Construction of expression vectors for Fusion proteins
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1) Conventional fusions

In order to express a fusion protein of two ORFs, we usually generate an expression vector by the following procedure:

Insert the N-terminal side ORF into the BamHI or NotI and EcoRI of the 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, the 2A peptide sequence was used in Ciona. This sequence allows us to express two ORFs from a single cis element without making a fusion of the two ORFs. This system can minimize the modifications to ORFs and subsequently would reduce the risk of impairing the activities of proteins by fusing to something.

We have established a systematic way to create 2A fusion expression vectors (e.g. Krasovec et al., 2022, Sci. Adv. 8, eabn3264). 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 a pSP vector compatible with the 2A system (such as pSP2AeGFP; Figure 1). The primers for amplifying the ORF will be as follows:

```
GATCCCCTTGCGGCCATGxxxxxxx
CCTGATCCTGCGGCCGCxxxxxxx (Please delete the stop
codon.)
```

The red ATG is the start codon of your ORF. The nucleotide length of the amplified

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

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

Insert your ORF at the EcoRI site of a pSP vector compatible with the 2A system (such as pSPeGFP2A; Figure 2). The primers for amplifying the ORF will be as follows:

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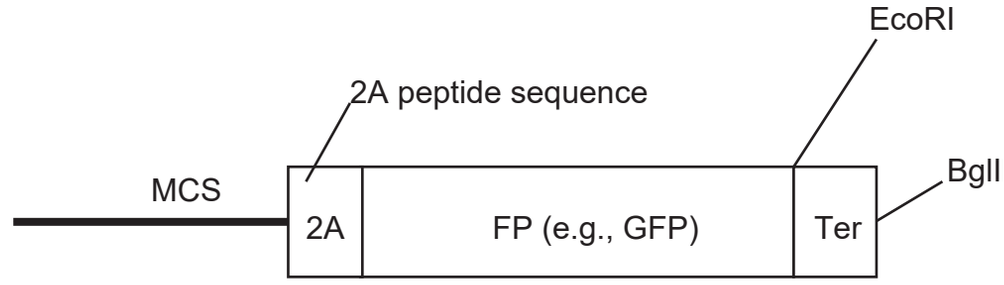
The red TTA corresponds to the stop codon (you can change the type of stop codon). The nucleotide length of the amplified ORFs should be a multiple of 3 (in order to adjust the reading frame). The red x must be the +1 of the codon. We usually delete the start codon from the amplified ORF to make sure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

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

Figure 1

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



MCS of pSPORF::2A::FP

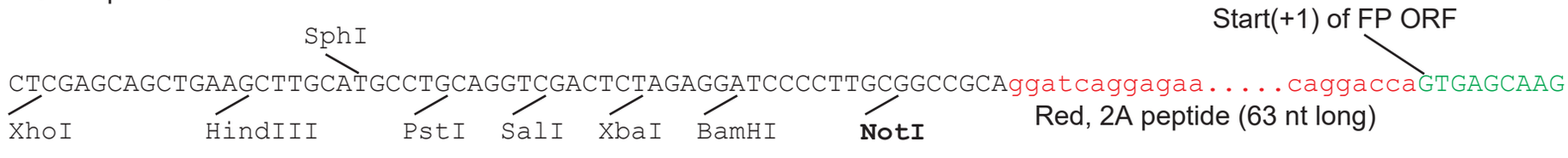
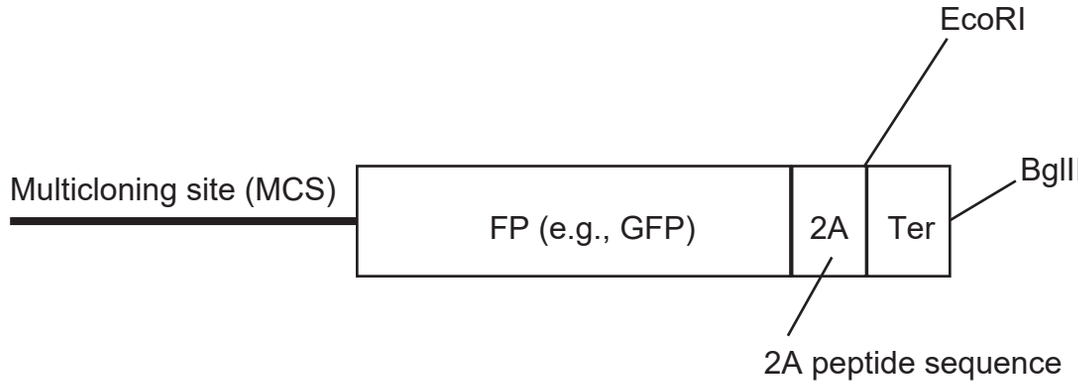
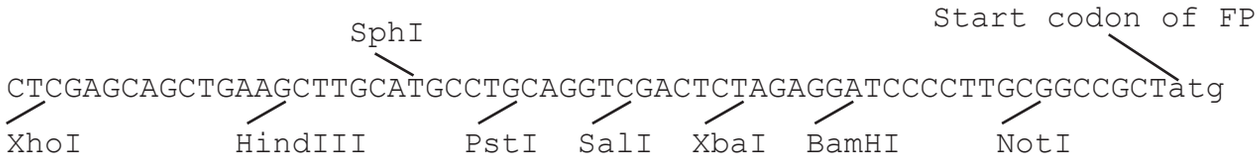


Figure 2

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



MCS of pSPFP::2A::ORF



Around the EcoRI site