modification (Sparkes *et al*., 2006). The transgenic clones (*A. tumefaciens* strain GV3101) were cultured at 28 °C overnight in 10 mL LB liquid

medium containing 25 mg/L kanamycin, 50 mg/L rifampicin, 10 mM MES and 40 μM acetosyringone. Then, clones were re-suspended with infiltration buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, and 150 μM acetosyringone) twice and diluted to a final concentration of OD_{600} = 0.8–1.0. The suspension was incubated at 25 °C for 4 h in the dark and then injected into the *N. benthamiana* leaves by needleless syringe-mediated infiltration. The phenotypes of transgenic *N. benthamiana* leaves were observed after 2 day.

Agrobacterium-mediated transformation of rice

The pYL1300H-eG, pYL1300H-CDeG, pYL1300H-CDGeG and pYL1300H-CDGAeG constructs were introduced into *A. tumefaciens* strain EHA105 by electroporation, respectively. Rice calli were induced from mature seeds of ZH11 and used for *A. tumefaciens* infection according to the previously described method (Nishimura *et al*., 2006). Hygromycin (50 mg/L) was used to select transformed rice calli.

qRT-PCR analysis

Total RNA was isolated from the transgenic *N. benthamiana* leaves using an RNA extraction kit (Promega catalogue no. LS1040, Madison, WI, USA) according to the manufacturer's instruction. The transcript levels of target genes were quantified by qRT-PCR with three biological replicates using SYBR qPCR Master Mix (Vazyme catalogue no. Q711-02, China). The *N. benthamiana NbL25* gene (Schmidt and Delaney, 2010) was utilized as the endogenous control for normalization.

Betanin extraction and quantification

Betanin was extracted from the transgenic *N. benthamiana* leaves using a betanin assay kit (COMIN, China) according to the manufacturer's protocol. Briefly, powder of *N. benthamiana* leaves (40 mg dry weight) was homogenized with extraction solution, the mixture was centrifuged, and the supernatant was harvested. Betanin content was calculated according to the absorbance value of supernatant at 525 nm measured with three biological replicates.

Accession numbers

The sequences of the TGSII-UNiE vectors have been submitted to GenBank with the accession numbers: pYL322d1-UNiE (OM223081), pYL322d2-UNiE (OM223082), pYLTAC380GW-UNiE (OM223083), pYLTAC380B-UNiE (OM223084), pYLTAC380N-UNiE (OM223085), pYLTAC380H-UNiE (OM223086), pYL1300H-UNiE (OM223087), pYL1300B-UNiE (OM223088) and pYL1300N-UNiE (OM223089). Sequences of genes used are shown in the supporting information.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Q.Z. conceived and supervised the project. Y.Z., J.H., J.T., Y.Y., S.L., Y.G., Y.L., W.X., Y.X., Y.H. and X.X. performed the experiments. Y.Z., J.H., J.T. and Q.Z. analysed data. Y.Z. wrote the draft. Y-G.L. and Q.Z. revised several versions of this manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Partial sequences of the UNiEDA-compatible TGSII-UNiE vectors.
Figure S2 Structural diagram of pYLTAC380GW-UNiE vector.
Figure S3 Optimization of the initial annealing temperature for thermo-cycling ligation of UNiEDA.
Figure S4 PCR analysis of pYL1300H- and pYLTAC380H-based constructs (eG, CDeG, CDGeG and CDGAeG) in *E. coli* and *A. tumefacines*.
Figure S5 Structural stability of pYLTAC380H-GSs/HPs/CDGAeG construct in *A. tumefaciens*.
Figure S6 Genotype of transgenic rice calli and plants.
Table S1 Unique nucleotide sequence (UNS) adapters designed for UNiEDA.
Table S2 UNS-guided PCR primers with 15-nt overlaps and Nb.BtsI nickase site for UNiEDA.
Table S3 Primers used in the study.
Note S1 Codon optimized sequences of four betanin biosynthetic genes.

