Construction of Expression vectors for Fusion proteins
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(Caution, please understand that our experience with the following system has not so much been accumulated when I wrote this chapter. Therefore, there might be a possibility a potential issue remains. If you find such an issue, please let us know)

1) Conventional fusions

In order to express a fusion protein of two ORFs, we usually generate the expression vector as follows:

Insert N terminal side ORF into the BamHI or NotI and EcoRI of pSP vector. EcoRI must remain after the insertion. The stop codon must be deleted from the ORF. Then insert the C terminal side ORF at the EcoRI site (by means of ligation or In-fusion).

NBRP provides pSPCFP-ter. The vector was used to make the eCFP::Hox1 fusion construct (Sasakura et al., 2012 Development 139, 2156-2160).

2) Using 2A peptide

Recently, 2A peptide sequence has been used in Ciona. This sequence allows us to express two ORFs from a single cis element without making fusions of the two ORFs. This system can minimize the modifications of ORFs and subsequently would reduce the risk to impair the activities of proteins by fusing something.

We have established the systematic way to create the 2A fusion expression vectors. The vector designs are shown in Figure 1 (ORF::2A::FP) and Figure 2 (FP::2A::ORF). FP means fluorescent protein.

2-1) making ORF::2A::FP constructs (Figure 1)

Insert your ORF at the NotI site of the pSP vectors compatible with the 2A system (such as pSP2AeGFP; Figure 1). The primers for amplifying the ORF will be as follows:

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GATCCCCTTGCGGCCATGxxxxxxx
CCTGATCCTGCGGCCGCxxxxxxxx (Please delete stop codon)
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The red ATG is the start codon of your ORF. The nucleotide length of the amplified ORFs should be a multiple number of 3 (in order to adjust the reading frame). By this reason the red x must be the +3 position of the codon. You must delete the stop codon from the amplified ORF. You can insert promoters at the upstream of the ORF using a restriction site. When you use the BamHI site, you can re-use the promoter fragment that is used in section 1. When a BamHI site is present in your ORF, you may firstly insert promoter and then insert ORF, or you may open the vector by inverse PCR.

2-2) making FP::2A::ORF constructs (Figure 2)

Insert your ORF at the EcoRI site of the pSP vectors compatible with the 2A system (such as pSPeGFP2A; Figure 2). The primers for amplifying the ORF will be as follows:
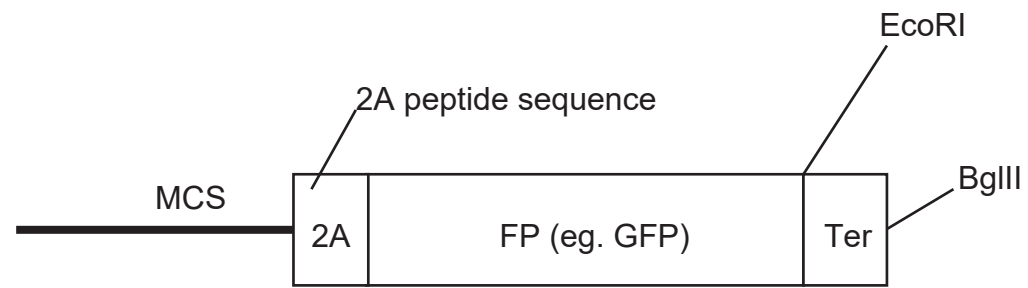
```
CCCAGGACCAGAATTCxxxxxxxxxx (Please delete start codon)
CGCTCAGCTGGAATTCTTAxxxxxxx
```

The red TTA corresponds to the stop codon (you can change the type of stop codon). The nucleotide length of the amplified ORFs should be a multiple number of 3 (in order to adjust the reading frame). The red x must be the +1 of the codon. We usually delete the start codon from the amplified ORF to make sure the use of the ATG at the first ORF. You can remove the inserted ORF and insert a new ORF with EcoRI.

You can insert promoters in the MCS. BamHI site is compatible with other pSP vector systems and you can use the primer sets mentioned the section 1-2. When a BamHI site is present in your ORF, please firstly insert promoter and then insert ORF, or you can open the FP::2A::ORF construct by inverse PCR.

Figure 1

The structure of pSP-2A peptide vector for fusing a ORF at the N-terminal side of a fluorescent protein
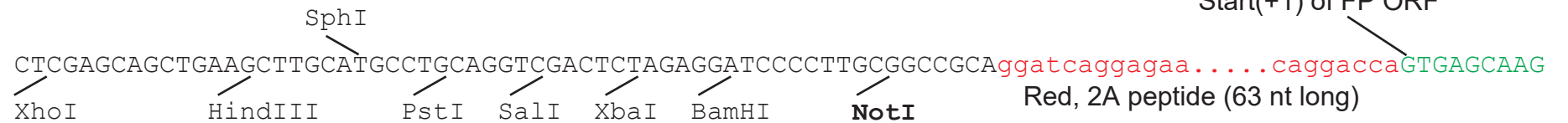(ORF::2A::FP)

EcoRI

2A peptide sequence

MCS

BglII

| 2A | FP (eg. GFP) | Ter |

MCS of pSPORF::2A::FP

Start(+1) of FP ORF

SphI

CTCGAGCAGCTGAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTTGCGGCCGCAggatcaggagaa.....caggaccaGTGAGCAAG

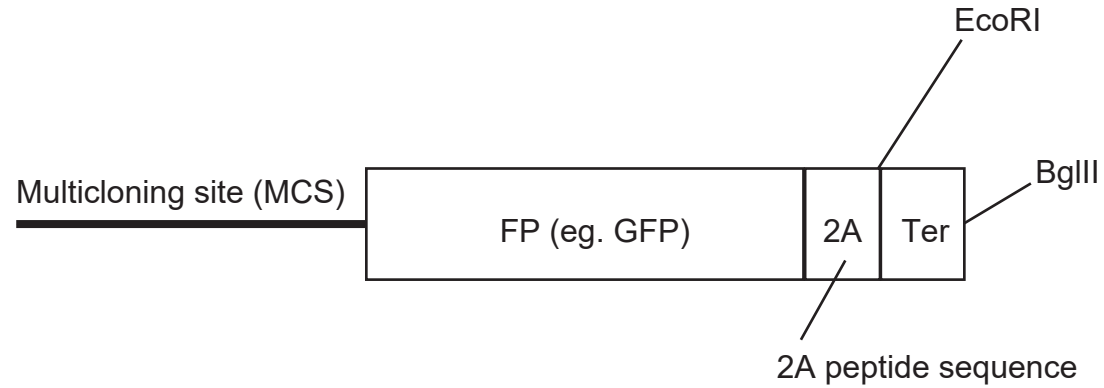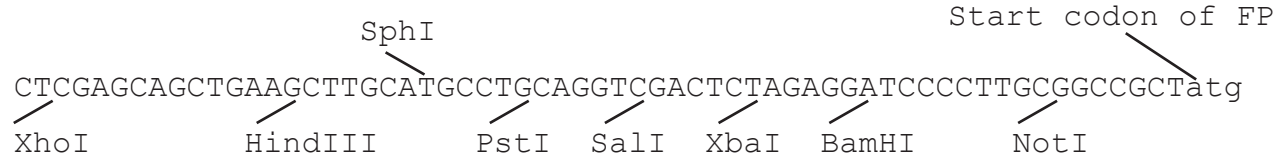XhoI   HindIII   PstI   SalI   XbaI   BamHI   NotI

Red, 2A peptide (63 nt long)

Figure 2

The structure of pSP vector for fusing a ORF at the C-terminal of a fluorescent protein
(FP::2A::ORF)

EcoRI

BglII

Multicloning site (MCS)

FP (eg. GFP)   2A   Ter

2A peptide sequence

MCS of pSPFP::2A::ORF

Start codon of FP

SphI

CTCGAGCAGCTGAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTTGCGGCCGCTatg

XhoI   HindIII   PstI SalI XbaI BamHI   NotI

Around the EcoRI site

EcoRI Termination sequence

aagGGATCAGGAGAAGGAAGAGGATCACTTCTTACATGTGGAGATGTTGAAGAAAACCCAGGACCAgaattcCAGCTGAGCGCCGGTCGCTAC...
Red, 2A peptide (63 nt long)

The end (+3) of FP (this case eGFP)