The TARGET System: Rapid Identification of Direct Targets of Transcription Factors by Gene Regulation in Plant Cells

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Abstract

The TARGET system allows for the rapid identification of direct regulated gene targets of transcription factors (TFs). It employs the transient transformation of plant protoplasts with inducible nuclear entry of the TF and subsequent transcriptomic and/or ChIP-seq analysis. The ability to separate direct TF-target gene regulatory interactions from indirect downstream responses and the significantly shorter amount of time required to perform the assay, compared to the generation of transgenics, makes this plant cell-based approach a valuable tool for a higher through-put approach to identify the genome-wide targets of multiple TFs, to build validated transcriptional networks in plants. Here, we describe the use of the TARGET system in Arabidopsis seedling root protoplasts to map the gene regulatory network downstream of transcription factors-of-interest.

Key words

Transcription factor, Gene regulatory network, Protoplast, Fluorescence activated cell sorting, RNA-seq

1. Introduction

The TARGET system (Transient Assay Reporting Genome-wide Effects of Transcription factors) was developed to enable the rapid identification of genes directly regulated by a transcription factor (TF)-of-interest [1]. The plant cell-based system makes use of transient transformation of isolated plant cell protoplasts [2] with a vector (pBeacon_GR vector series, Figure 1) containing a TF fused to the glucocorticoid receptor (GR). The GR-TF fusion protein is held in the cytoplasm by association with a heat shock protein (HSP) complex. The addition of the dexamethasone ligand displaces the GR-HSP association, which makes possible the induced nuclear translocation of the TF [3]. Concurrent application of the translational inhibitor cycloheximide ensures that only direct transcriptional targets are affected by the TF nuclear import, and precludes the action of secondary TFs that may be regulated by the primary TF. In addition, the pBeacon_GR vectors make use of positive fluorescent selection, which allows for the use of fluorescence activated cell sorting (FACS) to isolate the successfully transformed plant cells. This leads to reduction of transcriptomic background noise which aids in the detection of differentially expressed genes [4, 5].

TARGET has been used to study numerous TFs either individually or in groups [1, 6–13]. While this methods paper is focused on conducting TARGET in seedling root protoplasts, the assay has also been adapted to shoot protoplasts [9]. In addition, a new version of the TARGET assay that uses 2 different vectors (pBeaconRFP_GR and pBeaconGFP_GR), has led to a higher through-put TARGET assay of up to

24 TF assays/cycle [10]. Furthermore, the TARGET assay has been used in conjunction with other techniques for characterization of DNA binding, including chromatin-immunoprecipitation (ChIP-seq), DNA adenine methyltransferase identification (DamID-seq), or capture by 4tU-affinity labeling of TF-regulated nascent mRNAs [6, 14, 15, 11]. Additionally, ~50 TFs assayed in TARGET can now be integrated with a large collection of published TF-target datasets housed in the new ConnecTF software platform & database (https://connectf.org), which enables analyses, refinement, and visualization of extensive gene regulatory networks and their potential physiological relevance [12].

Aside from its use in fully-sequenced model plants, like Arabidopsis, the TARGET system can potentially also be used in crop species as well as in species where the genome sequence is unavailable or incomplete. Furthermore, the TARGET approach, which uses transient transformation of plant cell protoplasts, may be applicable to species where transgenic approaches to study transcriptional regulation are not feasible. Importantly, the TARGET system can be deployed in a much shorter time-frame, and at higher through-put compared to transgenic plant approaches.

In this chapter, we describe the use of the TARGET system (Figure 2) in Arabidopsis seedling root protoplasts; reviewing protoplast isolation, transient transformation, and treatment, as well as transcript analysis. Of note, these techniques can be used in other plant tissues such as shoot protoplast [9] and other species with minor modifications.

2. Materials

Prepare all solutions using ultra-pure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ/cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all regulations when disposing of waste materials.

2.1 Plant growth

1. Plant growth medium: 2.2 g/l MS salts + vitamins (Murashige and Skoog [16]), 1% w/v sucrose, 1% w/v agar. Adjust to pH 5.8 with KOH. Autoclave at 120 °C to sterilize. Pour into 10 x 10 cm square plates. Store at 4 °C.

2. 70% v/v ethanol, 20% v/v bleach (1.21% w/v sodium hypochlorite), and sterile water.

3. 100 µm nylon mesh (Sefar Filtration, USA), sterile transfer pipettes, ¼" micropore tape.

4. Plant growth chamber, 100 µmol m^{-2} s^{-1} PAR and 22 °C with 18 h-light/6 h-dark.

2.2 Generation of root cell protoplasts

1. Enzyme solution: 1.25% w/v Cellulase R-10 (Kanematsu USA), 0.3% w/v Macerozyme R-10 (Kanematsu USA), 0.4 M mannitol, 20 mM MES (2-(N-morpholino)ethanesulfonic acid), 20 mM potassium chloride, 0.1% w/v bovine serum albumin, 10 mM calcium chloride, 5 mM β-mercaptoethanol. Adjust to pH 5.7 with 1 M TRIS hydrochloride pH 7.5. Heat the solution to 55 °C for 10 min (the solution should become clear), cool to room temperature. Store at -20 °C. Filter sterilize with 0.22 µm filters. (*see* **Note 1 and 2**)

2. Scalpel, rotary shaker, 250 ml flasks, 40 µm cell strainer (BD Falcon, USA), 15 ml conical tubes, swing-bucket centrifuge (500 G), hemacytometer.

2.3 Transformation of root cell protoplasts

1. Plasmids: pBeaconRFP_GR, pBeaconGFP_GR, pBeaconRFP_C'GR, pBeaconGFP_C'GR, pBeaconCherry_DamGR (Table 1, Figure 1).

2. MMg solution: 0.4 M mannitol, 15 mM magnesium chloride hexahydrate, 4 mM MES. Adjust to pH 5.7 with 1 M potassium hydroxide solution. Make fresh. Filter sterilize with 0.22 µm filters.

3. MIDIprep kit (QIAGEN, USA). Store DNA at -20 °C. (*see* **Note 3**)

4. PEG solution: 40% w/v polyethylene glycol 4000, 0.4 M mannitol, 0.1 M calcium chloride. Make fresh. Filter sterilize with 0.22 µm filters. (*see* **Note 4**)

5. W5 solution: 154 mM sodium chloride, 125 mM calcium chloride, 5 mM potassium chloride, 5 mM MES. Adjust to pH 5.7 with 1 M potassium hydroxide solution. Store at room temperature. Autoclave or filter sterilize with 0.22 µm filters.

6. 24-well plates, epifluorescence microscope equipped with GFP and RFP (or equivalent) filters.

2.4 Treatment of protoplasts

1. Dexamethasone: 10 mM stock dissolved in 96% v/v ethanol. Store at -20 °C.

2. Cycloheximide: 35 mM stock dissolved in 96% v/v ethanol. Store at -20 °C.

2.5 Sorting of protoplasts

1. FACSAria (BD, USA) or equivalent sorter with PBS (Phosphate Buffered Saline) as a sheath fluid and a 100 µm nozzle.

2. RNeasy micro kit (QIAGEN, USA).

2.6 Transcript analysis

1. Dynabeads mRNA Purification Kit (Invitrogen/Thermo Fisher, USA).

2. NEBNext Ultra II RNA Library Prep Kit (New England BioLabs, USA).

3. Methods

Carry out all procedures at room temperature unless otherwise specified. Use aseptic techniques for all procedures. (*see* **Note 2**)

3.1 Plant growth

1. Sterilize 1 ml of dry Arabidopsis seed (approximately $35*10^3$ seeds for Col-0) in a 50 ml tube by a five min incubation with 10 ml 70% ethanol, followed by a ten min incubation with 10 ml 20% bleach, and rinsing three times with 50 ml sterile water.

2. Plate seeds in two rows on top of 100 µm nylon mesh in square plates with plant growth medium using a sterile transfer pipette (Figure 3). 1 ml of seed is divided over ten plates. Plates are sealed with micropore tape and placed vertically in a growth chamber.

3.2 Generation of protoplasts

1. Harvest the roots of 7- to 10-day-old seedlings using a scalpel and transfer to a 250 ml flask with 50 ml enzyme solution.

2. Shake the flask with the roots in enzyme solution at 75 rpm at room temperature for 3 h.

3. Filter the protoplasts by passing them over a 40 µm cell strainer into a fresh flask. (*see* **Note 5**)

4. Transfer the filtered protoplast suspension to 15 ml conical tubes and spin them down for 5 min at 500 G with no brake. Remove the supernatant by aspirating. (*see* **Note 6**)

3.3 Transformation of protoplasts

1. Wash the protoplasts by resuspending the protoplast pellet in 15 ml MMg solution and spinning them down for 5 min at 500 G with no brake. Remove the supernatant by aspirating.

2. Resuspend the protoplasts in a volume appropriate for quantification of protoplast density using a hemacytometer. Assess the protoplast density and adjust the volume of the MMg solution to achieve a density of $4*10^6$ protoplasts per ml. (*see* **Note 7**)

3. Prepare a 15 ml conical tube for each transformation (and one mock transformation with no DNA) by labeling them and adding 50 µg plasmid DNA to the bottom of the tube.

4. Add 250 µl ($1*10^6$) protoplasts to each tube. Add 250 µl PEG solution to each tube and mix well by vortexing for 5 seconds. (*see* **Note 8 and 9**)

5. Wash the protoplasts by adding 15 ml W5 solution to each tube and spinning them down for 5 min at 500 G with no brake. Remove the supernatant by aspirating. (*see* **Note 10**)

6. Resuspend the protoplasts in 1 ml W5 and transfer them to a 24-well plate.

7. Incubate the protoplasts overnight at room temperature in the dark while shaking at 50 rpm.

8. Inspect the protoplasts with an epifluorescence microscope equipped with GFP and RFP (or equivalent) filters to check for successful transformation. (*see* **Note 11**)

3.4 Treatment of root cell protoplasts

1. Divide each independent transformation over 4 wells; for mock, dexamethasone, cycloheximide, and dexamethasone + cycloheximide treatment. (*see* **Note 12-15**)

2. Treat the protoplasts with 35 µM cycloheximide and/or 10 µM dexamethasone. Cycloheximide is administered with a 20 min pretreatment and the subsequent dexamethasone treatment is incubated for 3 h. Stagger the start of treatments to account for the time it takes to sort individual samples (5-15 min).

3.5 Sorting of protoplasts

1. Set up the FACS with PBS as a sheath fluid and a 100 µm nozzle. (*see* **Note 10**)

2. Set up a dotplot for green (GFP) (488 nm excitation, 530/30 emission) vs. red (RFP) (561 nm excitation, 583/30 emission) fluorescence emission. (*see* **Note 16**)

3. Use the mock-transformed protoplasts to set up gates for RFP- and GFP-positive cells (Figure 4). (*see* **Note 17**)

4. Sort $20*10^3$ protoplasts into 350 µl RNA extraction buffer (RLT). Freeze samples (-20 °C) upon completion of the sort.

5. Extract the RNA according to the manufacturer's instructions, eluting with 50 µL of nuclease-free H_2O. (*see* **Note 18**)

3.6 Transcript analysis

1. Perform polyA mRNA purification from total RNA using the Dynabeads mRNA Purification Kit. For total RNA from $20*10^3$ cells, we typically use 15 µL of Oligo $(dT)_{25}$ beads per sample, combining the beads

needed for all samples in one tube for the initial bead wash step. Beads are resuspended in binding buffer and an equal volume (50 µL) is added to each total RNA sample.

2. Make sequencing libraries according to manufacturer's instructions using the NEBNext Ultra II RNA Library Prep Kit. (*see* **Note 19**)

3. Pool and sequence samples on an Illumina sequencer according to the manufacturer's specifications for single-end reads. Read lengths of 50-bp are sufficient to identify differentially expressed genes in Arabidopsis. Aim for between 5 and 10 million reads per library. (*see* **Note 20**)

4. Remove reads and bases of low quality and remove adapter sequences from raw reads. Align FASTQ files to the Arabidopsis genome (TAIR10) using a short-read mapping package (*e.g.* HISAT2 [17] or STAR [18]). (*see* **Note 21**)

5. Quantify read counts per gene using a program such as HTSeq-count [19] or featureCounts [20].

6. Identify genes that are differentially expressed in response to dexamethasone using DESeq2 [21] or another appropriate package for transcriptome analysis. Genes that respond to dexamethasone in the absence of cycloheximide include both indirect and direct target genes of the TF, while genes that respond to dexamethasone in the presence of cycloheximide are direct TF target genes. (*see* **Note 15**)

7. TF-target genes can be compared to published TF-target gene interactions using the ConnecTF platform [12] either via the public website or setting up a private instance of the tool.

4. Notes

1. The use of β-mercaptoethanol is optional. It has been reported to improve yield and/or viability but we have found no significant effect.

2. These experiments can be conducted under non-sterile conditions, since the experiment only takes 24-36 hours. This may speed up the process. However, for reproducibility, and especially if you are studying factors involved in plant-pathogenesis responses, it is recommended to perform the experiment using aseptic techniques. The enzyme solution may clog the 0.22 µm filters and require several extra filters to be used.

3. Resuspend the DNA pellet in a small volume of sterile de-ionized water (50 µl). Alternatively, use 10 mM Tris-HCl pH 8.0 (DNA pellets may dissolve better in slightly buffered water, note that EDTA inhibits transfections and it is in almost all kit elution buffers (TE)). It is critical to get a concentration between 1 and 4 µg/µl. Be sure to dilute plasmid DNA at least ten-fold before measuring concentration for accuracy. Other plasmid purification kits have been successfully used (*e.g*. ZymoPURE Plasmid Midiprep or Maxiprep kits (Zymo Research), PureLink™ HiPure Expi Plasmid Megaprep kit (Invitrogen)), however, the quality of the DNA is important and other purification methods/kits may need to be tested on a small scale first.

4. PEG of differing average molecular weight have been used successfully in our hands (1500 to 8000). Due to its viscosity, filter sterilization of the PEG solution may take more time.

5. Cell strainers can be washed, sterilized with 70% ethanol, and reused.

6. The use of 15 ml conical tubes ensures the formation of a compact pellet and prevents loss of protoplasts. Similarly, the use of centrifugation with no (or minimal) braking ensures maximum recovery of protoplasts.

7. Be gentle when resuspending the protoplasts. Large orifice pipette tips can be used to minimize shearing forces. A viability stain (*e.g.* fluorescein diacetate) can be used for a more secure count of live protoplasts.

8. Plasmid preps that contain too much salt will cause a precipitation of the DNA when mixed with the PEG solution.

9. Traditionally, the protoplast/PEG/DNA solution was incubated at room temperature for 15 min, but we have found that brief vortexing at moderate speed and immediate washing can work better and saves time.

10. W5 solution contains relatively high levels of calcium chloride which can, in the case of some cytometers, cause issues due to precipitation with the phosphate in the PBS sheath fluid that lead to clogging. An alternative is to use the enzyme solution base (enzyme solution without the enzymes added) as a wash and incubation solution. Another option is to use plain saline solution (or filtered tap water) as the sheath fluid.

11. We generally see protoplast transformation efficiencies ranging between 5% and 20% using Arabidopsis seedling root protoplasts.

12. For statistical analysis, we recommend a minimum of three independent transformations per tested TF.

13. In order to minimize the number of RNA-sequencing samples, the dexamethasone and cycloheximide alone treatments can be omitted. Include a control with an empty vector (*e.g.* pBeaconRFP_GR), or one with an insert without transcriptional activity (*e.g*. pBeaconRFP_GR-GUS).

14. For scaled-up TARGET assays (*e.g*. [10]), two separate protoplast transformations (one with a TF in pBeaconRFP_GR and one with another TF in pBeaconGFP_GR) can be combined and treated and sorted in unison. In this scaled-up assay, comparison of TF-transfected cells to Empty Vector – all in +DEX only, allows one to perform 24 TF in one assay. Vectors are available from the VIB-UGent Gateway vector collection (https://gatewayvectors.vib.be/).

15. If the experimental design includes an empty vector or GUS control in lieu of a combination of treatments, the model design for identifying TF regulated targets is only the vector used (*e.g.* empty vector vs. TF). When multiple TFs are examined across multiple days with the same treatments, the inclusion of the control allows the data to be analyzed simultaneously by including a Batch factor (*e.g.* TF + Batch) in the model, as in Brooks *et al.* 2019 [10].

16. We have successfully used 488 nm excitation for RFP detection. The excitation and emission frequencies listed here are not strictly the only ones that can be used.

17. A detailed methodology for setting up a FACS for sorting protoplasts is available [5].

18. We generally get about 85 ng of total RNA from $20*10^3$ root cells. A Bioanalyzer (Agilent, USA) with a 6000 RNA Pico Kit can be used to accurately assess RNA quantity and quality.

19. Library preparation kits from other manufactures can also be used, but ensure that they are optimized for low RNA input. Kits designed for 3' RNA sequencing can be used and do not require the purification of mRNA from total RNA (Step 3.6.1).

20. If using a kit designed for 3' RNA sequencing, the number of reads required to identify differentially expressed genes will be lower, between 3 and 6 million reads per sample, and allows pooling of more samples per run.

21. Standard quality control should be performed on each step in the bioinformatics analysis, beginning with the raw sequence data using a program such as MultiQC [22]. It should be noted that sequencing results from TARGET experiments often contain a large percentage of duplicate reads.

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Tables

Table 1. Vectors compatible with the TARGET System

Vector Name	Alternate names	Description	Ref.	Availability
pBeaconRFP_GR	pBOB11	RFP positive selection, N-terminal GR fusion, Gateway-compatible	[1]	VIB-UGent
pBeaconGFP_GR		GFP positive selection, N-terminal GR fusion, Gateway-compatible	[10]	VIB-UGent
pBeaconRFP_C'GR	pBOB11_C-Term	RFP positive selection, C-terminal GR fusion, Gateway-compatible	[11]	VIB-UGent
pBeaconGFP_C'GR		GFP positive selection, C-terminal GR fusion, Gateway-compatible	this work	To be deposited VIB-UGent
pBeaconCherry_DamGR	pDamBOB	mCherry positive selection, N-terminal Dam-GR fusion, BsaI (Goldengate) cloning	[11]	VIB-UGent

Figures

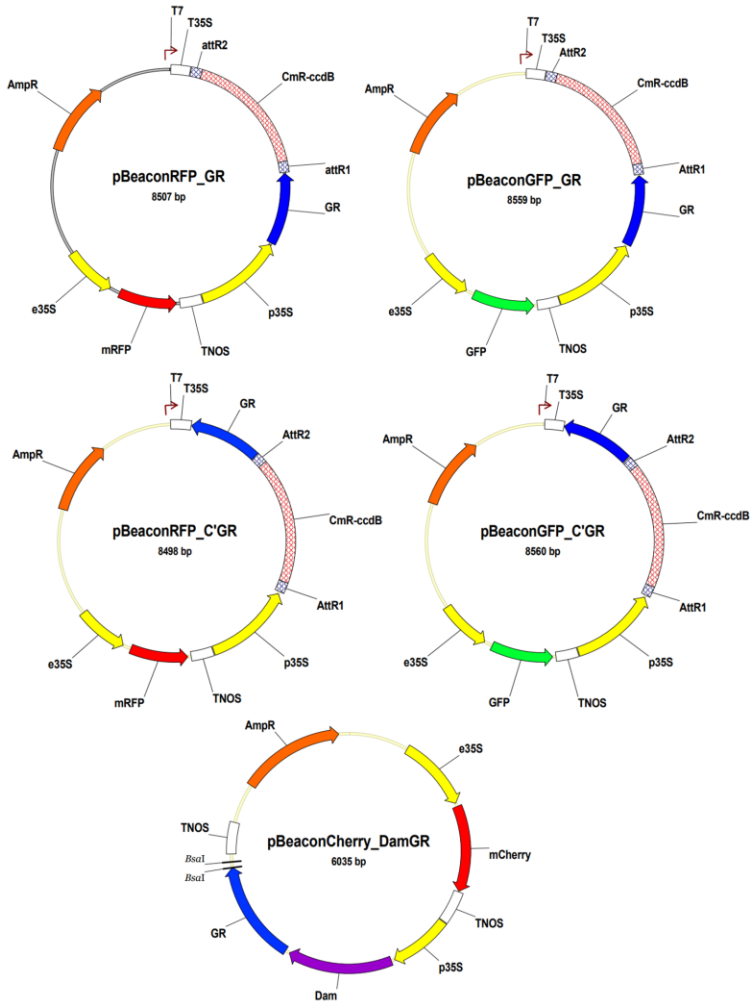

Figure 1. The pBeacon vector series for use in the TARGET system.

pBeaconRFP_GR, pBeaconGFP_GR, pBeaconRFP_C'GR, and pBeaconGFP_C'GR. These Gateway-compatible vectors vary in harboring the mRFP (monomeric Red Fluorescent Protein) or GFP (Green Fluorescent Protein) positive fluorescent selection markers as well as either an N- or C-terminal fusion with the glucocorticoid receptor (GR). The Goldengate-compatible pBeaconCherry_DamGR contains an mCherry positive selection marker and has an N-terminal fusion with DNA adenine methyltransferase (Dam) and GR. The TARGET vectors and full sequences are available from the VIB-UGent Gateway collection (https://gatewayvectors.vib.be/)(see Table 1).

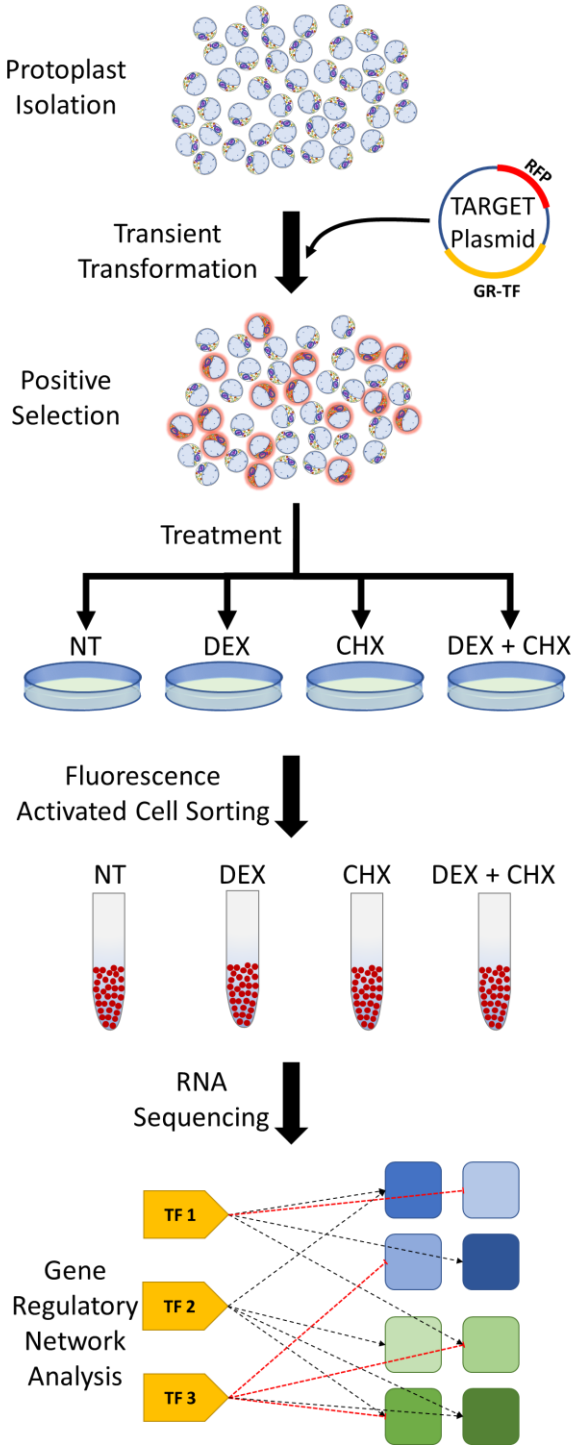

Figure 2. Schematic overview of the TARGET System.

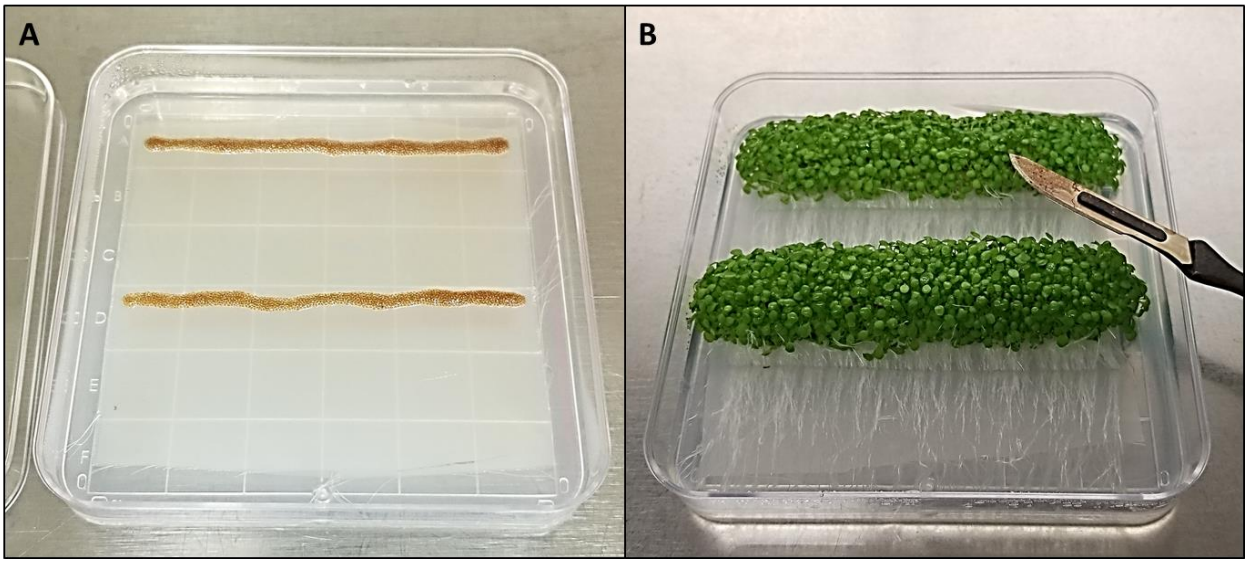

Figure 3. Arabidopsis seedling growth for harvesting of roots.

A. Sterilized seeds are plated on top of 100 µm nylon mesh in a 10 x 10 cm square plate with solidified plant growth medium. The plates are placed vertically in a growth chamber. **B.** After one week, the roots are harvested using a scalpel and used for the preparation of protoplasts. NB. The TARGET assay can also be performed on shoots [9].

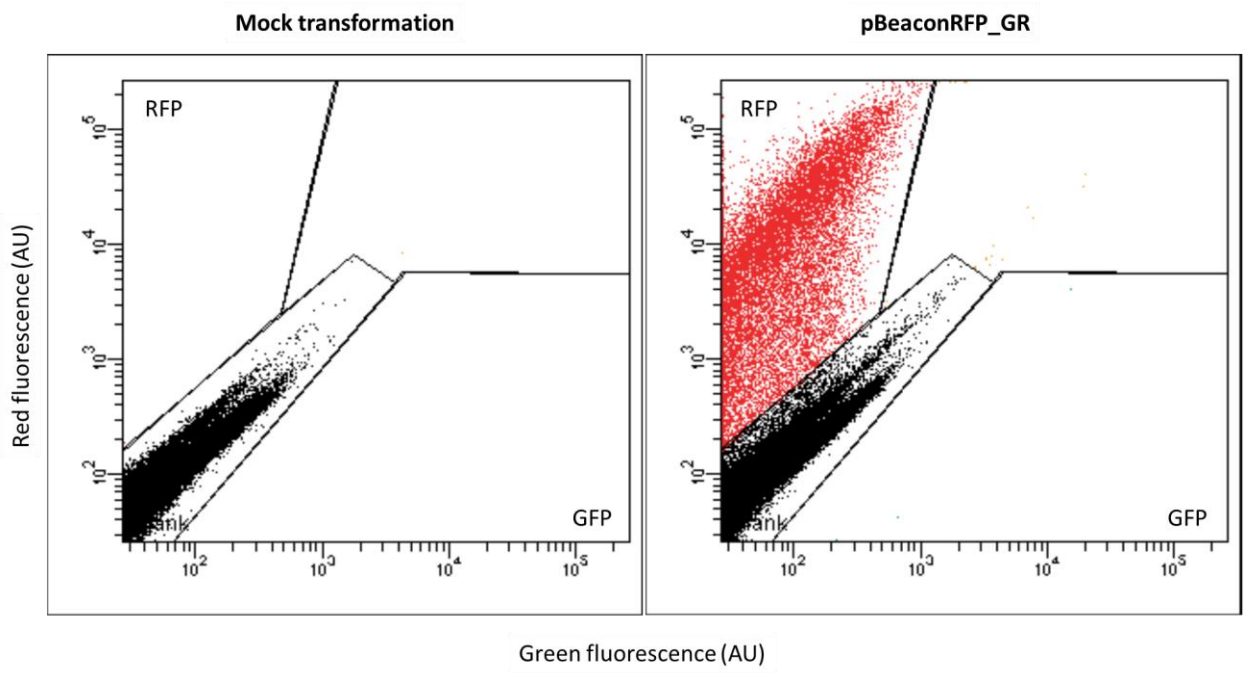

Figure 4. Cytometric FACS analysis of TARGET vector transformed root cell protoplasts.

Dotplots representing a mock transformation and protoplasts successfully transformed with pBeaconRFP_GR vector are shown. Red fluorescence is indicated on the Y-axis (in arbitrary units) and green fluorescence on the X-axis. Each dot represents an individual detection event. This data was generated with a FACSAria cell sorter.