

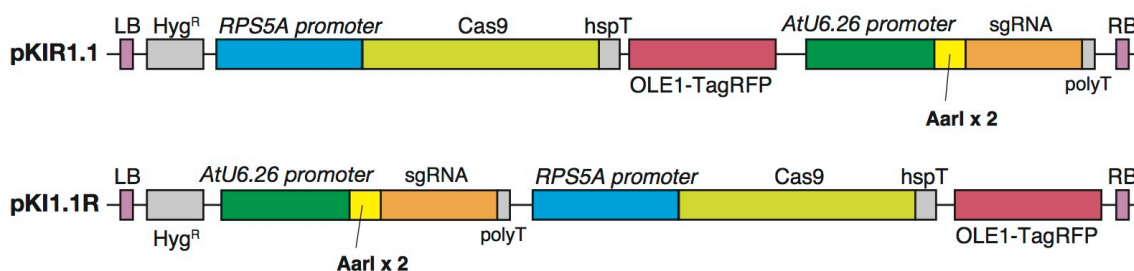
Details of CRISPR/Cas9 vectors (pKAMA-ITACHI)

as of 2016/12/16

	insert	antibiotics (bacteria)	plant selection
pKIR1.1	<i>RPS5Ap::Cas9:HspT</i> + <i>U6.26p::AarI_site:sgRNA</i>	Spec	Hyg or red fluorescence in seeds
pKI1.1R	<i>U6.26p::AarI_site:sgRNA</i> + <i>RPS5Ap::Cas9:HspT</i>	Spec	Hyg or red fluorescence in seeds

HspT, Heat shock protein terminator; sgRNA, single guide RNA; AarI, restriction enzyme;

Spec, Spectinomycin; Hyg, Hygromycin



As described in our paper entitled “pKAMA-ITACHI vectors for highly efficient CRISPR/Cas9-mediated gene knockout in *Arabidopsis thaliana*” in *Plant and Cell Physiology*, the *RPS5A* (*Ribosomal Protein Subunit 5A*) promoter-driven Cas9 efficiently induces knockout mutations. Our binary vectors pKAMA-ITACHI (pKI), pKIR1.1 and pKI1.1R carry *RPS5Ap::Cas9* and *U6.26p::sgRNA* within the T-DNA, and two AarI (restriction endonuclease) sites are inserted between U6.26 promoter and sgRNA scaffold.

Recently, we compared the mutation efficiencies of these two vectors. The results showed that pKI1.1R induced knockout phenotype more efficiently than pKIR1.1 in the T1 generations, partly because the pKIR1.1 T-DNA (at right border side) may be

(i) AarI digestion of pKI series and dephosphorylation

10x AarI Buf.	5.0 μ L	
pKIR1.1/pKI1.1R	x μ L	(= 1.5 μ g)
AarI	1.5 μ L	ThermoFisher, #ER1581
50x oligo	1.0 μ L	
<u>deionized water</u>	<u>42.5 - x μL</u>	
	50.0 μ L	

Incubate @ 37°C for 6 hrs. (Incubation over 16 hrs may cause star activity.)
then, add 1.0 μ L of rAPid (Alkaline Phosphatase, Roche, #04898133001)
and incubate @ 37°C for 30 min. and 75°C for 3min.

After the dephosphorylation, perform agarose gel electrophoresis and then cut out the digested vector and purify it by a column. (According to the manual of AarI, AarI may stay binding to DNA. To avoid this binding, we use DNA loading buffer with 1% SDS.)

(ii) Phosphorylation and annealing of DNA oligomers

Oligomer FWD (100 μ M)	1.0 μ L
Oligomer RVS (100 μ M)	1.0 μ L
10x T4 Ligase Buf. (NEB)	1.0 μ L
milliQ	6.5 μ L
<u>T4 Polynucleotide Kinase (NEB)</u>	<u>0.5 μL</u>
	10.0 μ L

37°C 30 min

95°C 5 min

↓ cool down, 5°C/min

25°C 5 min

4°C ∞

(iii) Ligation

Dilute the phosphorylated and annealed DNA to 250-fold with deionized water.

vector (AarI & rAPid-treated)	x μ L	(50 ng)
250-fold diluted hybridized DNA	1.0 μ L	
10x T4 Ligase Buf. (NEB)	1.0 μ L	
deionized water	7.0 – x μ L	
<u>T4 Ligase (NEB)</u>	<u>1.0 μL</u>	
	10.0 μ L	
16°C 30 min		

(iv) Plasmid Safe Exonuclease treatment

Ligation product	10.0 μ L	
10x Plasmid Safe Buf.	1.5 μ L	
25 mM ATP	0.6 μ L	
Plasmid Safe Exonuclease	1.0 μ L	epicentre, #E3101K
<u>deionized water</u>	<u>1.9 μL</u>	
	15.0 μ L	

37°C 30 min.

This treatment reduces the possibility that you get plasmids with an unwanted insertion.

Then, perform *E. coli* transformation

and culture it on an LB plate with 100 mg/L Spectinomycin.