Transient RNAi by in vitro transcribed dsRNA in protoplasts

In-vitro synthesis of dsRNA

DNA templates for the production of dsRNA (~250 bp) were produced by PCR using primers containing the 28-nucleotide minimal T7 promoter sequence (5'-TAATACGACTCACTATAGGGAGACCAC-3') at the 5' terminal followed by a 21-nucleotide gene-specific sequence.

After PCR, the dsDNA were purified and used as templates for in-vitro synthesis of dsRNA using the Ribomax Express kit (Promega).

The dsRNA were purified by phenol-chloroform-isopropanol extraction, dissolved in RNase-free water and quantified by UV spectrophotometry.

Protoplast transformation

Protoplast preparation methods should be optimized for the plant species used in the experiment. For example, protoplasts from *C. japonica* were prepared by cell-wall digestion solution containing 0.4% Onozuka Cellulase R10 (Yakult Pharmaceutical Ind. Co.), 0.2% Macerozyme R10 (Yakult Pharmaceutical Ind. Co.), 0.01% Pectolyase, 0.6 M sorbitol, 20 mM MES at pH 5.5, and 5 mM $MgCl_2$, which was pre-treated at 50° C for 30 minutes before filter-sterilization.

The introduction of dsRNA into the protoplasts was performed essentially as described by Chiu et al. Engineered GFP as a vital reporter in plants. *Curr. Biol.*, 6, 325-330 (1996). See also Sheen, J., A transient expression assay using Arabidopsis mesophyll protoplasts. http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html.

After being washed three times in W5 solution (154 mM NaCl, 125 mM $CaCl_2 \cdot 2H_2O$, 5

mM KCl and 5 mM glucose), the protoplasts were suspended in MaCaM solution (0.4M mannitol, 20 mM $CaCl_2 \cdot 2H_2O$ and 5 mM MES at pH 5.8) at 2 x 10^6 protoplasts/ml.

For the model RNAi experiments for luciferase gene, 1 μg of CaMV35S promoter driven *Photinus* LUC expression plasmid, 0.1 μg of CaMV35S promoter driven *Renilla* LUC expression plasmid, and 1-10 μg of *Photinus* LUC dsRNA were used for every 10^5 protoplasts. The amount of dsRNA should be optimized for the silencing of the endogenous genes.

The expression plasmids and dsRNA were gently mixed with the protoplasts in MaCaM solution.An equal volume of polyethylene glycol (PEG) solution [40% PEG in dissolved in 0.4 M mannitol and 0.1 M $Ca(NO_3)_2$] was then gradually added.

After gentle mixing, the protoplasts were incubated at 28˚C for 30 min., spun down, and re-suspended in 4ml of W5 solution.

After incubation at 28˚C at 40 rpm for 24 or 72 hours, the protoplasts were recovered to measure LUC activity.

Quantitation of luciferase activity (for the model experiment)

LUC activity was measured using a Dual-Luciferase assay system (Promega).

Cells were harvested by centrifugation and homogenized in 100 μl Passive Lysis Buffer (Promega). From 0.2 - 0.5 μl of the extracts were mixed with 100 μl LUC Assay Reagent II and the apparent luminescence was measured.

After the measurement of *Photinus* LUC acitivity, 50 μl of Stop & Glo reagent were added and the resultant *Renilla* activity was measured. Data were expressed as a ratio of the luminescence from *Photinus* divided by the luminescence value from *Renilla.*

<References>

1. Dubouzet JG, Morishige T, Fujii N, An CI, Fukusaki E, Ifuku K, Sato F. Transient RNA silencing of scoulerine 9-O-methyltransferase expression by double stranded RNA in Coptis japonica protoplasts. Biosci Biotechnol Biochem. 2005 Jan;69(1):63-70.

2. Kato N, Dubouzet E, Kokabu Y, Yoshida S, Taniguchi Y, Dubouzet JG, Yazaki K, Sato F. Identification of a WRKY protein as a transcriptional regulator of benzylisoquinoline alkaloid biosynthesis in Coptis japonica. Plant Cell Physiol. 2007 Jan;48(1):8-18.