Positive fluorescent selection in plant protoplasts

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Positive fluorescent selection permits precise, rapid and in-depth over-expression analysis in plant protoplasts

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ABSTRACT

Transient genetic modification of plant protoplasts is a straightforward and rapid technique for the study of numerous aspects of plant biology. Recent studies in metazoan systems have utilized cell-based assays to interrogate signal transduction pathways using high-throughput methods. Plant biologists could benefit from new tools that expand the use of cell culture for large-scale analysis of gene function. We have developed a system that employs fluorescent positive selection in combination with flow cytometric analysis and fluorescence activated cell sorting (FACS) to isolate responses in the transformed protoplasts exclusively. The system overcomes the drawback that transfected protoplast suspensions are often a heterogeneous mix of cells that have and have not been successfully transformed. This Gateway-compatible system enables high-throughput screening of genetic circuitry using over-expression. The incorporation of a red fluorescent protein (RFP) selection marker enables combined utilization with widely available green fluorescent protein (GFP) tools. For instance, such a dual labeling approach allows cytometric analysis of GFP reporter gene activation expressly in the transformed cells or FACS-mediated isolation and downstream examination of over-expression effects in a specific GFP-marked cell population. Here, as an example, novel uses of this system are applied to the study of auxin signaling, exploiting the RFP/GFP dual labeling capability. In response to manipulation of the auxin-response network through over-expression of dominant negative auxin-signaling components, we quantify effects on DR5::GFP reporter gene activation as well as profile genome-wide transcriptional changes specifically in cells expressing a root epidermal marker.
INTRODUCTION

It has been demonstrated that flow cytometric analysis and fluorescence activated cell sorting (FACS) of plant protoplasts is practicable, moreover, this technique has yielded valuable results in a number of different fields of research (Harkins and Galbraith, 1984; Galbraith et al., 1995; Sheen et al., 1995). For instance, FACS of protoplasts from Arabidopsis plants expressing tissue-specific fluorescent protein markers has been used to examine both basal and environmentally stimulated transcriptional profiles in particular cell types (Birnbaum et al., 2003; Brady et al., 2007; Gifford et al., 2008; Dinneny et al., 2008) and flow cytometry has been employed to analyze reactive oxygen species production and programmed cell death tobacco protoplasts (Nicotiana tabacum; Lin et al., 2006). A broad selection of fluorescent tools is available to study a plethora of physiological parameters in plants, e.g. cis-regulatory elements fused to fluorescent proteins (Haseloff and Siemering, 2006), genetically-encoded molecular sensors (Looger et al., 2005) or dye-based sensors (Haugland, 2002) can be used in combination with cytometry to measure diverse biological processes.

Here, we document the development of a protoplast transfection system that employs cytometry and a transient transformation vector harboring a fluorescent positive selection marker (pBeaconRFP; Fig. 1). The notable advantage of this system is that it allows for the exclusive analysis of the transformed cells and facilitates high-throughput dual color analysis. The new vector for use in this system is designed in such a way that it not only expresses a gene-of-interest but also expresses monomeric red fluorescent protein (mRFP). Furthermore, it is compatible with the Gateway recombinase-mediated cloning system, permitting fast and easy cloning. Because of its red emission spectrum, the mRFP marker can easily be used in combination with the commonly utilized green fluorescent protein (GFP). We present two examples of this system’s use in the analysis of an important signal transduction cascade involved in many aspects of plant development, namely the auxin perception pathway (Fig. 2; Guilfoyle and Hagen, 2007). Promising alternative uses of the system are further discussed.

Transient transformation of protoplasts is a widely utilized tool in plant research that is swift and unproblematic. The technique can be used, for example, to monitor the regulation of promoter elements, to analyze gene expression or enzymatic activity in response to a variety of stimuli, to examine the roles of transcription factors or signal-transduction cascade components or to study the subcellular localization of proteins (Sheen, 2001; Yoo et al., 2007). As opposed to stable transformation of plants (Arabidopsis thaliana being the most commonly used platform), which generally takes months and requires the use of a transfecting
agent (usually *Agrobacterium tumefaciens*), transfection of protoplasts can be achieved in just one day and entails only raw DNA and either a chemical- or electroporation-based transfection method. Additionally, transient transformation analyses can overcome problems encountered with stable over-expression such as pleiotropic developmental effects or non-viability, when a cell-based assay is appropriate. However, due to the fact that protoplast transformation efficiency is never 100%, results can be convoluted by the non-transformed cells.

Transformation efficiencies are often low and variable (*e.g.* Cummins *et al*., 2007; <10%) and depend on the employed method as well as properties of the protoplasts and DNA used. We usually get efficiencies ranging from 5 to 20% using *Arabidopsis* root protoplasts. Others in the field, however, have reported efficiencies of up to 90% using *Arabidopsis* mesophyll protoplasts (Sheen, 2001). Nonetheless, even a relatively small contamination with non-transformed cells can obscure effects and lead to a misinterpretation of results. For example, supposing one wanted to measure the ability of a dominant negative signaling component to inhibit the activation of downstream targets and one still sees a level of activation after transfection of the protoplasts; albeit significantly reduced as compared to a control, is the remaining activity due to a partial inhibition or is it only present in the non-transformed cells? If a way were to be found to select for successfully transformed cells, on the other hand, a much more precise measurement of the parameter of interest could be obtained.

As a first example we used the pBeaconRFP transient transformation system for the rapid analysis of a regulatory circuit by means of reporter gene readout. We over-express dominant negative mutant isoforms of the Aux/IAA transcription factors (IAAnmII; Fig. 2; Tiwari *et al*., 2001) in protoplasts derived from the roots of *Arabidopsis* seedlings stably transformed with the auxin sensitive reporter *DR5::GFP* (Fig. 3A; Ottenschläger *et al*., 2003; the pBeaconRFP system is also usable in mesophyll cells as mRFP is readily distinguishable from chlorophyll autofluorescence cytometrically [data not shown]). In this first experiment, we validate the system using the elegant experiments pioneered in the Guilfoyle lab (Ulmasov *et al*., 1997). A repeat of experiments performed previously (Tiwari *et al*., 2001), now using pBeaconRFP and flow cytometry, demonstrates it is possible to assess auxin-induced DR5 promoter activity exclusively in transformed protoplasts by measuring GFP signal intensity.

The second example of the application of the pBeaconRFP positive selection marker system involves examination of the transcriptional effects of the expression of IAAnmIIIs in a specifically marked cell population (Fig. 4A). We make use of protoplasts derived from a
cell-type-specific GFP marker line (P_{WER::GFP}, the WEREWOLF promoter fused to GFP), which expresses primarily in atrichoblasts (Lee and Schiefelbein, 1999), transfected with two different IAA\_nmII isoforms. Subsequent genome-wide transcriptional profiling of auxin-treated and mock-treated IAA\_nmII expressing cells makes it possible to distinguish distinctive patterns of gene expression regulated by the different mutant Aux/IAA isoforms.

The use of high-throughput cell-based screening methods in the study of regulatory networks has become a conventional and effective approach in animal systems (e.g. Müller et al., 2005; Palmer et al., 2006). Cytometric and FACS-based analyses have also been much more widespread and prolific in animal or microbiology research than in plant research. The combination of a selectable protoplast transformation system along with the use of cytometry now allows us to take these powerful techniques to a new level in plant research.

RESULTS

Modification of reporter gene activation by transient over-expression

In order to demonstrate the use of the pBeaconRFP system to study signal transduction, we took advantage of the auxin-sensitive DR5::GFP reporter gene. DR5 is a highly active synthetic auxin response element created by Ulmasov and coworkers (1997) and derived from the soybean (Glycine max) GH3 indole-3-acetic acid amido synthetase promoter. Upon treatment of seedlings or protoplasts harboring a DR5 reporter gene with auxin, the reporter is activated throughout the plant or protoplast suspension (Ulmasov et al., 1997).

In the original experiments (Ulmasov et al., 1997), carrot protoplast suspensions were transfected with three different plasmids: 1) a reporter containing DR5::GUS, 2) an effector expressing an Aux/IAA and 3) a transformation efficiency control expressing luciferase. Measurement of auxin-induced GUS activity relative to luciferase activity showed a reduced induction of GUS activity in protoplast suspensions transfected with the Aux/IAA effector plasmid as compared to those transfected with a control vector, indicative of the repressive effect on auxin responses of this family of transcription factors. A drawback of this initial system is that GUS activity induced in protoplasts that have been transformed with the reporter and not the effector will also be measured. The relative amount of protoplasts transformed with less than all three of the applied vectors will vary from experiment to experiment and among different effector plasmids. An improved version of this system, in which mesophyll protoplasts from a stably transformed DR5::GUS Arabidopsis line were utilized, avoided the need for the co-transfection with the reporter and allowed for an analysis
of the reporter in a more natural chromatin environment but did not address the issue of measuring response only in transformed cells (Tiwari et al., 2006).

It has been demonstrated previously that stabilizing mutations in Domain II of Aux/IAA proteins lead to a repression of auxin-responsive reporter gene activation (Fig. 2; Tiwari et al., 2001). These authors used the carrot protoplast system described above and presented results indicating that over-expression of these dominant negative mutant isoforms caused a marked reduction in reporter gene activation, although it appeared that the inhibition was incomplete.

Here, we have constructed an mRFP positive marker containing Gateway-compatible transient transformation vector, pBeaconRFP (Fig. 1), and have cloned the dominant negative Aux/IAA isoforms IAA7mII and IAA19mII, provided by the Guilfoyle lab, into this vector. pMON999_mRFP was utilized as a control vector, expressing only mRFP. These vectors were used to transfect protoplasts derived from the roots of 1-week-old DR5::GFP Arabidopsis seedlings (Fig. 3A). After an overnight incubation, giving the transformed protoplasts the opportunity to start expressing the IAA7mII and mRFP, protoplast suspensions were treated with 5 µM indole-3-acetic acid (IAA) or mock-treated with solvent and monitored cytometrically. Figure 3B shows the acquired cytometric data in a dot plot format. Mock-transfected protoplast suspensions (suspensions that encountered the polyethylene glycol transfection procedure without the addition of plasmid, see MATERIALS AND METHODS) displayed only a minor population expressing GFP when treated with solvent alone, this population likely represents protoplasts derived from the natural DR5::GFP-expressing auxin maxima of the root (i.e. the root tip and lateral root primordia). These suspensions exhibited a sizeable induction of GFP expression when treated with auxin, as expected. In suspensions transfected with the control vector, the induction of GFP expression was clearly apparent in both the non-transformed and RFP-positive, transformed protoplasts. In stark contrast, the auxin-induced GFP expression in suspensions over-expressing either IAA7mII or IAA19mII was only evident in the non-transformed cells and not perceptible in the RFP-positive protoplasts. Quantification of the GFP signal in RFP-positive cells (Fig. 3, C and D) demonstrates that there was an approximate 8-fold increase of GFP signal in protoplasts transformed with the control vector, whereas the protoplasts transformed with the dominant negative Aux/IAA isoforms exhibited no obvious induction. Interestingly, the quantification also showed that the GFP signal in mock-treated IAA7mII- and IAA19mII-expressing protoplasts was already less intense than in the protoplasts transformed with the control vector, a 1.7- and 2.6-fold repression, respectively (Fig. 3D).
independent experiment is presented, showing a time course analysis of GFP induction (Fig. 3E), reiterating the previous result and allowing examination of the kinetics of reporter gene activation.

These results corroborate previous results (Tiwari et al., 2001) and validate the pBeaconRFP system. Furthermore, they demonstrate that we were able to measure reporter gene activation specifically in the transformed cells and indicate that both IAA7mII and IAA19mII effectively repress auxin-induced DR5::GFP expression. This system permits a highly quantitative live analysis and has the potential for large scale screening of candidate genes for effects on reporter gene activation.

Transcriptional analysis of cell-type-specific transient over-expression

In order to demonstrate an entirely novel use for the system, we used pBeaconRFP in combination with a cell-type-specific GFP marker to isolate dually labeled cells by FACS and analyze the effects of over-expression in a particular cell population. An expansive collection of cell-type-specific fluorescent markers is available to the plant research community (Lee et al., 2006; http://www.plantsci.cam.ac.uk/Haseloff/construction/catalogFrame.html; http://enhancertraps.bio.upenn.edu/default.html). Furthermore, transcriptional changes in specific cell types in response to several environmental stimuli have been scrutinized and these studies have demonstrated that distinct cell types respond differentially to external cues (Gifford et al., 2008; Dinneny et al., 2008). Auxin responses are also expected to diverge between different cell types, this can be deduced from, among other evidence, the cell-type-specific expression of the different isoforms of the ARF-Aux/IAA auxin perception pathway (Weijers and Jürgens, 2004).

We have used pBeaconRFP to transiently express IAAnmIIs in protoplasts derived from the roots of PWER::GFP Arabidopsis seedlings. Following overnight incubation and a 3 hour treatment with IAA or solvent alone, dually labeled protoplasts were separated using FACS and transcriptionally profiled by means of microarray analysis (Fig. 4A). Protoplast suspensions were transfected with the pMON999_mRFP control vector, pBeaconRFP_IAA7mII or pBeaconRFP_IAA19mII. Figure 4B shows microscopic images of a PWER::GFP protoplast suspension transfected with the control vector, demonstrating that there are protoplasts present in all four expected categories: blank, PWER::GFP alone, pMON999_RFP alone and dually labeled. Untransfected wild-type and PWER::GFP protoplast suspensions as well as a wild-type protoplast suspension transfected with the control vector were employed to conservatively set up sorting gates in such a way that exclusively the dually
labeled protoplasts would be sorted (Fig. 4C). The experiment was performed in triplicate; 9 separate transfections, 18 treatments, sorts and microarrays. In corroboration with known auxin responses and our own data, the expression of Arabidopsis GH3.5, as measured by microarray, resembles the DR5::GFP expression measured in the previous experiment, displaying a drastically reduced auxin-induced increase in expression level and a basal repression of expression in protoplasts transformed with the IAA7mIIIs (Fig. 3, D and E; Fig. 4D). Furthermore, genes displaying a response to auxin in the protoplasts transformed with the control vector generally exhibited a dampened response in the protoplasts expressing IAA7mIIIs (Table I). Analysis of the data as a whole showed that the protoplasts transformed with the IAA7mIIIs were fundamentally already very different compared to the protoplasts transformed with the control vector. Interestingly, although they were more similar to each other than to the control, there was also a substantial amount of statistically significant gene expression differences between protoplasts expressing IAA7mII and IAA19mII (Fig. 4D; Table I).

These results provide a proof of concept for the feasibility of transcriptional profiling after transient protoplast transformation. This is now possible due to the fact that the system eliminates any contaminating effects of non-transformed cells. Furthermore, the dual-color cell sorting approach makes it possible to analyze the effect of over-expression in a specific population of cells. In this case, the system allowed us to compare the outcome of expression of two highly homologous signal transduction cascade components and the results indicate that IAA7 and IAA19 have both overlapping and unique downstream consequences in protoplasts derived from the Arabidopsis root epidermis. These results can be pursued to investigate mechanisms that lead to the specificity of auxin signal transduction. This demonstrates how the pBeaconRFP system can be used as a tool for rapid and high-throughput as well as in-depth analysis of genetic circuitry.

DISCUSSION

The system described here, making use of the pBeaconRFP positive selection marker vector in combination with flow cytometry and FACS, has several advantages over traditional protoplast transient transformation techniques. 1) The vectors containing a fluorescent positive marker make it possible to examine effects exclusively in the transformed protoplasts, thereby avoiding confounding of the results by non-transformed cells. 2) Functional data can be obtained even with protoplasts from tissues or species with intrinsically low transformation efficiencies. 3) The use of RFP as a positive marker allows
cytometric analysis of transient gene expression in combination with systems employing any distinguishable fluorophores as readout. 4) FACS-based collection of transformed protoplasts also enables accurate use of any other non-fluorometric downstream analyses. 5) The system is Gateway-compatible, making it quick and easy to clone genes-of-interest and amenable to high-throughput approaches (e.g. Sutter et al., 2005).

Making use of the pBeaconRFP vector and FACS-based collection of cells permits analysis not only of effects on GFP-reporter gene activation or transcriptional profiles, as demonstrated here, but also of any other measurable parameters, such as enzymatic activities or metabolite levels. In combination with cell-identity markers, this system now also makes it possible to quickly analyze over-expression effects in a cell-type-specific manner. Additionally, measuring effects in a particular cell population, as opposed to a heterogeneous mix of protoplasts, allows for a more defined and specific analysis. Moreover, there is the potential of measuring multiple parameters at once, for instance, one could measure the effect of manipulation of upstream signal transduction elements both on mitogen activated protein kinase activation and its ultimate downstream transcriptional responses. Of course this system does not have to be used exclusively with flow cytometry or FACS; for example, it could also be used to select transformed protoplasts for individual analyses such as patch clamping or subcellular protein localization studies. Alternatively, a use in combination with more basic fluorometric analyses could be envisioned, such as microscopic analyses or assays performed with plate readers. Lastly, the system is conceptually well suited for high-throughput screening purposes (e.g. looking for genes that activate or inhibit activation of a favorite reporter gene or complementation screens in mutant backgrounds). In conclusion, the technique described here opens up a wide field of possibilities not previously feasible in plant research.

Further development and enhancement of this system is ongoing. A transient silencing vector containing a positive selection marker will allow for RNAi manipulations. Enhancement with glucocorticoid receptor protein fusion or a transcriptionally inducible system will make it possible to time the activation or over-expression of one’s gene-of-interest (Moore et al., 2006). A Gateway-compatible multicolor protein tagging set will give the possibility of high-throughput protein localization studies as well as protein interaction screens. Additionally, vectors with alternate positive selection markers, such as GFP or other fluorescent proteins, will permit analysis of protoplasts transformed with multiple effectors. Lastly, the development of low-stress-eliciting protoplast transfection procedures will allow examination of protoplasts that more closely resemble their natural state.
The pBeaconRFP vector will be made available through the Flanders Institute of Biotechnology (VIB, http://www.psb.ugent.be/gateway/), where the backbone originated. The microarray data has been deposited in the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database under accession GSE13783.

MATERIALS AND METHODS

Plant materials and treatment
Seed from wild-type (Col-0), DR5::GFP (Col-0, obtained from the Arabidopsis Biological Resource Center [ABRC], stock# CS9361) and PWER::GFP (Col-0, obtained from Dr. John Schiefelbein, University of Michigan, USA) Arabidopsis plants was sterilized by 5 min incubation with 96% ethanol followed by 20 min incubation with 50% household bleach and rinsing with sterile water. Seeds were plated on square 10x10 cm plates (Fisher Scientific) with MS-agar (2.2 g/l Murashige and Skoog Salts [Sigma-Aldrich], 1% [w/v] sucrose, 1% [w/v] agar, 0.5 g/l MES hydrate [Sigma-Aldrich], pH 5.7 with KOH) on top of a sterile nylon mesh (NITEX 03-100/47, Sefar filtration Inc.) to facilitate harvesting of the roots. Seeds were plated in two rows of approximately 150 seeds. Plates were vernalized for 2 days at 4° C in the dark and placed vertically in an Advanced Intellus environmental controller (Percival) set to 35 µmol/m²*sec⁻¹ and 22° C with an 18h-light/6h-dark regime. Protoplast suspensions were treated with 5 µM indole-3-acetic acid (IAA; Sigma-Aldrich) or mock-treated with solvent alone. A 20 mM IAA stock was dissolved in ethanol and stored at -20° C.

Vector construction
pMON999_mRFP was obtained from Dr. Joop Vermeer (Universiteit van Amsterdam, The Netherlands). pBeaconRFP was constructed by PCR amplification of the 35S::mRFP::TNOS cassette from pMON999_mRFP with primers mRFP_F2 gaattgcatatgcttcaagcttctgcagg and mRFP_R ttaatacatatgcctcggattagatce, both with an NdeI restriction site (in bold), using Phusion polymerase (New England Biolabs). The PCR product was ligated into the NdeI site of p2GW7.0 (http://www.psb.ugent.be/gateway/). The orientation of the insert was checked by PCR.

A pZP211 plasmid containing 35S::HA-IAA7mII and a pUC18 plasmid containing PIAA19::HA-IAA19mII were obtained from Dr. Thomas Guilfoyle (University of Missouri, USA). IAA7mII and IAA19mII were PCR amplified with primers IAA7_AttB1 aaaaagcaggctatgatcggccaacttatgaac, IAA7.AttB2 agaaagctgggttcaagatcttgttctgcagg, IAA19.AttB1 aaaaagcaggctatgagagagagagagagagac and IAA19.AttB2 aaaaagcaggctatggagaaggaaggactc.
agaagctgggtcactgctactctctctag and subsequently re-amplified with primers AttB1 ggggacaagttgtgacaaaagcgct and AttB2 ggggaccaccttgtacaagaagctgggt using Phusion polymerase. The PCR products were recombined into pDONR221 using BP clonase and subsequently shuttled into pBeaconRFP with LR clonase (Invitrogen).

Protoplast isolation and transfection
Protoplast isolation and polyethylene glycol (PEG) -mediated transfection was performed basically as described by the Sheen lab (http://genetics.mgh.harvard.edu/sheenweb/). Roots of 1-week-old seedlings were harvested with a scalpel and placed into a gently shaking flask with 100 ml protoplasting solution for 3 h. Protoplasting solution was prepared as follows: 1.25% (w/v) cellulase (Yakult), 0.3% (w/v) macerozyme (Yakult), 0.4 M mannitol, 20 mM MES, 20 mM KCl, pH 5.7 with Tris/HCl pH 7.5; heat for 10 min at 55° C, cool to room temperature; 0.1% (w/v) BSA, 10 mM CaCl2, 5 mM β-mercapto ethanol. The protoplast solution was filtered through 40 µm cell-strainer (BD Falcon, USA), divided over 15 ml conical tubes and centrifuged for 10 min at 150 G. Pellets were washed once with transfection solution (0.4 M mannitol, 15 mM MgCl2 hexahydrate, 4 mM MES, pH 5.7 with KOH), centrifuged again and resuspended in transfection solution with a final concentration of 4x10^6 protoplasts/ml (generally, we obtain between 8x10^3 and 1x10^4 protoplasts from 20 plates). 15 ml conical tubes were prepared for each transfection with 50 µg of plasmid DNA (10-20 µl) and 250 µl of protoplasts in transfection solution. 250 µl of PEG solution (40% [w/v] PEG 4000, 0.4 M mannitol, 0.1 M CaCl2) was added and the suspension was mixed by flicking the tube repeatedly. Suspensions were incubated for 15 min, after which the protoplasts were washed with 15 ml of incubation solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM MES, pH 5.7 with KOH), centrifuged and resuspended in 1 ml of incubation solution. Protoplast suspensions were incubated overnight in 24-well plates in the dark.

Flow cytometry and FACS
Protoplast suspensions were cytometrically analyzed and sorted with a FACSARia (BD Biosciences) fitted with a 100 µm nozzle and using PBS as a sheath fluid. The sheath pressure was set at 20 p.s.i and the defection plate voltage at 5000 V (default “Low” setting). A 488 nm Coherent Sapphire Solid State laser was used for excitation and emission was measured at 530/30 nm for GFP and 610/20 nm for RFP. The photomultiplier tube voltage was set at 60 V for forward scatter, 159 V for side scatter, 350 V for GFP and 335 V for RFP. The threshold value for event detection was set at 8835 on forward scattering. The drop drive frequency was
set to approximately 30 kHz and the amplitude was set to approximately 45 V; the drop delay value was approximately 27 (these settings will vary slightly with day-to-day operation of the FACSAria). Data was processed using the FACSDiva 5.0.2 software (BD Biosciences). Compensation constraints were set to adjust for spectral overlap between GFP and RFP (GFP: minus 0.50% RFP and RFP: minus 17.91% GFP). Gates for sorting dually labeled protoplasts were set up using blank (wild type), RFP-only (pMON999_mRFP-transfected wild type) and GFP-only \((P_{WER}::GFP)\) protoplast suspensions in such a way that the sorted dually labeled protoplasts in the “Double” gate would not be contaminated by blank, RFP-only or GFP-only protoplasts (see Fig. 4C).

**Microscopy**

Microscopic images of protoplasts mounted in a Bright-Line Hemacytometer (Hausser Scientific) were obtained with DIC, GFP- and Texas Red-filters on an Eclipse 90i microscope (Nikon) running on Metamorph software (Molecular Devices Corporation).

**Microarray analysis**

Protoplasts were sorted directly into RNA extraction buffer and RNA was extracted using an RNeasy Micro Kit with RNase-free DNase Set according to the manufacturer’s instructions (QIAGEN). RNA was quantified with a Bioanalyzer (Agilent Technologies) and amplified and labeled with WT-Ovation Pico RNA Amplification System and FL-Ovation cDNA Biotin Module V2, respectively (NuGEN). The labeled cDNA was hybridized, washed and stained on an ATH-121501 *Arabidopsis* full genome microarray using a Hybridization Control Kit, a GeneChip Hybridization, Wash, and Stain Kit, a GeneChip Fluidics Station 450 and a GeneChip Scanner (Affymetrix).

Data was normalized using the MAS 5.0 method with a scaling factor of 250. Statistical analysis was performed as follows. We first filtered genes that showed expression below noise levels by removing genes whose average expression signal (among three replicates) never exceeded a threshold of 75 in any experiment. The data was subjected to a Two-Way ANOVA (treatment x transient genetic background) and all genes that showed a significant effect at \(p<0.05\) at any level, including the interaction level, were taken as showing some response to experimental conditions \((n=7,145)\). These genes are shown on the heatmap, which was generated with a log2 transformation of the data followed by row normalization. The heatmap was generated in Matlab 7.6.0 (The Mathworks). To test for gene expression differences in individual comparisons between the different treatments, we used a procedure
that accounts for multiple testing (Significance Analysis of Microarrays, two-class unpaired test, Wilcoxon statistic; q<10% False Discovery Rate). In order to assess the effects of IAA\text{mII} expression on auxin responses, the transcripts that showed a significant difference between mock-treated control vector and IAA-treated control vector (basal auxin response, n=809) were then tested for their fold-change response in experiments in which protoplasts were transiently transformed with pBeaconRFP\_IAA\text{mII} and mock-treated or treated with auxin. Increases and decreases in average expression were converted to an absolute fold change to measure the overall effect of the over-expression on the basal auxin response. IAA7 and IAA19 were removed from analysis in their respective over-expressor samples.

ACKNOWLEDGEMENTS
The authors would like to thank Joop Vermeer for the pMON999\_mRFP plasmid, Tom Guilfoyle for the 35S::HA-IAA7\text{mII} and P\text{IAA19}'::HA-IAA19\text{mII} plasmids and John Schiefelbein for the P\text{WER}'::GFP line.
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** The pBeaconRFP transient transformation system. A schematic representation of the control vector pMON999_mRFP and pBeaconRFP, a high copy number plasmid containing a 35S-driven mRFP positive marker and Gateway cassette.

**Figure 2.** The ARF-Aux/IAA auxin response pathway. In the absence of auxin, Aux/IAAs repress the activity of ARF transcription factors. Upon perception of auxin, Aux/IAAs are ubiquitinated and degraded in a proteasome-dependent manner. Dominant negative mutant isoforms of Aux/IAAs (e.g. IAA7mII and IAA19mII) can no longer be ubiquitinated and effect a stable repression of ARF function.

**Figure 3.** Repression of DR5::GFP activation by IAA7mII and IAA19mII. A, A schematic representation of the experiment. Protoplasts derived from the roots of 1-week-old DR5::GFP Arabidopsis seedlings were transfected with either pMON999_mRFP, expressing only mRFP, or pBeaconRFP expressing IAA7mII or IAA19mII. After an overnight incubation, protoplast suspensions were treated for 10 hours with 5 µM indole-3-acetic-acid (IAA) or solvent alone. B, Flow cytometric analysis of transfected and treated protoplast suspensions. The GFP and RFP intensities for individual protoplasts were recorded and are represented in dot plots, 10,000 events are displayed in each plot. Gates were defined to separate blank, GFP- and RFP-positive events. C, A frequency distribution of the GFP signal of events falling within the “RFP” gates in panel B. D, Quantification of the mean GFP signal in RFP-positive cells for auxin- and mock-treated protoplasts transformed with the control vector or pBeaconRFP with IAA7mII or IAA19mII. Data is presented in a histogram +/- s.e.m. (n=798-2447). E, A 6-hour time course of GFP quantification in an independent experiment. Data is presented in a line graph +/- s.e.m., (n=579-2390).

**Figure 4.** Transcriptional analysis of cell-type-specific IAA7mII and IAA19mII expression. A, A schematic representation of the experiment. Protoplasts derived from the roots of 1-week-old PWER::GFP seedlings were transfected with either pMON999_mRFP, expressing only mRFP, or pBeaconRFP expressing IAA7mII or IAA19mII. After an overnight incubation, protoplast suspensions were treated for 3 hours with 5 µM IAA or solvent alone. Dually labeled protoplasts were isolated by FACS and used for microarray analysis. B, Microscopic examination of protoplasts derived from the roots of 1-week-old PWER::GFP seedlings that were transfected with pMON999_mRFP. Scale bar represents 50 µm. C, Fluorescence activated cell sorting of the transfected protoplast suspensions. Dot plots are shown depicting the controls used to set up the gates: an untransfected protoplast suspension derived from wild-type roots (blank), an untransfected protoplast suspension derived from PWER::GFP roots, a protoplast suspension derived from PWER::GFP roots and a protoplast suspension derived from wild-type roots transfected with pMON999_mRFP. In addition, a dot plot depicting a protoplast suspension derived from PWER::GFP roots transfected with pMON999_mRFP is shown; protoplasts falling within the gate marked “Double” were sorted and used for microarray analysis. 100,000 events are displayed in each dot plot. D, Transcriptional analysis of sorted protoplasts. A log-scale heatmap and a histogram quantifying the differences in gene expression between the various collected protoplasts are shown. The heatmap displays all the genes that exhibit any significant difference between either mock- and auxin-treatment, between transformation with the different vectors or on an interaction level (see MATERIALS AND METHODS) as measured by microarray analysis, rows represent genes and columns represent treatment and transformation vectors (n=3). The histogram presents the difference in GH3.5 expression (as measured by microarray) between the various collected protoplasts +/- s.d. (n=3).
TABLES

Table I  Transcriptional changes induced by auxin-treatment and IAA
mII over-expression. The average fold-change in expression of the 809
genes responsive to auxin treatment in the pMON999_mRFP control vector
is presented for protoplasts transformed with the control vector as
well as protoplasts transformed with the IAA7mII and IAA19mII over-expressors. The number of genes
with statistically significant differences (see MATERIALS AND METHODS) in expression between all
vectors and treatments are given.

<table>
<thead>
<tr>
<th>Average fold-change in expression of pMON999_mRFP</th>
<th>pMON999_mRFP</th>
<th>pBeaconRFP_IAA7mII</th>
<th>pBeaconRFP_IAA19mII</th>
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<tr>
<td>Number of genes with significant changes in expression</td>
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<td>IAA</td>
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*The average is given +/- s.e.m. (n=809)
*Statistically significant difference compared to pMON999_mRFP as determined by a two-tailed unpaired t-test (p<10^-4)