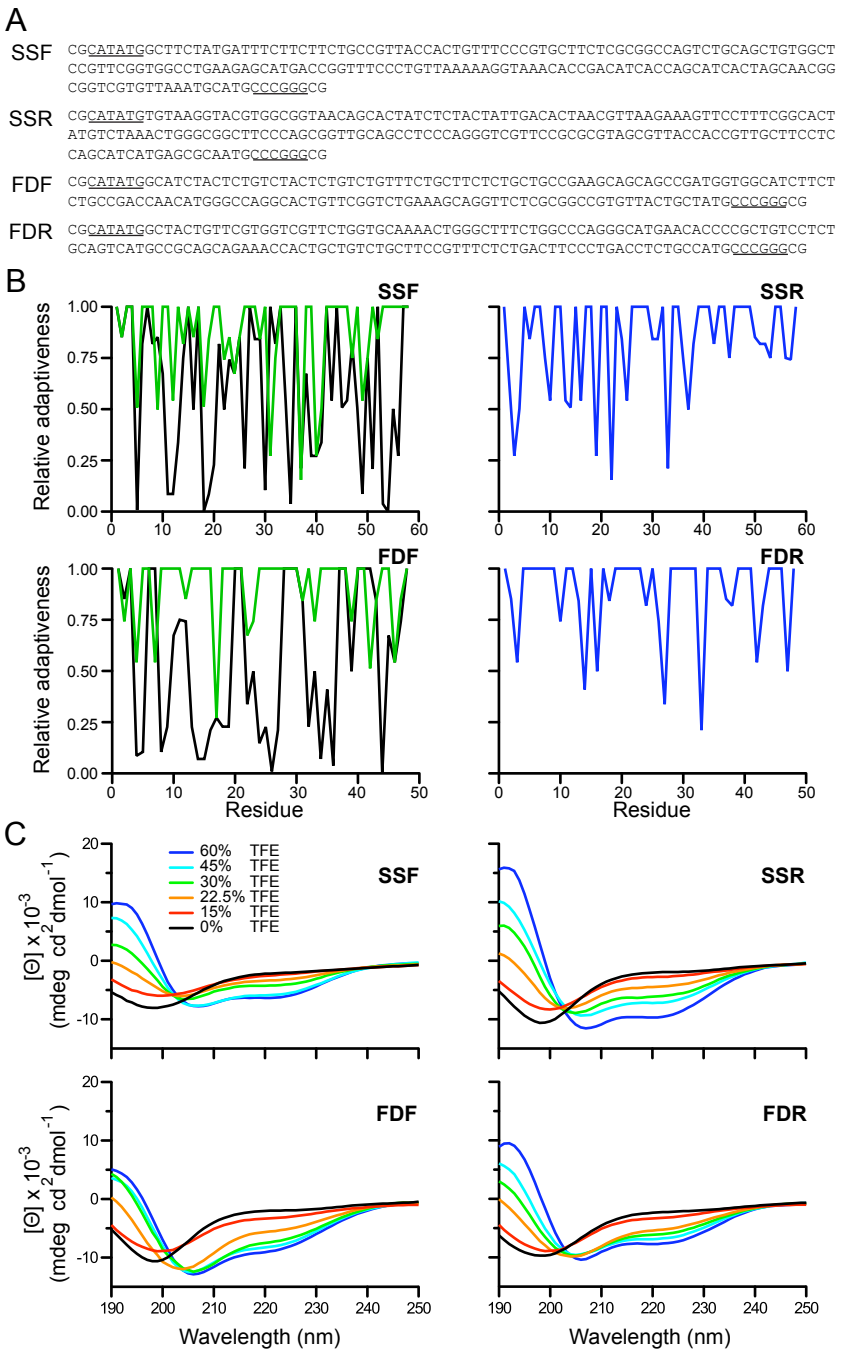
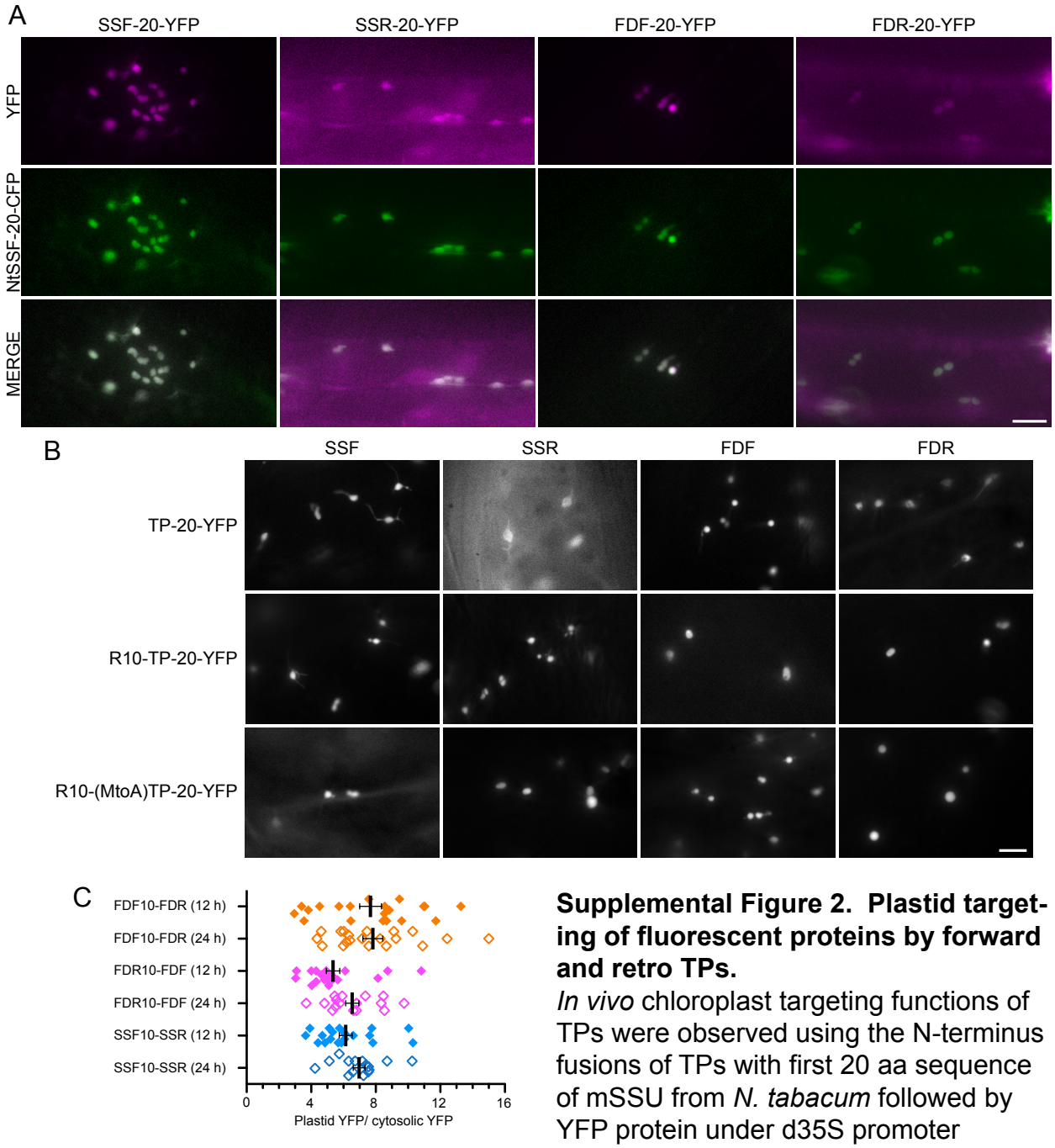


Supplemental Figure 1. Codon optimization and far UV CD spectra of forward and retro peptides.

(A) The sequences of synthetic codon optimized TPs. The restriction sites are underlined.

(B) The relative adaptiveness (w) of the codons from SSF, SSR, FDF, and FDR TPs. Black and green lines represent the w prior to and after codon optimization, respectively. Since the reverse sequences do not exist in nature, there is no black trace.

(C) CD spectra of SSF, SSR, FDF and FDR. The line colors represents percent TFE. Data was collected at 25ºC.

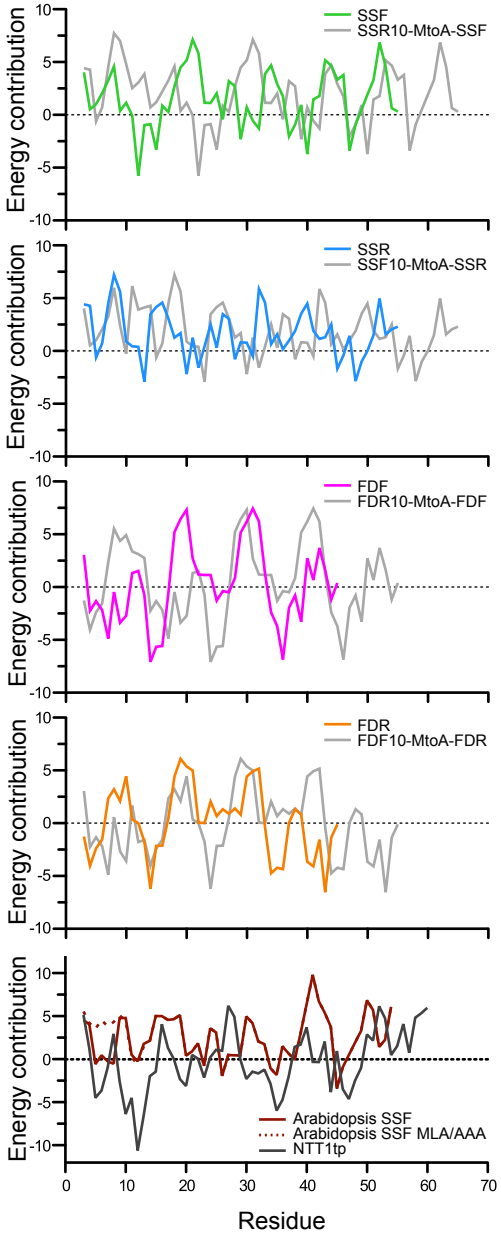


Supplemental Figure 2. Plastid targeting of fluorescent proteins by forward and retro TPs.

In vivo chloroplast targeting functions of TPs were observed using the N-terminus fusions of TPs with first 20 aa sequence of mSSU from *N. tabacum* followed by YFP protein under d35S promoter

(A) Localization patterns of transiently expressed TP-YFP fusion proteins in *Arabidopsis* seedlings were compared to the CFP plastid marker constructs (NtSSF-20-CFP). Forward peptide driven YFP proteins showed strong localization in the chloroplasts, whereas retro-peptide driven YFP proteins stayed mostly outside of the chloroplasts. The results are similar to those from onion epidermis and tobacco leaves (Figure 5A and C). Bar, 10 μm. (continue next page)

Supplemental Figure 2. (Continue)
(B) Plastid targeting of fluorescence proteins by extended forward and retro TP constructs. The extension 10 aa sequences representing the first 10 aa sequence of the opposite TP were added at the N-terminus of each TP-YFP fusion constructs. In vivo plastid targeting was observed in transiently expressed onion epidermis. Left labels indicate the types of fusion proteins. TP indicates TP as shown on the top labels. R10 marked the first 10 aa sequence from the opposite TP. MtoA marked the substitution of internal Met with Ala. Ratio of plastid YFP/ cytosolic YFP intensities were showed in Figure 6A. Bars, 10 μm.
(C) Accumulation of the plastid targeted YFP proteins increase slightly over time. Ratio of plastid/ cytosolic YFP intensities after 12 and 24 h after transformations were shown. Left labels show the TP in the constructs. Suffix 10 indicated only first 10 aa sequence. Data was collected from 20 cells except SSF10-SSR (24 h) and FDR10-FDF (24 h)



Supplemental Figure 3. Hsp70 Binding Site Prediction. Hsp70 binding site prediction results based on algorithms developed by Rudiger et al. (1997). NTT1tp is the TP of *Arabidopsis* nucleotide transporter 1.

Supplemental Table 1. Codon adaptive indices of the competitors based on three codon usage tables of *E. coli*.

DNA Sequences	Hénaut & Danchin[a]	Carbone et al.[b]	Nakamura et al.[c]	Mean	SD
Wild-type FDF	0.32557	0.16813	0.58672	0.36014	0.21143
Optimized FDF	0.88172	0.84682	0.91045	0.87967	0.03186
Optimized FDR	0.84668	0.75238	0.91127	0.83678	0.07991
Wild-type SSF	0.33722	0.21405	0.54256	0.36461	0.16596
Optimized SSF	0.82290	0.74577	0.85286	0.80718	0.05525
Optimized SSR	0.78089	0.67800	0.83168	0.76352	0.07830

[a] Hénaut , A., and A. Danchin. 1996. Analysis and predictions from *Escherichia coli* sequences, or *E. coli in silico*. *In Escherichia coli* and *Salmonella*: cellular and molecular biology. Vol. 2. F. Neidhardt and R. Curtiss, editors. ASM Press Washington, D.C. 2047-2066.

[b] Carbone, A., A. Zinovyev, and F. Kepes. 2003. Codon adaptation index as a measure of dominating codon bias. *Bioinformatics*. 19:2005-15.

[c] Nakamura, Y., T. Gojobori, and T. Ikemura. 2000. Codon usage tabulated from international DNA sequence databases: status for the year 2000. *Nucleic Acids Res*. 28:292.

Supplemental Table 2. Subcellular targeting predictions of forward and reverse transit peptides. [a,b]

Program	Prediction	SSF	SSR	FDF	FDR
ChloroP 1.1	Chloroplast localization	**0.573**	**0.559**	**0.556**	**0.548**
	Cleavage site	56	56	46	46
iPSORT	Localization	**Cpst**	n/a	**Cpst**	Mito
	Target sequence	ASMISSS	n/a	MASTLS	MATVRG
Predotar	Chloroplast localization	**1**	0.04	**0.8**	0
	Mitochondria localization	0.13	0.02	0.02	0.4
	ER localization	0	0.01	0.01	0.04
	Other localization	0.03	0.93	0.23	0.58
PredSL	Chloroplast localization	**1**	**0.99**	**1**	0.1129
	Mitochondria localization	0	0.0039	0.0002	0.6538
	Secreted protein	0.0004	0.0019	0.0005	0.0994
	Cleavage site	33	38	47	55
ProtComp	Chloroplast localization	**3**	**4.6**	**2.9**	**2.2**
	Mitochondria localization	0	0.15	0.03	0
	Plasma membrane	0	0	0.08	0
	Nuclear localization	0	0.04	0	0
	Peroxisome localization	0	0	0	0.77
Protein Prowler	Chloroplast localization	**1**	0.25	**1**	0.16
	Mitochondria localization	0.02	0.3	0.01	0.82
	Secreted protein	0	0.03	0	0
	Other localization	0	0.42	0	0.01
PSORT	Chloroplast stroma	**0.81**	0	**0.77**	0.2
	Thylakoid space	0.965	0	0.957	0
	Thylakoid membrane	0.651	0	0.574	0.2
	Mitochondria matrix	0.853	0.1	0.461	n/a
	Nucleus	0	0.3	0	0.3
	Peroxisome	0	0.3	0	0.3

[a] Predictive scores shown in bold indicate the prediction is above threshold necessary for confident prediction.
[b] Abbreviations used: Cpst, chloroplast; Mito, mitochondria; n/a, not applicable.

Supplemental Table 3. Rules applied during development of the heuristic FGLK motif detection.

Rule	Definition [a]
1	F|W AND P|G AND K|R AND A|L AND S|T
2	F|W AND P|G AND K|R AND A|L
3	Any 4 of (F|W, P|G, K|R, A|L, T)
4	F|W required, any 3 of (P|G, K|R, A|L, T)
5	F|W required, any 3 of (P|G, K|R, A|L, S)
6	F|W|Y, P|G
7	[FWPGKRALST] +, variable window, minimum 4
8	F|W AND P|G AND K|R AND A|L AND S
9	[FWYPGKRALST] +, variable window, minimum 4
10	F|W AND P|G AND K|R|N AND A|L AND S|T
11	F|W AND P|G AND K|R|N AND A|L|V AND S variable window
12	F|W AND P|G AND K|R|N AND A|L AND S variable window
13	F|W AND P|G AND K|R|N AND A|L|V AND S|T
14	F|W AND P|G AND K|R|N AND A|L|V
15	F|W|L|V AND P|G AND K|R|N AND optional S|T variable window
16	F|W|L|V AND P|G AND K|R|N AND S|T
17	F|W|Y AND P|G variable window
18	F (to generate logoplot for context)
19	F|W|Y AND P|G
20	F|W|Y AND P|G
21	F|W AND P|G AND K|R|N|Q AND A|L|I|V
22	F AND P|G AND K|R AND A|L|V
23	F|W AND P|G AND K|R AND A|L
24	F|W AND P|G AND K|R AND A|L|V AND S
25	F|W AND P|G AND K|R AND A|L|V AND S|T
26	F AND P|G AND K|R AND A|L
27	F AND P|G AND K|R AND A|L|V AND S
28	F AND P|G AND K|R AND A|L|V AND S|T
29	F AND P|G AND K|R AND A|L AND S|T
30	F AND A|L AND P|G AND K|R AND S|T|W|Y|I|M
31	F AND A|L AND P|G AND K|R|W|Y|I|M
32	F AND L AND P|G AND K|R AND S|T|W|Y|I|M
33	F AND P|G AND K AND A|L AND S|T
34	F AND P AND K|R AND A|L AND S|T
35	F AND P AND K AND L AND S|T
36	F AND A|L AND P|G AND K|R AND S|T

[a] All rules exclude D and E; vertical bar '|' indicates alternation, any single residue; brackets indicate 'any of'; '+' means one or more

Supplemental Table 4. Curve fitting parameters for prSSU homologous binding data.

Fitted Values [a]	Datasets		
	30 nM [b]	100 nM [b]	Global
K_d (nM) [c]	153.8 (153.1)[d]	153.8 (153.1)[d]	153.8 (153.1)[d]
95% CI of K_d (nM) [c]	89.95 – 263.1 (92.12 – 254.4)	89.95 – 263.1 (92.12 – 254.4)	89.95 – 263.1 (92.12 – 254.4)
Nonspecific binding (%) [c]	0.3622 (0.3586)	0.3622 (0.3586)	0.3622 (0.3586)
Bottom plateau (%) [c,e]	36.22 (35.86)	10.87 (10.76)	-
R^2	0.9209 (0.9141)	0.8397 (0.7655)	0.9626 (0.9490)
R^2 [f]	0.9208 (0.9142)	0.8398 (0.7647)	-

[a.] Model: Total binding = $((B_{max} \times [Hot])/([Hot]+[Cold]+K_d))+(\text{nonspecific binding} \times [Hot])$.
[b.] Concentration of ^{35}S-prSSU.
[c.] Values are shared between 2 datasets.
[d.] Values from scintillation counting and autoradiograph (in parentheses).
[e.] Bottom plateau = nonspecific binding x [Hot].
[f.] R^2 generated from swapping the data and fitted parameters from scintillation counting and autoradiograph.

Supplemental Table 5. Curve fitting parameters for competitive binding data.

Fitted Parameters	Competitors				
	FDF	**FDR**	**SSF**	**SSR**	**mSSU**
K_i (nM)[a]	2220	3735	2537	3091	No Inhibition
95% CI of K_i (nM)	1516 – 3252	2587 – 5392	1752 – 3672	2023 – 4723	-
R^2	0.8763	0.8580	0.8565	0.8264	-
IC_{50} (nM)[b]	3663	6164	4186	5101	No Inhibition
95% CI of IC_{50} (nM)	2501 – 5366	4270 – 8898	2891-6060	3338-7794	-
R^2	0.8763	0.8580	0.8565	0.8264	-

[a.] Model: Total binding = Bottom + (Top-Bottom)/(1+$10^{([Cold] - logIC50)}$). Where $logIC_{50}$ = log (10^{logKi} x (1 + [Hot]/K_d)).
[b.] Model: Total binding = Bottom + (Top-Bottom)/(1+$10^{([Cold] - logIC50)}$).

Supplemental Table 6. Sequence of oligonucleotides used in cloning.

Generated Construct	Oligonucleotide Name	Sequence [a]
pBS-SSF-YFP	SSF_NheI_F	GTTGTTGCTAGCATGGCTTCTATGATTTCTTCTTCTG
	SSF_MscI_R	GTTGTTTGGCCACACCTGCATGCATTTAACACGACCGCCGTTGCTG
pBS-SSR-YFP	SSR_NheI_F	GTTGTTGCTAGCATGTGTAAGGTACGTGGCGGTAACAGCACTATCTC
	SSR_MscI_R	GTTGTTTGGCCACACCTGC ATTGCGCTCATGATGCTGGAGGAAGC
pBS-FDF-YFP	FDF_NheI_F	GTTGTT GCTAGCATGGCATCTACTCTGTCTACTCTGTCTG
	FDF_MscI_R	GTTGTTTGGCCACACCTGC ATAGCAGTAACACGGCCGCGAGAACC
pBS-FDR-YFP	FDR_NheI_F	GTTGTTGCTAGCATGGCTACTGTTCGTGGTCGTTCTG
	FDR_MscI_R	GTTGTTTGGCCACAC CTGCATGGCAGAGGTCAGGGAAGTC
pBS-SSF10-SSR-YFP	SSF10_F	CTAGCATGGCTTCTATGATTTCTTCTTCTGCCGTTG
	SSF10_R	CTAGCAACGGCAGAAGAAGAAATCATAGAAGCCATG
pBS-SSR10-SSF-YFP	SSR10_F	CTAGCATGTGTAAGGTACGTGGCGGTAACAGCACTG
	SSR10_R	CTAGCAGTGCTGTTACCGCCACGTACCTTACACATG
pBS-FDF10-FDR-YFP	FDF10_F	CTAGCATGGCATCTACTCTGTCTACTCTGTCTGTTG
	FDF10_R	CTAGCAACAGACAGAGTAGACAGAGTAGATGCCATG
pBS-FDR10-FDR-YFP	T7 Universal	TAATACGACTCACTATAGGG
	FDR10_XbaI_R	GGTGGTTCTAGATGCACCAGAACGACCACG
All of MtoA constructs	M13 Forward	CGCCAGGGTTTTCCCAGTCACGAC
	Nos_R	CTTAACGTAATTCAACAGAA
pBS-SSF10-MtoA-SSR-YFP	SSF10_MtoA_SSR_F	GCTTCTATGATTTCTTCTTCTGCCGTTGCGTGTAAGGTACGTGG
	SSF10_MtoA_SSR_R	CCACGTACCTTACACGCAACGGCAGAAGAAGAAATCATAGAAGC
pBS-SSR10-MtoA-SSF-YFP	SSR10_MtoA_SSF_F	CGGTAACAGCACTGCGGCTTCTAGCATTTCTTCTTCTGCC
	SSR10_MtoA_SSF_R	GGCAGAAGAAGAAATGCTAGAAGCCGCAGTGCTGTTACCG
pBS-FDF10-MtoA-FDR-YFP	FDF10_MtoA_FDR_F	CTCTGTCTACTCTGTCTGTTGCGGCTACTGTTCGTGGTCG
	FDF10_MtoA_FDR_R	CGACCACGAACAGTAGCC GCAACAGACAGAGTAGACAGAG
pBS-FDR10-MtoA-FDF-YFP	FDR10_MtoA_FDF_F	GGTCGTTCTGGTGCAGCGGCATCTACTCTGTCTACTCTG
	FDR10_MtoA_FDF_R	CAGAGTAGACAGAGTAGATGCC GCTGCACCAGAACGACC
pET-SSF-YFP	SSF_NdeI_F	GGTGGTCATATGGCTTCTATGATTTCTTCTTCTGC
pET-SSR-YFP	SSR_NdeI_F	GGTGGTCATATGTGTAAGGTACGTGGCGGTAACAGC
pET-FDF-YFP	FDF_NdeI_F	GGTGGTCATATGGCATCTACTCTGTCTACTCTGTCTG
pET-FDR-YFP	FDR_NdeI_F	GGTGGTCATATGGCTACTGTTCGTGGTCGTTCTGG
pET-NtSSF-YFP	NtSSF_NdeI_F	GGTAGATACATATGGCTTCCTCAGTTC
All of pET constructs	M13 Reverse	TCACACAGGAAACAGCTATGAC

[a] The restriction sites are underlined.