

TARGET: A Transient Transformation System for Genome-Wide Transcription Factor Target Discovery

Dear Editor,

Determining the fundamental structure of gene regulatory networks (GRN) is a major challenge of systems biology. In particular, inferring GRN structure from comprehensive gene expression and transcription factor (TF)–promoter interaction data sets has become an increasingly sought-after aim in both fundamental and agronomical research in plant biology (Bonneau et al., 2007; Ruffel et al., 2010). A crucial step for the assessment of GRN is the identification of the direct TF–target genes. Transgenic plant lines expressing tagged versions of the TF-of-interest can be used together with transcriptomic and DNA-binding analyses to obtain high-confidence lists of direct targets (e.g. Mönke et al., 2012). However, the generation of such transgenics can be a limiting factor, especially in large-scale studies or in non-model species. Transient transformation of protoplasts is therefore often employed for the study of TF–promoter interactions, using co-expression of effector constructs with a TF-of-interest and reporter constructs with a promoter-of-interest. We have developed a rapid technique to study the genome-wide effects of TF activation in protoplasts that uses transient expression of a glucocorticoid receptor (GR)-tagged TF. We demonstrate here that this system can be used to rapidly retrieve information on direct target genes in less than 2 weeks' time. As a proof-of-principle candidate, we used the well-studied TF, ABSCISIC ACID INSENSITIVE 3 (ABI3; Koornneef et al., 1989; Mönke et al., 2012) and established the *de novo* identification of the abscisic acid response element (ABRE) and a majority of the previously classified direct targets. We have named this technique *TARGET*, for *Transient Assay Reporting Genome-wide Effects of Transcription factors*.

Technically, plant protoplasts are transfected with a plasmid (pBeaconRFP_GR) that expresses the TF-of-interest fused to GR, which allows the controlled entry of the chimeric GR–TF into the nucleus by addition of the GR–ligand dexamethasone (DEX; Schena and Yamamoto, 1988). In addition, the vector contains a separate expression cassette with a positive fluorescent selection marker (red fluorescent protein; RFP) which enables fluorescence-activated cell sorting (FACS) of successfully transformed protoplasts (Figure 1A; Bargmann and Birnbaum, 2009). This purification step allows reliable qPCR or transcriptomic analysis of multiple independent transfections, which would otherwise be hampered by the presence of a population of untransformed cells that varies from experiment to experiment. Lastly, the effect of target gene induction by DEX treatment is

measured in the presence or absence of the translation inhibitor cycloheximide (CHX), allowing for the distinction of direct and indirect target genes of the TF under study (Supplementary Data). pBeaconRFP_GR–ABI3 was used to transfect protoplasts prepared from the roots of *Arabidopsis* seedlings, where ABI3, known largely for its role in seed development, has also been shown to be involved in development (Brady et al., 2003).

As a first test of the *TARGET* system, the expression of known direct ABI3 targets *PER1* and *CRU3* was assayed by qPCR. Compared to control gene expression, both *PER1* and *CRU3* showed significant induction of transcript levels upon DEX treatment in the ABI3–GR-transfected protoplasts in the presence of CHX (Figure 1B and 1C, and Supplementary Data). *PER1* and *CRU3* expression in protoplasts transformed with an empty vector control showed no significant induction by DEX treatment (Figure 1B and 1C). Significant induction of *CRU3* expression could only be measured when CHX was present, indicating that the effects of CHX may in some cases facilitate ABI3 function. Enhancement of ABA signaling output by protein synthesis inhibitors that could explain this phenomenon has been noted before by independent studies (Reeves et al., 2011). For the transcriptomic analysis, using ATH1 Genome Array chips, a two-way analysis of variance (ANOVA) was performed, followed by a Tukey *post hoc* test to identify genes whose expression is differentially regulated in response to DEX treatment in the absence or presence of CHX ($p < 0.05$, fold change > 1.5 ; Supplementary Data). Genes found to be significantly regulated by DEX treatment in the empty vector control were omitted from further analysis. This analysis yielded a total of 668 unique genes whose expression was affected by DEX-induced nuclear localization of ABI3, 227 regulated genes without CHX, and 458 regulated genes with CHX (microarray results were validated by qPCR; Supplementary Data). There was just a 17-gene overlap with and without CHX, reiterating that (as was seen for *CRU3* in preliminary qPCR analysis) there are many genes whose response to GR–ABI3 was facilitated by the presence of the protein synthesis inhibitor CHX. The 210 genes regulated only in the absence of CHX were categorized

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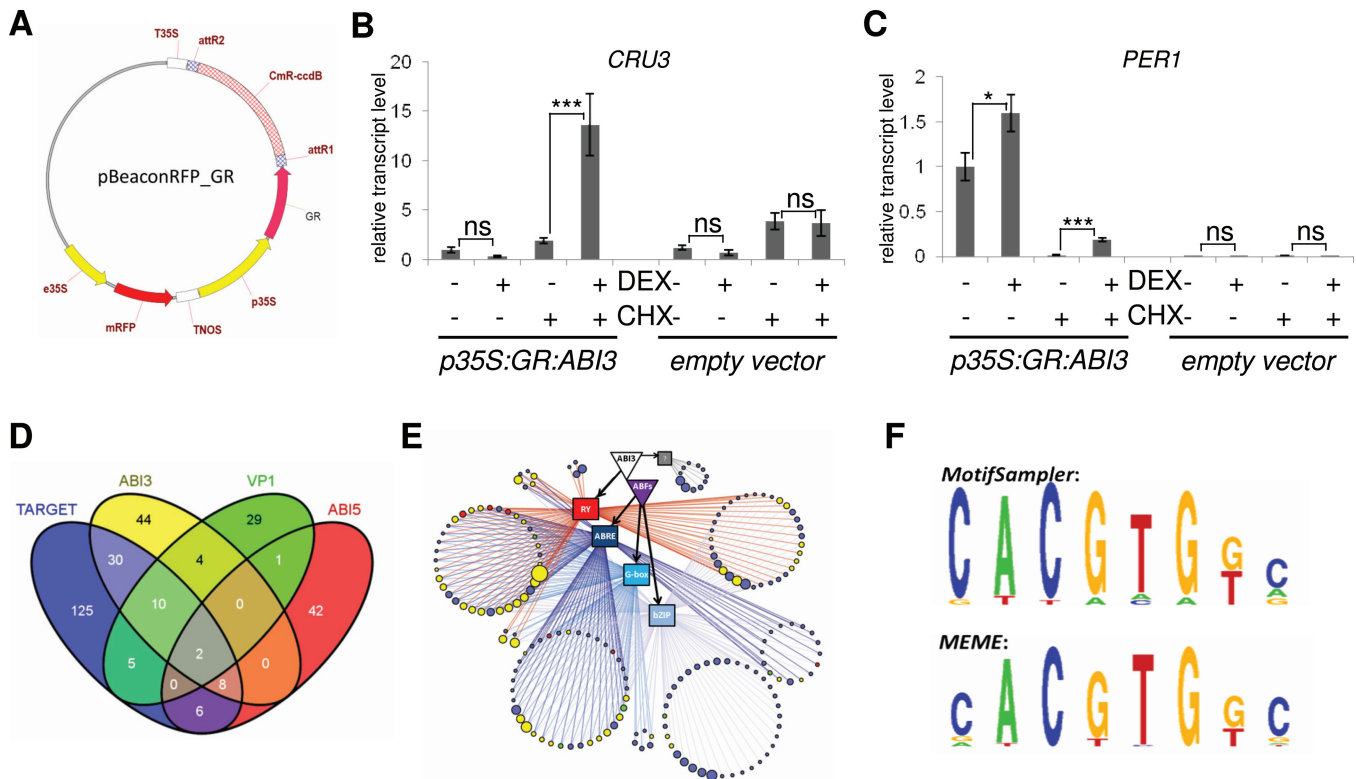


Figure 1. The *TARGET* System for Rapid TF-Target Identification in Plant Protoplasts.

(A) The pBeaconRFP_GR vector contains a red fluorescent protein (RFP) positive selection cassette and a Gateway recombination cassette that is in frame with the rat glucocorticoid receptor (GR) fusion protein. The plasmid is used to transfect protoplast suspensions, followed by treatment with dexamethasone and/or cycloheximide and cell sorting of successful transformants for transcriptomic analysis.

(B, C) qPCR quantification of *PER1* and *CRU3* transcript levels in protoplasts transformed with pBeaconRFP_GR-ABI3 or an empty vector control and treated with DEX and/or CHX. Averages \pm SEM are presented. ns, not significant; * $p < 0.05$; *** $p < 0.001$. *t*-test DEX treatment $n = 3$.

(D) The intersection of 186 genes identified by *TARGET* (blue) as directly up-regulated by ABI3 and genes identified by previous studies as direct up-regulated targets of ABI3 (98 genes; yellow), up-regulated targets of VP1 (51 genes; green), and ABI5 (59 genes; red).

(E) Network model of putative ABI3 (white triangle) connections to its direct up-regulated target genes via the RY-repeat motif (red square; CATGCA) and through interaction with ABRE binding factors (ABFs; purple triangle) and ABRE (dark blue square; ACGTGKC) or the more degenerate G-box (blue square; CACGTG) and bZIP core (light-blue square; ACGTG) elements. Target genes (circles) are sized according to their strength of induction and colored according to the overlap in (D).

(F) Weight matrix representation of the ABRE-like (CACGTGKC) motif retrieved by the *MotifSampler* and *MEME* algorithms from the 1 kb upstream of the transcription start sites of the top 50 direct up-regulated ABI3 targets, $Z_e = 7.19$ and $Z_e = 7.11$, respectively. See [Supplementary Data](#).

as putative indirect targets of ABI3, whereas the 458 genes regulated in the presence of CHX (186 induced and 272 repressed genes) were designated as putative direct targets of ABI3.

The list of 186 putative direct up-regulated genes was highly significantly enriched for genes previously identified as direct targets of ABI3 in whole-plant studies ($Z_e = 54.3$), as well as targets of the maize homolog VIVIPAROUS1 ($Z_e = 20.8$) and co-regulator ABI5 ($Z_e = 20.9$) (Figure 1D and 1E, and [Supplementary Data](#); Suzuki et al., 2003; Reeves et al., 2011; Mönke et al., 2012). These significant intersections indicate that the activation of ABI3 in protoplasts reflects the effects attributed to this transcriptional regulator in *in planta* studies. The list also showed a significant overrepresentation of GO-terms, including *response to ABA*, *response to water deprivation*, *lipid storage*, and *embryo development* (no significant overlap or enrichments were found in the lists of indirect targets or direct down-regulated targets;

[Supplementary Data](#)). Furthermore, promoter analysis of the 50 most strongly induced direct up-regulated genes found significant enrichment of previously identified ABRE-like elements and the RY-repeat motif (Figure 1E and [Supplementary Data](#)). *De novo* searches for recurring motifs within these promoters (using two independent algorithms, *MEME* and *MotifSampler*) yielded the recovery of the CACGTGKC ABRE (Figure 1F and [Supplementary Data](#)). These results show the *TARGET* system can be used successfully to investigate TF function in protoplasts with significance to whole plants.

One advantage of the *TARGET* system lies in the speed at which identification of genome-wide TF targets can be performed. A candidate TF can now be scrutinized for its target genes in a genome in a matter of weeks rather than the months required for the generation of stable transgenic plant lines. The *TARGET* transient transformation system can also be used purely

as a verification of specific TF-target interactions by qPCR, much as yeast one-hybrid (Y1H) assays are often used, but now in the context of endogenous gene activation in plant cells rather than promoter binding in a yeast strain. The *TARGET* approach brings the convenience of microbiological systems like Y1H to the genome-wide transcriptomic capabilities of *in planta* studies. Another advantage of the use of protoplast transformation in the *TARGET* system is that it can be done in a wide range of species where the generation of transgenic plant lines is either impossible or problematic and more time-consuming (Sheen, 2001). The *TARGET* system combined with RNA sequencing can enable rapid and systematic assessment of TF function in numerous plant species, such as in important crop model species.

This system is not a replacement for in-depth studies using transcriptional and chromatin immuno-precipitation (ChIP) analyses in transgenic plants. Rather, *TARGET* is rapid tool for GRN investigations that may have uses in particular circumstances. There are considerations associated with the use of this system. On its own, a genome-wide analysis will yield results that contain false-positives and false-negatives. Identification of direct regulated genes by *TARGET* is therefore not unequivocal; additional assays for direct TF-target interaction (e.g. ChIP, Y1H, gel shift assays) are required for definitive identification of TF targets. The functionality of the chimeric GR-TF is not tested in this system, other than by the substance of the results. CHX treatment by itself may have effects on transcription that influence the DEX effect on certain direct target genes. Lastly, the cellular dissociation procedure itself may induce gene expression responses that could conceal the effects of TF activation. We therefore envisage two ways of using the *TARGET* system: either in combination with other techniques to get high-confidence target lists for a particular TF or as a high-throughput analysis of numerous TFs in a given GRN to get a broad view of putative interactions.

Overall, the results presented here demonstrate that *TARGET* represents a novel and rapid transient system for TF investigation that can be used to help map GRN. Important indications of TF operation, such as direct target genes, biological function by GO-term associations, and *cis*-regulatory elements involved in its action can be obtained in a rapid and straightforward manner. The proof-of-principle analysis with ABI3 offers a new dataset of transcripts affected by this TF, adding to our understanding of the downstream significance of this central regulator.

The pBeaconRFP_GR vector will be made available through the VIB website (<http://gateway.psb.ugent.be/>).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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