BBF RFC 106: A Standard Type IIS Syntax for Plants

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1. Purpose

Here we define a standard syntax for assembling standard parts for expression in plant cells, extensible to all other eukaryotes. Variations of the Type IIS mediated cloning method known as Golden Gate Cloning[1], most notably Golden Braid (GB2.0)[2] and Golden Gate Modular Cloning (MoClo)[3] are in common use, particularly for the assembly of plasmids for delivery to plant cells. Many characterised plant parts compatible with Type IIS mediated assembly are available outside of the Registry of Standard Parts, as well as plasmids with the features necessary for delivery of DNA to plants cells via the shuttle chassis, *Agrobacterium tumefaciens*[4]. This RFC describes a consensus Type IIS syntax for plant parts to allow assembly into complete eukaryotic transcriptional units in plasmid vectors that contain the necessary features for transfection of plant chassis. We use *Marchantia polymorpha*, a primitive and easy-to-engineer liverwort and *Nicotiana benthamiana* a model plant as exemplar chassis.

2. Relation to other BBF RFCs

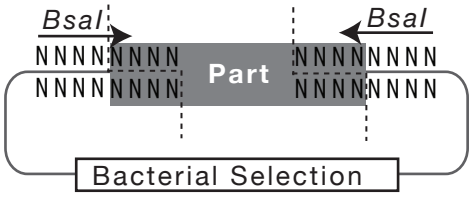
Assembly standards that used Type IIS restriction enzymes have been described in RFC28, RFC53 and RFC61. RFC88 proposed a Type IIS standard for the *Saccharomyces cerevisiae* chassis. RFC92 proposed the inclusion of parts compatible with both BBF RFC10 and Type IIS enzymes in the Registry. This RFC describes a standard Type IIS syntax for the assembly of parts for plants, extensible to all eukaryotes.

3. Copyright Notice

4. Introduction

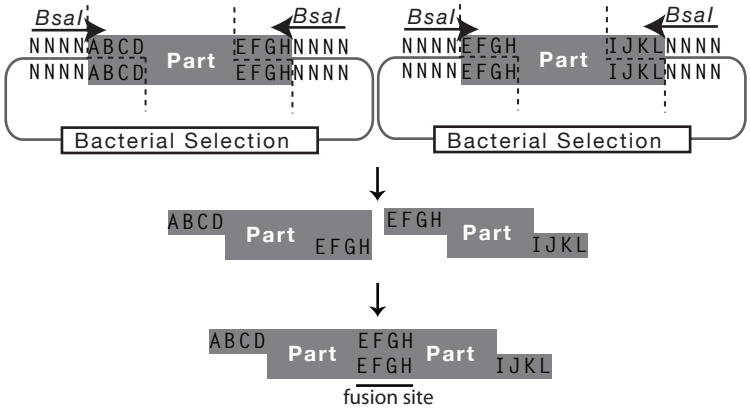
Type IIS restriction enzymes cleave downstream of their recognition site. They can be used to assemble multiple standard parts housed in separate plasmids into a single plasmid in a defined order in a single one-pot, one-step reaction as follows:

i. Standard parts MUST be housed in plasmids flanked by a pair of convergent recognition sequences for a Type IIS enzyme such as *BsaI*.

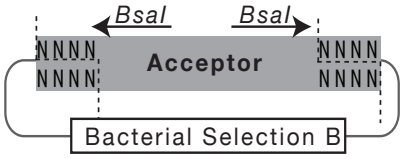


ii. The overhangs created by digestion MAY be of any identity and can therefore be standardized so that parts of particular types, for example coding sequences are flanked with the same sequences.

iii. The terminating overhang fusion site of a part MUST be identical to the starting overhang of the part that will follow it in the final assembly. This creates a fusion site:



iv. The accepting plasmid into which parts will be assembled MUST contain pair of divergent recognition sequences for the same enzyme.



v. The parts themselves, and all plasmid backbones, MUST otherwise be free of recognition sequences for this enzyme.

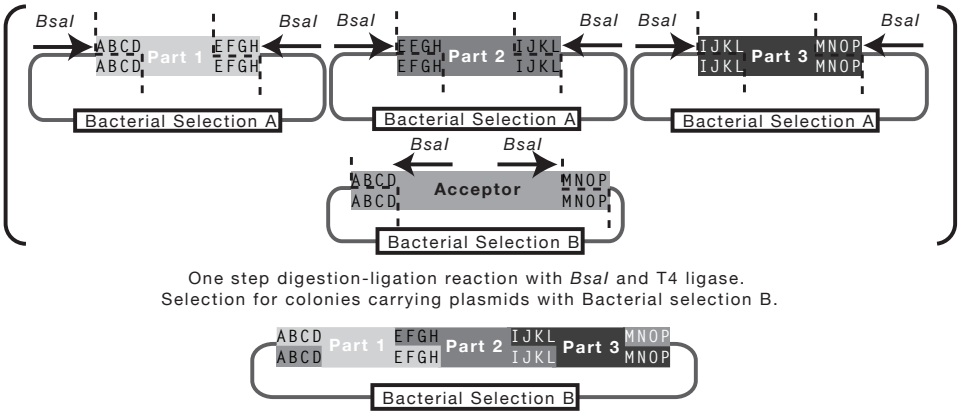
vi. The parts MUST not be housed in a plasmid backbone with the same antibiotic resistance as the accepting plasmid into which parts will be assembled.

vii. Each fusion site in the final assembly MUST be unique.

viii. All standard parts and the accepting plasmid can be combined in a single reaction mix containing the Type IIS enzyme and DNA ligase.

ix. The reaction mix is incubated at a temperature suitable for digestion followed by a temperature suitable for ligation. Since the final product does not contain any the Type IIS recognition sequence, the reaction can be cycled for high efficiency.

x. The reaction is transformed and plated on media containing the antibiotic to which the accepting plasmid carries resistance:

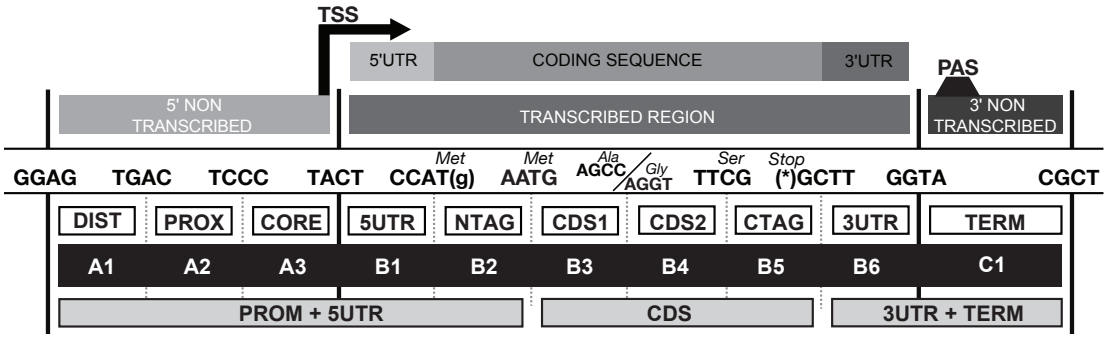


One step digestion-ligation reaction with *BsaI* and T4 ligase.
Selection for colonies carrying plasmids with Bacterial selection B.

5. A Standard Type IIS Syntax for Plants - Extensible to all Eukaryotes.

i. Standard Parts

i. A standard syntax for eukaryotic genes has been defined and eleven fusion points defined. Standard parts are sequences that correspond to one or more adjacent functional regions of a eukaryotic gene and are flanked by the defined standard fusion sites for those regions.



POSITION	NAME	FUNCTION	5' OVERHANG	3' OVERHANG
A1	DIST	Distal promoter region, *cis* regulator or transcriptional enhancer	GGAG	TGAC
A2	PROX	Proximal promoter region, *cis* regulator or transcriptional enhancer	TGAC	TCCC
A3	CORE	Minimal promoter region, including transcription start site (TSS)	TCCC	TACT
A4	5UTR	5' untranslated region	TACT	CCAT
B2	NTAG	N terminal coding region	CCAT	AATG
B3	CDS1	Coding region - optional N terminal coding region	AATG	AGCC /AGGT
B4	CDS2	Coding region no start or stop codon	AGCC /AGGT	TTCG
B5	CTAG	C terminal coding region	TTCG	GCTT
B6	3UTR	3' untranslated region	GCTT	GGTA
C1	TERM	Transcription terminator including polyadenylation signal (PAS)	GGTA	CGCT

ii. Standard parts MUST be housed in plasmid backbones free of ampicillin/carbenicillin and kanamycin bacterial resistance genes.

iii. Standard parts MUST be flanked with a pair of convergent *BsaI* recognition sequences. On digestion with *BsaI* the standard part MUST be released from the plasmid backbone with the flanking four base pair 5' overhangs that define its function.

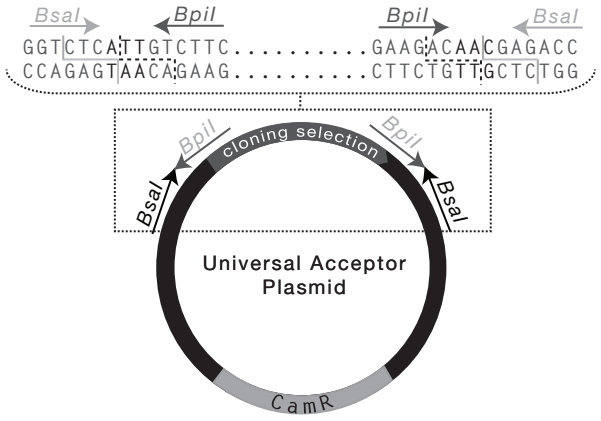
iv. Additional *BsaI* recognition sites MUST not be present within parts or plasmid backbones, except for the flanking convergent pair. The OPTIONAL additional removal of *BpiI* and *BsmBI* recognition sites allows subsequent multi-gene assembly using either the MoClo or GB2.0 plasmid systems:

TYPE	ENZYME	SEQUENCE (4bp overhang underlined)	COMPATIBILITY
ILLEGAL	**BsaI**	CCACGANNNNN	**MoClo/GB2.0**
AVOID	**BpiI**	GAAGACNNNNNN	**MoClo/GB2.0**
AVOID	**BsmBI**	CGTCTCNNNNN	**MoClo/GB2.0**

Cloning New Standard Parts

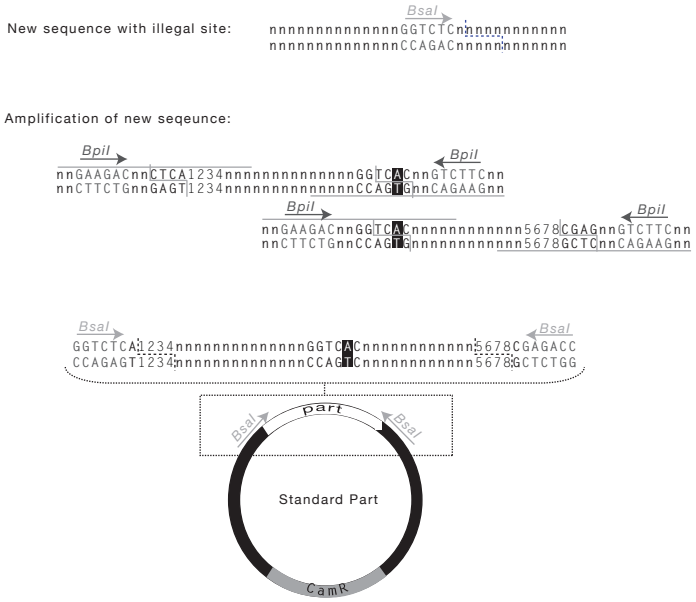
v. New standard parts MUST be cloned into a Universal Acceptor Plasmid (UAP). The plasmid backbone of UAPs is pSB1C3.

vi. Empty UAPs contain a cloning site consisting of a pair of divergent TypeIIS recognition sequences (e.g. *BpiI,* as depicted here, or *BsmBI*) flanked by overlapping convergent *BsaI* recognition sequences.



vii. Sequences containing no illegal sites can be amplified with oligonucleotide primers with 5' overhangs that add (i) Add Type IIS recognition sequences (e.g. *BpiI,* as depicted, or *BsmBI*) and fusion sites to allow one step digestion-ligation into the universal acceptor and (ii) add the desired fusion sites that will flank the part when re-released from the UAP backbone with *BsaI.*

viii. A sequence containing a single illegal *BsaI* recognition sequence can be amplified in two fragments using oligonucleotide primers with 5' overhangs that (i) introduces a mutation to destroy the illegal site (ii) adds TypeIIS recognition sequences (e.g. *BpiI,* as depicted, or *BsmBI*) and fusion sites to allow one step digestion-ligation into the universal acceptor and (iii) adds the desired fusion sites that will flank the part when re-released from the UAP backbone with *BsaI:*



6. Accepting plasmids for standard parts

For transfer to plants standard parts may be assembled into plasmids with the features necessary for delivery to the cell. For plants, sequences are often delivered via a shuttle chassis, *A. tumefaciens.*

a. Introduction to Agrobacterium-mediated Delivery

i. *A. tumefaciens* is a soil phytopathogen that naturally infects plants, injecting a portion of its DNA known as the 'transferred (T)-DNA' into the host plant's nuclear genome, causing tumour-like crown gall disease. The T-DNA is delivered via the bacterial type IV secretion system[5,6].

ii. The molecular transfer of DNA is dependent on the expression of virulence genes resident on the tumour-inducing (Ti) plasmid. The T-DNA is defined and recognized by conserved, flanking T-DNA borders known as the Left (LB) and Right Border (RB). These polarized border sequences serve as the target for the Vir endonucleases, which subsequently recognise proteins in the plant to enable integration of the T-DNA in the plant genome [5–8].

iii. For ease of engineering, scientists have separated the virulence genes and the T-DNA on separate plasmids to create a binary vector system: consisting of a vir-helper plasmid containing the virulence genes and T-plasmid containing the T-DNA flanked by the conserved borders. The T-plasmid commonly contains an additional origin of replication for *E.coli* for easy replication and assembly of the T-DNA region. T-regions as large as 200 kbp have been successfully mobilized with increasing efficiency with smaller T-regions[6,9].

b. Plasmid Features for Agrobacterium-mediated Delivery

i. The T-plasmid comprises a conserved backbone with origins of replication for *E. coli* and *A. tumefaciens* and an antibiotic resistance gene cassette for selection in bacteria.
ii. It also contains a region known as the T-DNA, flanked by the LB and RB sequences. The T-DNA sequence is variable and it is in this region that sequences to be transferred to the plant nuclear genome are cloned.
iii. For assembly of standard parts into a binary vector plasmid, within the T-DNA, adjacent to LB and RB the accepting plasmid contains divergent *BsaI* recognition sites and the vector must be otherwise free of such sites.
iv. Optionally, the binary vector plasmid can also carry additional convergent Type IIS recognition sequences to allow the complete transcriptional units to be released and assembled with other transcriptional units into a multi-gene assembly in a new T-plasmid backbone.
v. There are two commonly used plasmid vector systems with features for delivery to plants via *A. tumefaciens* that can be used to assemble transcriptional units into multi-gene constructs using Type IIS enzymes: GoldenBraid and MoClo[2,10]. The MoClo system is patented [EP2395087 A1].

7. *Marchantia polymorpha*, a New Model Organism for Plant Synthetic Biology

i. Introduction to the Chassis

i. Liverworts are descendants of the earliest terrestrial plants. Their small and modular morphological characteristic is a reflection of their simple genomic structures. Recent developments in transformation techniques can yield within 13 days a large number of stably transformed plants. This ease of transformation, combined with an increasing amount of genome characterisation place *M. polymorpha* as an ideal experimental chassis. Contrary to historical model species, the choice of *M. polymorpha* has preceded its establishment, allowing the community to set standards in a coherent and united way paving the way for faster and more efficient sharing of information and parts.

ii. Agrobacterium-mediated Transformation Protocol

i. Stable integration of foreign DNA in *M. polymorpha* can be achieved in 10 days and selected by 13.
ii. *M. polymorpha* spores are sterilized and plated. The purified T-plasmid is electroporated into *A. tumefaciens. Transformed A. tumefaciens* are selected on LB-agar plates containing the appropriate antibiotics at 30°C for 2-3 days. Isolated colonies are used to start 10 mL cultures in LB liquid medium containing the appropriate antibiotics and grown for 24h at 28°C. The cultures are centrifuged at 3000 g for 10 min and the pellet is re-suspended in approximately 2 mL (adjusted to OD600 1.5-2) of 1/2 GB media, 5% sucrose, 100 µM acetosyringone and incubated for six hours with agitation. The now 5 day old spores are scraped from the plates in 25 ml ½ GB, 5% sucrose and 100 µM acetosyringone solution and co-cultivated with 1 ml of the Agro solution for 1.5 days with agitation under full illumination at RT. The spores are collected with a 40µm cell strainer, washed with 150 mL sterilized ddH20 and 100 µg/mL cefotaximine

and plated on ½ GB media with 10µg/mL hygromycin + 100 µg/mL cefotaximine. *M. polymorpha* transformants can be screened within 3 days.

iii. Plantlets can be repropagated on 1/2GB media with the appropriate antibiotics, for accelerated growth.

iv. To produce isogenic lines, cells from gemmae cups, asexual reproductive organ present on thalli from 4 weeks on, are collected and plated on selective media.

v. For selection of stable transformants in *M. polymorpha*, a hygromycin resistance cassette is included adjacent to the LB.

vi. The hygromycin resistance gene is followed by the 3' untranslated sequence and terminator from the nopaline synthase gene (nos) from *A. tumefaciens* and promoted by MPEF1α. MPEF1α is an endogenous *M. polymorpha* promoter active from early in development, and its use can lead to high transformation efficiency.

vii. Wild type spores are not vulnerable to standard concentrations of kanamycin so this should be avoided as a selection agent.

8. *Nicotiana benthamiana*: A Model Dicotyledenous Plant Chassis

i. Description of the chassis

i. *N. benthamiana* is a widely used experimental plant from the solanaceous group of flowering plants that includes tomatoes, potatoes and capsicums. It is widely used in plant pathology due to the large number of plant pathogens (viruses, bacteria, fungi, oomycetes etc) that can successfully infect it. *N. benthamiana* it is easily genetically transformed and regenerated and amenable to virus-induced gene silencing and transient protein expression, including the production of therapeutic compounds and pharmaceuticals. The genome sequence is available at the Solgenomics network [http://solgenomics.net].

ii. Type IIS resources for model dicotyledenous plants

i. A resource for plant synthetic biologists, comprising a set of cloning vectors for the domestication of new parts, assembly into mulitigene constructs and 96 standardized parts to enable facile construction of multigene constructs for plant transformation was published[4]. Parts include promoters, untranslated sequences, reporters, antigenic tags, localization signals, selectable markers, and terminators. The comparative performance of regulatory parts was performed in *N. benthamiana.* Several other laboratories have also characterized parts for plants that can be assembled using Type IIS assembly[2,11,12] including parts for engineering chloroplast genomes[13].

iii. Agrobacterium-mediated Transformation protocol

i. Transient expression of foreign DNA can be detected within 28 hours of transfection of young *N. benthamina* leaves infiltrated with *A. tumefaciens*. *A. tumefaciens* are selected on LB-agar plates containing the appropriate antibiotics at 28°C for 2-3 days.

ii. Isolated colonies are used to start 5 mL pre-cultures in LB liquid medium containing the appropriate antibiotics and grown for 24 hours at 28°C.

iii. A larger culture is made by adding 500 µL of pre-culture to 50 mL LB liquid medium with the appropriate antibiotics. The flask is incubated for 16 hours at 28°C with agitation.
iv. The bacterial cultures are centrifuged at 3200 g for 10 min to pellet the cells. The supernatant is discarded and each pellet is re-suspended in 5 mL of MMA (10 mM MgCL2, 10 mM MES/KOH pH 5.6; 150 µM acetosyringone). The cells are incubated in MMA for two hours in the dark at room temperature. The optical density of the culture at 600 nm is measured with a spectrophotometer and adjusted to 0.8 by diluting with extra MMA.
v. For the co-infiltration of multiple *A. tumefaciens* cultures, equal volumes of the 0.8 OD_{600nm} *A. tumefaciens* cultures in MMA are mixed together.
vi. For transient expression holes are pierced in the underside of leaves of 3 weeks old *N. benthamiana* plants using a sterile pipette tip. One millilitre needle-less syringes are then used to infiltrate the entire leaves with the *A. tumefaciens* cultures. Tissues are collected approximately 6 days after agro-infiltration (depending on the genes expressed) and can be directly used for analysis, freeze-dried or frozen in liquid nitrogen prior to storage at -80°C.
vii. For stable integration of the T-DNA, pieces of leave are sterilised and co-cultivated with *A. tumefaciens* cultures in the dark for 48 hours. The pieces of leaf are then transferred to selection media where they form callus at the cut edges. Resistant callus are removed to media containing hormones to induce shoot elongation and, subsequently, resistant shoots are moved to media to allow the production of roots. Plantlets that form roots are transplanted to soil.

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