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Design and construction of multigenic constructs for plant biotechnology using the GoldenBraid cloning strategy.

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Summary/Abstract

GoldenBraid (GB) is an iterative and standardized DNA assembling system specially designed for Multigene Engineering in Plant Synthetic Biology. GB is based on restriction-ligation reactions using type IIS restriction enzymes. GB comprises a collection of standard DNA pieces named “GB parts” and a set of destination plasmids (pDGBs) that incorporate the multipartite assembly of standardized DNA parts. GB-reactions are extremely efficient: two transcriptional units (TU) can be assembled from several basic GBparts in one T-DNA less than 24h. Moreover, larger assemblies comprising 4-5 TUs are routinely built in less than two working weeks. Here we provide a detailed view of the GB methodology. As a practical example, a Bimolecular Fluorescence Complementation (BiFC) construct comprising four TUs in a 12 kb DNA fragment is presented.

ii. Key Words

Synthetic Biology; DNA assembly; Type IIS enzymes; Plant Biotechnology; Multigene Constructs; Multigene Engeneering.

1. Introduction

Synthetic Biology aims to engineer living systems with functions not found in nature, thus incorporating new features to existing living organisms ***(1,2)***. Complex protein production, metabolic engineering or genetic networks often need the integration of several transgenes into the biological model, what requires more than single transcriptional units (TU) building but the construction of complex multigenic structures. Custom DNA synthesis ***(3)*** as well as other strategies involving homologous and site-specific recombination ***(4,5,6)*** have been reported as successful methodologies for multigene engineering. However, neither they produce reusable units nor facilitate the set up of combinatorial strategies. An alternative approach is the modular construction of genetic devices using standardized DNA parts. Modular design facilitates combinatorial engineering, as standard DNA parts can be easily exchanged improving the possibilities of the building process.

GoldenBraid (GB) ***(7, 8)*** is a cloning system that follows a modular construction strategy. GB allows the binary combination of multipartite assemblies using an extremely simple set of rules. GB makes use of the multipartite Golden Gate cloning method ***(Chapter X, 9, 10)*** to generate a multipartite assembly of standardized basic DNA parts, which are then incorporated to a double loop cloning design that allows binary assembly of multipartite constructs. In this way, GB technology enables the standardization of Golden Gate for its use in Synthetic Biology. This is achieved with a small toolbox consisting of a maximum of only eight destination plasmids and a limited number of simple assembly rules. This new cloning system facilitates the reusability of DNA parts and assembled devices to efficiently built complex constructs.

In this chapter we describe in depth the methodology of the GB cloning system, giving the details on the reactions to be performed and providing special hints to facilitate the assembly of multigene constructs. As an example, we show here the design and assembly of four genes/TUs in one binary destination vector and its use to perform a Bimolecular Fluorescence Complementation (BiFC) analysis ***(11)***. In order to validate the BiFC GB constructs, the interaction between the *Arabidopsis thaliana* transcriptional factors FRUTITFULL (FUL) and SUPRESSOR OF OVEREXPRESSION OF CO (SOC1) will be tested ***(12,13)***. The final BiFC GB constructs comprise 4 transcriptional units: in addition to the usual BiFC units (N and C

terminal portions of the Yellow fluorescent Protein)), we have incorporated a "monitoring/silencing suppressor module" to improve the performance of transient expression assays in *Nicotiana bentahmiana*. This special module contains the reporter *Renilla* ***(13)*** used as internal reporter to normalize the efficiency of the transient transformation, and the Tomato Bushy Stunt Virus silencing suppressor p19 ***(14)*** used to inhibit the effects of gene silencing.

2. Materials

2.1. GoldenBraid Destination Vectors (pDGBs) and Cloning Methodology

Previously described assembly systems allow standardization but the resulting units cannot be easily re-used in subsequent assembly reactions. A solution to this limitation, described as GB ***(7)***, was to insert a loop (braid) in the cloning design, so that the expression plasmids from first level become entry plasmids for second level assemblies and vice versa. To this end, two types of destination plasmids were designed, namely level α and level Ω. The key point in GB design is that, while all plasmids contain two restriction/recognition sites for two different type IIS enzymes, level α and level Ω plasmids are designed to have their sites in inverted orientations. Plasmids also carry different resistance markers for efficient counter selection.

Although in the most basic setup only four pDGBs are needed to establish the double loop, we built eight different pDGBs in order to make possible the assembly of TUs in reverse orientation. Numbers and letters serve to identify each destination plasmid according to the flanking overhangs left by BsaI and BsmBI digestion, respectively:

A. Level α plasmids are used as destination plasmids in BsaI GB-reactions. These are pDGB_A12C, pDGB_C12B, pDGB_A21C, pDGB_ C21B. In most cases only pDGB_A12C or pDGB_C12B will be used unless there is any interest in assembling TUs in reverse orientation (in this case the choice would be pDGB_A21C or pDGB_C21B). This group of plasmids contains kanamycin as resistance marker and is both used for the multipartite assembly of GBparts and for the binary combination of TUs.

B. Level Ω plasmids are used as destination plasmids in BsmBI-GB reactions. These are pDGB_1AB3, pDGB_3AB2, pDGB 1BA3 and pDGB 3BA2. Regularly, only pDGB_1AB3 or pDGB_3AB2 will be selected by users unless they need to assemble TUs in reverse orientation. This group of plasmids incorporates spectinomycin as bacterial resistance.

The mechanism of GB is shown in figure 1. Standard parts are normally assembled in level α plasmids, for example into pDGB_A12C. The resulting composite parts can be combined with other structures assembled in the complementary pDGB_C12B using any of the level Ω plasmids as destination vectors for the assembly. In a second assembly round, composite parts assembled using level Ω plasmids can be combined together inside a level α plasmids provided that they share a common sticky end. As it can be observed, GB works as an endless iteration of binary assemblies keeping the ability of incorporating more units, with the only theoretical limit of the capacity of the destination vector backbone.

2.2. GoldenBraid Parts

"GBparts" are functional DNA fragments flanked by fixed 4 nt overhangs which are generated by cleavage with the type IIS enzyme BsaI. As described in Golden Gate assembly ***(Chapter X, 10)***, the overhangs determine the relative position of a GBpart in a multipartite assembly. In the most general case, we considered three functional standard categories when building TUs: promoters (which include the transcription origin and 5´ UTR), coding regions (CDS), and terminators (which include 3´ UTR and polyA site and transcriptional stop signal). Each category corresponds to a relative position in the assembly (although users can define additional intermediate categories). Therefore we arbitrarily assigned flanking 4 nt to each of the three basic categories (see figure 1B) that have to be used in every GBpart to ensure compatibility between different users.

Basic parts normally come in the form of a circular plasmid, and the insert corresponds to the part itself. Upon BsaI digestion, the part is released from the plasmid leaving 4 nt overhangs, ready to be assembled together with other parts in a BsaI GB-reaction. A large collection of

ready to-assemble standard GBparts has been established and can be consulted at www.gbcloning.org

2.3. Materials for PCR Amplification and TA-Cloning of DNA pieces

1. Gene-specific Oligonucleotides with GB extensions (see section 3.1).
2. Phusion® High-Fidelity DNA Polymerase (ThermoScientific).
3. dNTP mixture (10 mM each dNTP).
4. Mili-Q sterilized water.
5. Biotools DNA Polymerase (Biotools).
6. Agarose electrophoresis gel: 1% agarose TAE 1X (40 mM Tris–acetate and 1 mM EDTA).
7. QIAquick PCR Purification Kit (Qiagen).
8. pGEM®-T Easy Vector System (Promega).
9. Thermocycler.
10. 50% Glycerol for storing the correct assemblies.

2.4. Escherichia coli Cell Transformation and Culture

1. House-made competent Cells, One Shot® TOP10 or One Shot® Mach1™ T1R chemically competent *Escherichia coli* kit (Invitrogen).
2. Electroporator and 1mm electroporation cuvettes.
3. Sterile SOC medium: 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose.
4. Sterile Lysogeny Broth (LB) medium: 1% tryptone, 0.5% yeast extract, 1% NaCL.
5. Lysogeny Agar (LA) plates: 1% tryptone, 0.5% yeast extract, 1% NaCL, 1.5% agar. Plates contain the appropriate antibiotics (kanamycin at 50 µg mL^{-1}, ampicillin and spectinomycin at 100 µg mL^{-1}), IPTG (0.5 mM) and X-Gal (40 µg mL^{-1}).
6. A shaker and growing chamber at 37ºC.

7. E.Z.N.A. Plasmid Mini Kit (Omega Bio-tek).

2.5. Materials for Multipartite Assembly Reactions

1. Miniprep-purified GBparts.
2. α -level GB destination vectors.
3. T4 DNA ligase (Promega, Madison, USA), BsaI restriction enzyme (New England Biolabs).
4. Thermocycler
5. 50% Glycerol for storing the correct assemblies.
6.

2.6. Materials for Binary Assembly Reactions

1. Miniprep-purified composite parts.
2. α-level and/or Ω-level GB destination vectors.
3. T4 DNA ligase (Promega), BsaI, BsmBI restriction enzyme (New England Biolabs).
4. Thermocycler.
5. 50% Glycerol for storing the correct assemblies.
6.

2.7. Agrobacterium tumefaciens Cell Transformation and Culture

1. House-made pSOUP *Agrobacterium tumefaciens* strain GV3101 electrocompetent cells.
2. Electroporator and 1mm electroporation cuvettes..
3. Sterile SOC medium (2% w/v tryptone, 0.5% w/v Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl, 10mM MgSO4 and 20 mM Glucose)
4. Sterile LB supplemented with 2 mM $MgSO_4$ and 2 mM sucrose.
5. LA plates containing the appropriate antibiotics (tetracycline at 12.5 µg mL^{-1}; gentamicin at 30 µg mL^{-1}, rifampicin and kanamycin at 50 µg mL^{-1}, ampicillin and spectinomycin at 100 µg mL^{-1}).
6. QIAprep Spin Miniprep Kit (Qiagen).

7. Lysozyme (Sigma).

2.8. Plant Transient Transformation

1. Agroinfiltration MES Buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, 200 µM acetosyringone).
2. Spectrophotometer set at a wavelength of 600 nm and transparent plastic cuvettes.
3. Sterile 1 mL Plastikpak syringes without needle.
4. 30-35 days old *Nicotiana benthamiana* plants (growing conditions: 24°C day/20°C night in a 16 h light/8 h dark cycle).

2.9. Bifluorescence Complementation Analysis

1. Confocal microscope, such as TCS SL (Leica).
2. Microscope slides and cover slips.
3. Scalpel.
4. Mounting media for microscopy (Leica Biosystems).

2.10. House made DH5α electrocompetent cells

1. Day 1: Streak out frozen glycerol stock of bacterial cells onto an LB plate without antibiotics and grow overnight at 37ºC.
2. Day 2:
    a. Media Preparation: 2L of ddH20. 1L of 10% v/v glycerol, 1,5L LB. Chill overnight at 4ºC.
    b. Pick a single colony of *E.coli* from the fresh LB plate and inoculate a 15 mL starte cuture of LB without antibiotics. Grow overnight at 37ºC.
3. Day 3:
    a. Inoculate 1,5 L of LB media with the 15 mL starter culture and grow for about 3h in a 37°C shaker.
    b. Check the OD600 and when it reaches 0.4, chill on ice for 30 minutes. Chill also the centrifuge bottles.

c. Distribute the culture in 6 centrifuge tubes. Harvest the cells by centrifugation at 5000 rpm for 15 minutes at 4°C.
d. Resuspend each pellet in 250 mL of ice cold water. Shake smoothly. Centrifuge at 5000 rpm for 15 minutes at 4°C.
e. Decant the supernatant and resuspend each pellet in half the volume so the final volume of the culture is reduced to 750 mL and it can be combined in three centrifuge tubes. Harvest the cells by centrifugation at 5000 rpm.
f. Decant the supernatant and resuspend the pellets in 10 mL 10% glycerol. Transfer the final volume to smaller centrifuge tubes.
g. Centrifuge the tubes at 5000 rpm for 15 minutes at 4°C and decant the supernantant. Resuspend the final pellets in 2 mL of ice cold 10% glycerol. Aliquot in ≈50µl into 1.5 mL tubes and freeze with liquid nitrogen. Store at -80ºC.

2.11. House made Agrobacterium tumefaciens with pSOUP electrocompetent cells

1. Day 1: Streak out frozen glycerol stock of bacterial cells onto an LB plate with rifampicin and tetracycline and grow at 28ºC for two days.
2. Day 3:
   a. Media Preparation: 2L of water, 1L of 10% v/v glycerol, 1,5L LB. Chill at 4ºC.
   b. Pick a single colony from the plate late and inoculate a 5 mL starte cuture of LB without rifampicin and tetracycline. Grow for two days at 28ºC to saturation..
3. Day 5:
   a. Inoculate 1,5 L of LB media with 1:200 saturated *A. tumefaciens* culture. Grow overnight for about 16h in a 28°C shaker. The final OD should be around 1.5.
   b. Distribute the culture in 6 centrifuge tubes. Harvest the cells by centrifugation at 5000 rpm for 15 minutes at 4°C.
   c. Resuspend each pellet in 250 mL of ice cold 10% Glycerol ddH20. Centrifuge at 5000 rpm for 15 minutes at 4°C.
   d. Decant the supernatant and resuspend each pellet in half the volume so the final volume of the culture is reduced to 750 mL and it can be combined in three centrifuge tubes. Harvest the cells by centrifugation at 5000 rpm.

e. Decant the supernatant and resuspend the pellets in 10 mL 10% glycerol. Transfer the final volume to smaller centrifuge tubes.

f. Centrifuge the tubes at 5000 rpm for 15 minutes at 4°C and decant the supernantant. Resuspend the final pellets in 2 mL of ice cold 10% glycerol. Aliquot into 1.5 mL tubes (≈50µl each) and freeze with liquid nitrogen. Store at -80ºC.

3. Methods

3.1. Domestication of GoldenBraid Basic Parts

A crucial aspect of Synthetic Biology is standardization, which can only be achieved through concerted community effort. In order to ensure that GB users can exchange their parts, user´s need to use the same overhangs for the same part categories. We call “domestication” to the adaptation of basic DNA parts to the GB rules. It comprises not only the addition of flanking BsaI sites as indicated earlier, but also the removal of internal BsaI and BsmBI sites. We strongly recommend building BsaI and BsmBI-free parts to facilitate GB-reactions, especially for big assemblies (see further in this section for instructions). As GB reactions are extremely efficient, assemblies can still be successful even if the sites are not eliminated. However, this will produce less correct colonies, and for large assemblies involving many pieces your efficiency may be lower. Furthermore, if assembled TUs are going to be reused to build more complex devices, the presence of undomesticated pieces will eventually cause troubles.

As it was described in section 2.2, there are three basic GB categories. In this example, we will build one Terminator-GBPart (pE_T35s; the35S terminator of the Coliflower Mosaic Virus), two CDS-GBparts (pE_FUL and pE_SOC1) and two special GBparts that share overhangs with standard promoters but include the split fragments of YFP to enable BiFC experiments (pE_35s:YFN and pE_35s:YFC, consisting on constitutive 35S promoter of CaMV fused to the N-/C-terminal half of YFP). To build these GB parts, follow these steps:

1. Design GB oligonucleotides, incorporating the indicated extensions listed in Table 1, including 20-22 nt from the sequence to be amplified. Note that the overhang preceding a CDS includes the starting ATG codon, and therefore the rest of the gene should be designed in frame with this ATG. (Note 1)

2. If the part to be domesticated contains no BsaI or BsmBI internal sites, continue this protocol in step 5. If any internal BsaI or BsmBI sites have to be removed, continue the protocol in Step 3.
3. The process for removing internal type IIS sites is depicted in Figure 2A where an internal BsmBI site from FUL is eliminated, following a standard overlap extension PCR protocol (OE-PCR). In the case of one internal site, two pairs of primers are required. Design the two external primers (FUL.F1 and FUL.R2 in Figure 2A) as described above (section 3.1.1, using extensions shown in Table 1) Design the second (internal) pair of primers (FUL.R1 and FUL.F2) incorporating a nucleotide mismatch so as to mutate the internal Type IIS BsmBI site. Keep in mind that for CDSs, the open reading frame should be maintained (see primers designed for FUL in Table 2). The internal primers must overlap at least 20 nt. Perform the OE-PCR as follows:
    a. Prepare the first pair of reactions using primer pairs FUL.F1-FUL.R1 and FUL.F2-FUL.R2 (Note 2) and a suitable template. Run an electrophoresis gel to verify the success of the PCR (use only 1/5 of the volume) and purify the rest of the reaction using QIAquick PCR Purification Kit. (Note 3)
    b. Prepare the overlapping reaction with an equimolar ratio of both PCR fragments as template. Do not add any primers at the beginning. Set your PCR in two different steps:
        i. Step 1: 10 cycles with an annealing temperature determined by the overlapping region between the two fragments. (Note 4)
        ii. Step 2: Add 10 µM of the external primers (FUL.F1 and FUL.R2). Set the reaction for 25 cycles with the annealing temperature determined by the external pair of primers. At the end, check the PCR by agarose gel electrophoresis (Figure 2C, Lane 5). Once it is correct, proceed to step 6.
4. If the internal restriction site is close enough to the 5’ or 3’ ends of the GBpart (see example for the GB part SOC1 in Figure 2B), the situation is solved by making the GB oligo longer, and introducing a mutation in the recognition sequence of the restriction

enzyme. Keep mind that in the case of a CDS, the open reading frame should be maintained. Proceed to Step 5.

5. PCR the GBpart using a suitable template and specially designed GB primers. Verify the correct amplification by agarose gel electrophoresis. Primers used for the amplifications are listed in Table 2. (Figure 2C, Lanes 2, 3, 4 and 6).
6. Purify the PCR products using QIAquick PCR Purification Kit. (Note 5)
7. Add 3' A overhangs with Taq DNA Polymerase. Set the reaction by mixing 17 µL of the purified PCR, 2 µL 10X reaction buffer, 0.5 µL dNTPs and 0.5 µL Taq Polymerase.
8. Proceed to ligate the resulting amplicons in pGEMT by adding: 50 ng of pGEMT, 150 ng of the purified PCR, 3u T4 DNA ligase and 5 µL of the ligase buffer, in a 10 µL reaction. (Note 6)
9. Incubate the ligation reaction during 1 h at room temperature.
10. Transform 1µL of the reaction into 50 µL *E.coli* electrocompetent cells, outgrow by adding 500 µL SOC shaking during 1 h in a shaker set in a 37ºC growing chamber. Spread two aliquots (50 µL and 500 µL) in LB plates containing ampicylin, IPTG and X-Gal. Incubate overnight in a 37 ºC growing chamber.
11. Pick four white colonies and grow them overnight in liquid LB containing ampicillin.
12. Miniprep the cultures and check that the cloned part is correct by restriction digestion analysis. Correct clones from pE_35s:YFC, pE_35s:YFN, pE_FUL, pE_SOC1 and pE_T35s are shown in Figure 2D.(Note 7)
13. Sequence the GBpart using M13 forward and reverse universal primers.
14. Store the cells (containing the GBpart) in the form of glycerol stock (15% glycerol) and DNA miniprep.

3.2. *Single TU Assembly in α-Level Plasmids (Multipartite Reaction)*

GB constructs are based on restriction-ligation reactions. GBparts have to be combined with the α-level vectors to assemble the functional TU. In this chapter we show two examples of multipartite assembly, which lead to the construction of two TUs, namely 35s:YFN::FUL:T35s and 35s:YFC::SOC1:T35s, from their constitutive GBparts. The remaining two TUs needed to

complete a luciferase-monitored BiFC system (namely the “monitoring/silencing suppressor module” comprising constitutively-expressed P19 and luciferase TUs) were assembled separately, stored in the GB Collection (you can check the GBparts we have in the collection in www.gbcloning.org), and incorporated to the BiFC system as fully reusable composite parts (see binary assembly section).

For the Split Fluorescence units (35s:YFN::FUL:T35s and 35s:YFC::SOC1:T35s), three domesticated parts are assembled into the complementary level α vectors as depicted in figure 3A and 3B. The following protocol has to be followed:

1. Prepare the assembly by dispensing in a 10 µL reaction 75 ng of the α-level destination vector, 75 ng of the DNA parts to be assembled, 3u BsaI and 3u T4 DNA ligase.
2. Set the reaction in a thermocycler: 25 cycles x (37ºC 2 min, 16ºC 5 min).
3. Transform 1µL of the reaction into 50 µL *E. coli* electrocompetent cells, outgrow by adding 500 µL SOC shaking during 1 h in a shaker set in a 37ºC growing chamber. Spread two dilutions (50 µL and 500 µL) in LB plates containing kanamycin, IPTG and X-Gal. Incubate overnight in at 37ºC growing chamber.
4. Once colonies are visible, it is possible to distinguish between those carrying intact vectors (blue) and those transformed with your construction (white). Pick four white colonies and grow them overnight in LB containing kanamycin.
5. Isolate DNA by a miniprep method and digest the obtained plasmids. A correct clone from the example constructs A-35s:YFN::FUL:T35s-C and C-35s:YFC:SOC1:T35s are shown in Figure 3C. (Note 8)
6. Store the composite units in the form of bacterial glycerol stock (15% glycerol) and DNA miniprep.

3.3. *Multigene Assembly in Ω Level Plasmids (Binary Reaction)*

Any composite part GB-assembled in α-level plasmids can be combined in Ω-level plasmids. To combine two TUs into a Ω-level plasmid, the right entry and destination plasmids have to carefully been chosen so the sticky ends are compatible. In an example (see figure 3B), pEGB_

A-35s:YFN::FUL:T35s-C and pEGB_C-35s:YFC::SOC1:T35s-B can be assembled into pDGB 1AB3 as the share a C sticky end.

1. Prepare the assembly reaction by dispensing 75 ng of the Ω-level destination vector, 75 ng of the TUs to be assembled, 3u BsmBI and 3u T4 DNA ligase in a final volume of 10 µL.
2. Set the reaction in a thermocycler: 25 cycles x (37ºC 2 min, 16ºC 5 min).
3. Transform 1µL of the reaction into 50 µL *E.coli* electrocompetent cells, outgrow by adding 500 µL SOC shaking during 1 h in a shaker set in a 37ºC growing chamber. Spread two dilutions (50 µL and 500 µL) in LB plates containing spectinomycin, IPTG and X-Gal. Incubate overnight in a 37ºC growing chamber.
4. Pick four white colonies (blue colonies will contain the intact destination vector) and grow them overnight in LB containing spectinomycin. Obtain DNA Minipreps from them and check the assembly by restriction digestion analysis. The digestion of a correct clone from the construct 1- pEGB_ 1-35s:YFN::FUL:T35s-35s:YFC::SOC1:T35s-3 is shown in Figure 3E. (Note 5)
5. Store the DNA construct in the form of bacterial glycerol stock (15% glycerol) and DNA miniprep.

3.4. *Multigene Assembly in α-Level Plasmids (Binary Reaction)*

Composite parts GB-assembled in Ω-level plasmids can be combined in α-level plasmids as long as entry plasmids are compatible. The previously assembled BiFC TUs (section 3.3) can be combined with a previously assembled, reusable “monitoring/silencing suppressor module” to perform transient expression assays in *Nicotiana benthamiana*. This special module comprises two TUs, the reporter *Renilla* ***(14)*** and the TBSV silencing suppressor p19 ***(15)*** (in both cases the expression is directed by the 35S promoter and the Nopaline Synthase terminator). This special module prevents silencing effects and allows monitoring the transformation efficiency using commercial Renilla assay systems. The renilla-p19 module was previously assembled in a similar fashion as it is described in sections 3.2 and 3.3 and was subsequently added to the stored GB collection. The ability to re-use GB devices is one of the strengths of GB system.

To assemble together the YFN-TFC module with the “monitoring/silencing suppressor” module, one, the following steps are followed (depicted in figure 4):

1. Prepare the assembly reaction by dispensing 75 ng of the α-destination vector, 75 ng of the TUs to be assembled, 3u BsaI and 3u T4 Ligase in a final volume of 10 µL.
2. Set your reaction in a thermocycler: 25 cycles x (37º 2 min, 16º 5 min).
3. Transform 1µL of the reaction into 50 µL *E. coli* electrocompetent cells, outgrow by adding 500 µL SOC shaking during 1 hour in a shaker set in a 37ºC growing chamber. Spread two dilutions (50 µL and 500 µL) in LB plates containing kanamycin, IPTG and X-Gal. Incubate overnight in a 37ºC growing chamber.
4. Pick four white colonies and grow them overnight in LB containing kanamycin.
5. Check that the construct is correctly assembled by restriction digestion analysis using DNA miniprep from the colonies. The final construct pEGB_A-35s:YFN::FUL:T35s-35s:YFC::SOC1:T35s-35s:Renilla:TNos-35s:P19:Tnos-C is ready (see figure 4B). (Note 5)
6. Keep a stock of the final construct in the form of bacterial glycerol stock (15% glycerol) and DNA miniprep.

3.5. *Agrobacterium GV3101 Transformation*

Once the multigene construct is ready, it has to be transformed into *Agrobacterium tumefaciens*. Some GB destination vectors are based in pGreenII ***(16)***, so if that is the case, the *Agrobacterium* strain should carry the helper plasmid pSOUP (Note 9).

3.1. Transform 15 ng of plasmid to 50 µL *A. tumefaciens* electrocompetent cells. Add 500 µL of SOC medium and incubate in a 15 mL tube at 28ºC for 2 h with agitation. Spread two dilutions (50 µL and 500 µL) in LB plates containing kanamycin (or spectinomycin, according to the resistance of the plasmid), tetracycline and rifampicin. Incubate for 48 h in a 28ºC growing chamber.

3.2. Pick four colonies and inoculate 5 mL of LB medium containing the appropriate antibiotics and incubate for 24-36 h.

3.3. Collect cells by centrifugation and perform a miniprep DNA isolation (Note 10).

3.4. Check correct clones by restriction digestion analysis (Note 11) and store the strain in the form of glycerol stock (15% glycerol).

3.6. *Agrobacterium-mediated Transient Expression Protocol for BiFC Assays in Nicotiana benthamiana Leaves*

For BiFC experiments ***(11)***, a transient expression experiment in *Nicotiana benthamiana* leaves can be performed. It is important to include a negative control construct that prevents the reassembly of the fluorescent protein and results only in background fluorescence. The negative construct was also built following the same procedure as the FUL-SOC1 positive construct described in sections 3.3, 3.4 and 3.5 (not shown). The pair of non-interacting proteins was FUL and the *Antirrhinum majus* Rosea1 transcription factors ***(17)***. Agroinfiltration **(18)**, a frequently used technique for transient gene expression, was used for BiFC monitoring. The assay can be performed as follows:

1. Pick the selected clones (positive and negative BiFC) and inoculate a 50 mL culture tube containing 5 mL LB medium supplemented with 2 mM $MgSO_4$, 2 mM sucrose and the appropriate antibiotics. Grow the cultures at 28ºC with shaking for 48 h.
2. Subculture the clones into a new tube by adding 50 µL of the saturated culture into 5 mL fresh LB medium supplemented with 2 mM MgSO4, 2mM sucrose and the appropriate antibiotics. Grow overnight in the same conditions.
3. Pellet the cells (20 minutes at 3000 rpm) and resuspend them with Agroinfiltration buffer to an optical density at 600 nm of 0.4.
4. Incubate the cultures at room temperature for 2 h with agitation.
5. *Nicotiana benthamiana* leaves are inoculated by syringe-agroinfiltration in leaves of 30-35 days old plants. After 4-5 days, the tissue can be harvested and the expression of the transgenes analyzed.
6. Cut a 0.5 cm x 0.5 cm piece of the agroinfiltrated leaves and prepare it using the mounting media on the slide for the Confocal Microscopy TCS SL (Leica) and visualize the positive and negative probes (Figure 4C).

4. Notes

Note 1: GB- oligonuleotides comprise between 30 and 40 nt. There is no need to use highly purified primers if your supplier guarantees you a low error rate.

Note 2: When using Phusion Polymerase, primers greater than 20 nt in length do the annealing for 10–30 seconds at 3°C above the melting temperature (Tm) of the lowest primer Tm. However, manufacturer's Tm calculations are not valid for PCRs based on GB oligos as they have an extension that should not be included in Tm calculation. To calculate Tm, you can use the formula Tm = 4(GC) + 2(AT), where GC represents the number of guanine and cytosine, and AT represents the number of adenine and thymine.

Note 3: This purification step is essential for removing primers as the second reaction only uses the external pair of primers.

Note 4: The first annealing temperature for the OE-PCR second step can be calculated using the formula Ta = 3(GC) + 2(AT), where GC represents the number of guanine and cytosine, and AT represents the number of adenine and thymine.

Note 5: It is very important to remove all the Phusion DNA Polymerase as the proofreading activity in Phusion DNA Polymerase is very strong. Any remaining Phusion DNA Polymerase will degrade the A overhangs, thus creating blunt ends again.

Note 6: For optimal ligation efficiency it is recommended to use fresh PCR products, since 3´A-overhangs will gradually be lost during storage.

Note 7: A BsaI restriction reaction will release the desired GBpart as a single fragment. BsaI is a relatively expensive restriction enzyme so we suggest screening the colonies using EcoRI (pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites EcoRI and NotI, providing a cheap digestion for release of the insert) and to verify BsaI sites only with positive clones.

Note 8: Although each TU assembly will require a different restriction reaction, BglII can be considered as a universal enzyme for pDGBs based on pGreenII as it flanks the GB cassette in both 5' and 3' ends.

Note 9: If electrocompetent cells without pSOUP are not available, it is possible to co-transform the final pEGB and pSOUP at the same time but the efficiency will be lower. pGreenII/pSOUP is a binary vector system described by Hellens et al. (2005) pSOUP is the helper plasmid that provides the replicase function for the pSa replication origin of pGreen. pGreen will not replicate in *Agrobacterium* if pSOUP is not present.

Note 10: We recommend the use of Quiagen Kit for *Agrobacterium* minipreps. Adding 10 µL of lysozyme together with Solution I and incubating for 10 minutes at 37ºC will improve the final plasmid yield.

Note 11: To select the restriction enzymes for digesting the A*grobacterium* minipreps, it is important to consider that both pSoup and the pEGB will be present. We suggest the use of BglII/EcoRV because it will linearize pSOUP (9.2 kb) and will make at least two cuts in the desired pEGB.

5. Acknowledgments

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6. Figures

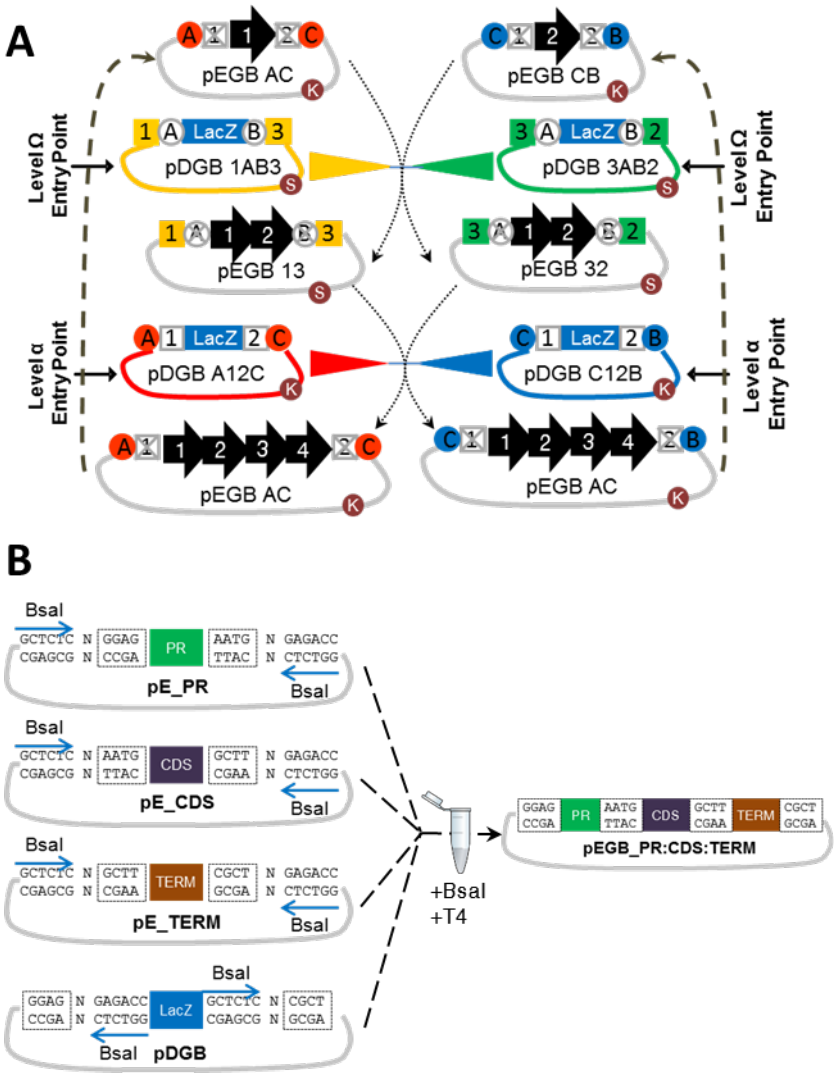

FIGURE 1. The mechanism of GoldenBraid. (A) TUs (e.g., 1, 2) assembled in complementary level α plasmids can be used as entry vectors for a level Ω binary assembly, provided that they share a common BsmBI sticky end (encircled C). Similarly, constructs assembled using paired level Ω plasmids can be used as entry vectors for a subsequent level α assembly, as they share a BsaI sticky end (squared 3). Level α and level Ω can alternate indefinitely creating increasingly complex structures, as depicted by the arrows closing the double loop. Encircled K and S represent kanamycin resistance gene and spectinomycin resistance gene, respectively. (B) Standard parts flanked by fixed BsaI cleavage sites (boxed) are multipartite assembled using level α plasmids. Upon assembly, the newly assembled

TU(Promoter:CodingSequence:Terminator) remains flanked by BsmBI cleavable sites (not depicted here).

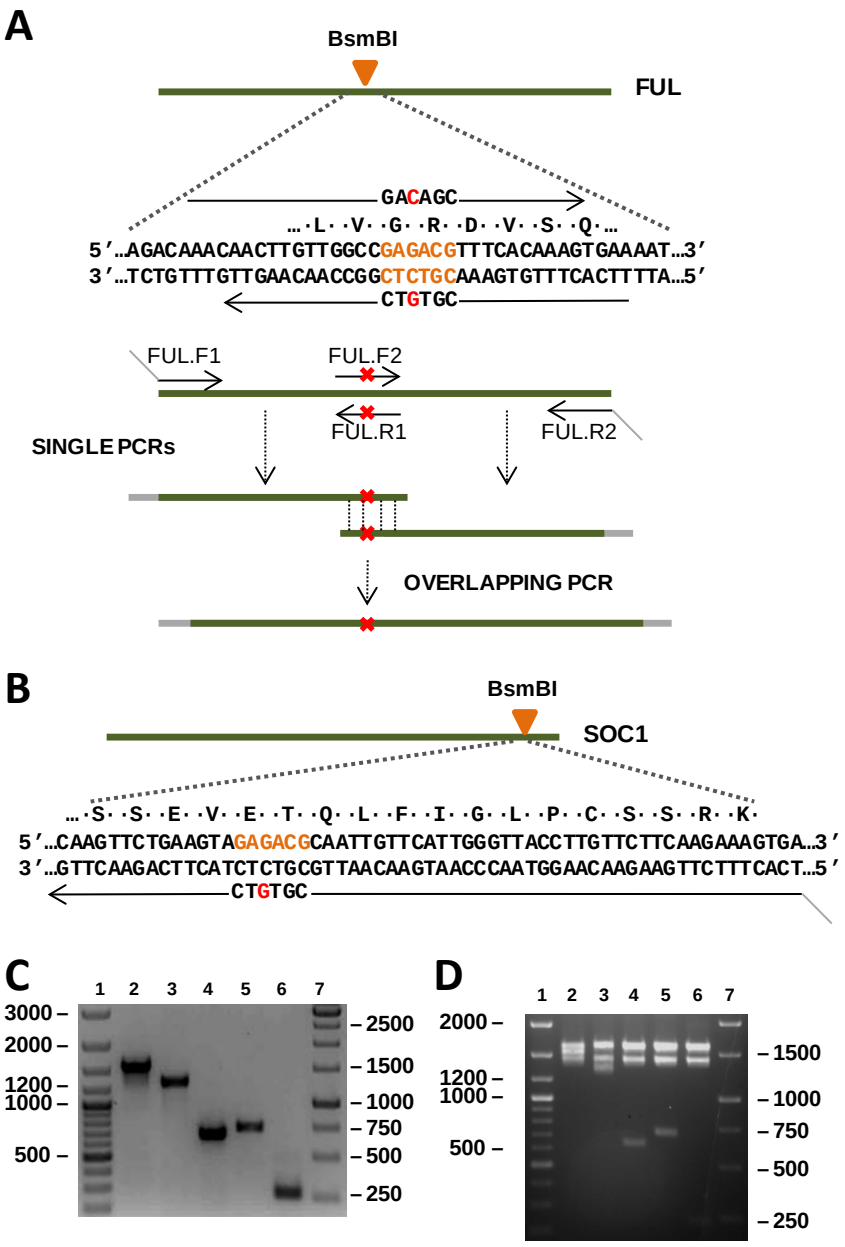

FIGURE 2: Domestication of GBparts (A) Overlap Extension PCR strategy for FUL domestication through the silent mutation of an internal BsmBI site. Primers FUL.F1 and FUL.R2 are designed to introduce the appropriate BsaI flanking sites. A second pair of primers (FUL.F2 and FUL.R1) are designed to introduce a single nucleotide mismatch (G>C) producing a silent mutation and eliminating the internal BsmBI site. In a first reaction, two fragments sharing 20-25 nt are PCR amplified using primer pairs FUL.F1-FUL.R1 and FUL.F2-FUL.R2; in a second step an overlapping PCR using both PRC products as templates and using only FUL.F1 and FUL.R2 primers is performed. The final GB-FUL PCR lacks the internal BsmBI site.

(B) Removal of a Type IIS site close to the 3’ end of SOC1 by mutating the site on the reverse primer of the GBpart, making it longer than usual. (C) Agarose gel electrophoresis of the PCRs for the 5 GBparts described. Lane 1: DNA Marker; Lane 2: 35s:YFN; Lane 3: 35s:YFC; Lane 4: SOC1; Lane 5: FUL; Lane 6: T35s; Lane 7: DNA Marker (D) BsaI digestion of the 5 GB parts generated. Each of them has three bands, two of them from pGEMT (1622 and 1433 pb) and a third one corresponding to the GB part. Lane 1: DNA Marker; Lane 2: pE35s:YFN; Lane 3: pE_35s:YFC; Lane 4: pE_SOC1; Lane 5: pE_FUL; Lane 6: pE_T35s; Lane 7: DNA Marker.

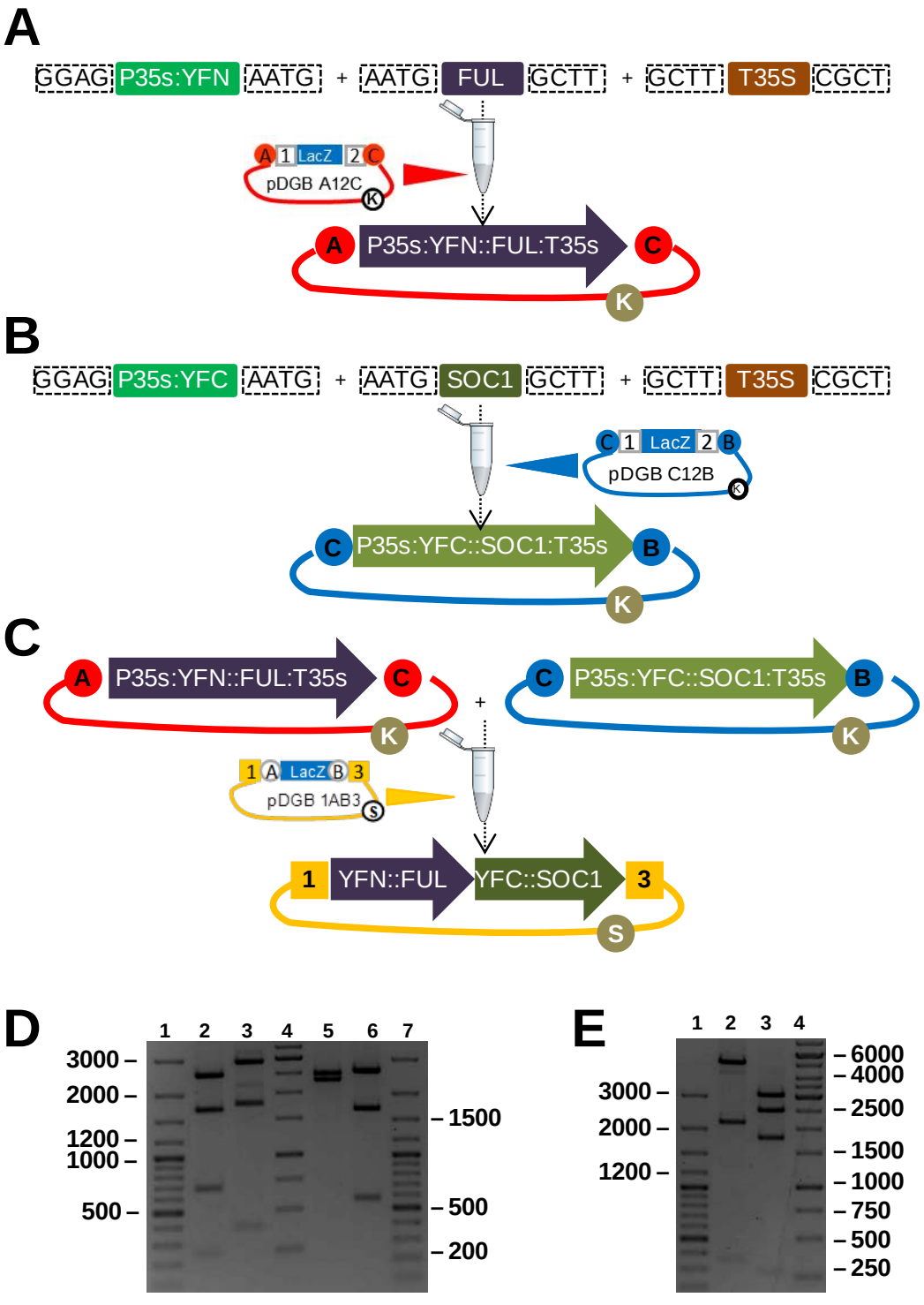

FIGURE 3: Multipartite assembly of a transcriptional unit in a level α plasmid and Binary combination of two units in a level Ω plasmid. (A) Combination GBparts (pE:35s:YFN, pE_FUL and pE_T35s) to build the TU pEGB_35s:YFN::FUL:T35s in the vector pDGBA12C. (B) Combination GBparts (pE:35s:YFC, pE_SOC1 and pE_T35s) to build the TU

pEGB_35s:YFC::SOC1:T35s in the vector pDGBC12B. (C) Assembly of the complementary TUs pEGB_A- 35s:YFN::FUL:T35s-C and pEGB_C-35s:YFC::SOC1:T35s-B in the destination vector pDGB1AB3. (D) Digestion of correct clones of the multipartite assemblies pEGB_A-35s:YFN::FUL:T35s-C (Lane 2: BglII; Lane 23: PvuI+NcoI) and pEGB_C-35s:YFC::SOC1:T35s-B (Lane 5: BglII; Lane 6: HindIII) (E) BanI (Lane 2) and BglI (Lane 3) digestion of a correct clone of the assembly of the two TUss pEGB_ 1-35s:YFN::FUL:T35s-35s:YFC::SOC1:T35s-3.

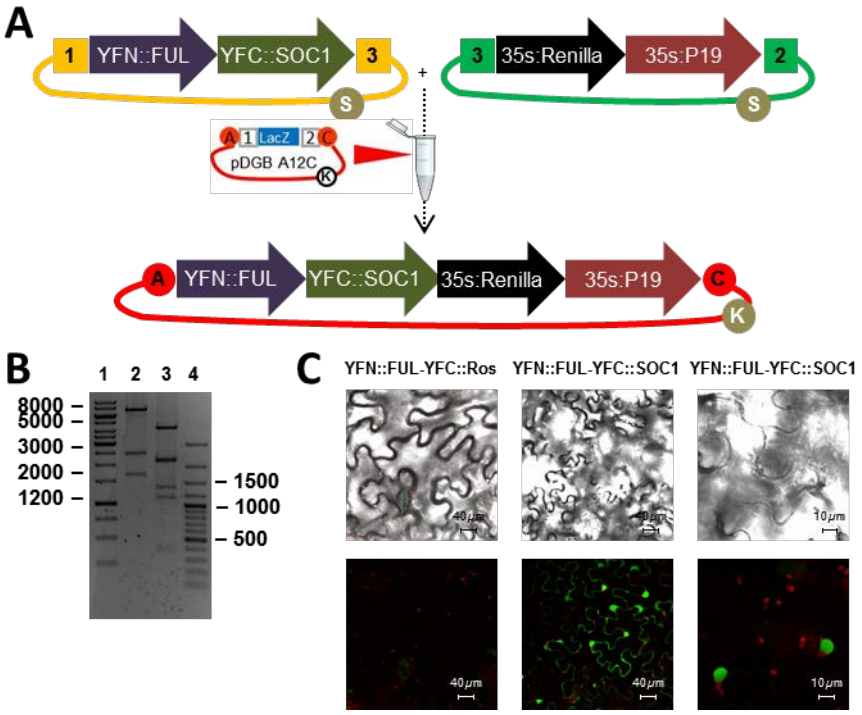

FIGURE 4: Multigenic constructs for BiFC. (A) Assembly of 4 TUs in one T-DNA comprising the two units for BiFC and the "monitoring/silencing suppressor module". (B) BglII (Lane 2) and BanI (Lane3) digestion of the final multigenic construct pEGB_A-35s:YFN::FUL:T35s-35s:YFC::SOC1:T35s-35s:Renilla:TNos-35s:P19:Tnos-C. (C) Confocal microscopy expression patterns of the negative (YFN::FUL-YFN:Ros) and the positive (YFN::FUL-YFN:SOC1) BiFC constructs, this latter at two magnifications.

7. Tables

Category	Foward	Reverse
Promoter (including 35s:YFC and 35s:YFN)	5' GG**GGTCTC**A*GGAG*-GSP 3'	5' GG**GGTCTC**A*AATC*-GSP 3'
CDS (including FUL and SOC)	5' GG**GGTCTC**A*AATG*-GSP 3'	5' GG**GGTCTC**A*AAGC*-GSP 3'
Terminator (including T35s)	5' GG**GGTCTC**A*GCTT*-GSP 3'	5' GG**GGTCTC**A*AGCG*-GSP 3'

TABLE 1. GB Extensions for the three main categories in multigenic assemblies. BsaI recognition and cutting sites (corresponding to GB overhangs) are marked in **bold** and *italics* respectively. GSP: Gene Specific Primers..

GB PART	PRIMER	SEQUENCE
35s:YFN and 35s:YFC	35s.F1	5' **GGGGTCTCAGGAG**ACTAGAGCCAAGCTGATCTC 3'
	Linker.	5' **GGGGTCTCACATT**AGCGATCCACCTCCACCAGAT 3'
SOC	SOC.F1	5' **GGGGTCTCAAATG**GTGAGGGGCAAAACTCA 3'
	SOC.R1	5' **GGGGTCTCAAAGC**TCACTTTCTTGAAGAACAAGGTAACCCAATGAACAATTGTGTCTCTACTTCAGAAC 3'
FUL	FUL.F1	5' **GGGGTCTCAAATG**GGAAGAGGTAGGGTTCA 3'
	FUL.R1	5' CAAACAACTTGTTGGCCGCGACGTTTCACAAAGTG 3'
	FUL.F2	5' TTTCACTTTGTGAAACGTCGCGGCCAACAAGTTG 3'
	FUL.R2	5' **GGGGTCTCAAAGC**TCACTCGTTCGTAGTGGTAGGAC 3'
T35s	T35s.F1	5' **GGGGTCTCAGCTT**CGGCCATGCTAGAGTCCGCAAA 3'
	T35s.R1	5' **GGGGTCTCAAGCG**AGGTCACTGGATTTTGGTTT 3'

TABLE 2. GB Oligonucleotides for the amplification of the GBparts used in this chapter. GB Extensions are marked in **bold**. 35s:YFN and 35s:YFC are amplified using the same pair of oligos as these oligos bind to the 35s and to the linker located after the YFP half.