TECHNICAL ADVANCE

A simplified method for the analysis of transcription factor–promoter interactions that allows high-throughput data generation

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Summary

Transient expression systems are intensively used to study the transactivation potential of transcription factors and to confirm target promoters. Here we present a novel system based on the high-efficiency transformation of cultured *Arabidopsis thaliana* cells by agrobacteria. To demonstrate the potential of this system, we compared it with a commonly used protoplast transfection assay, and studied the regulation of phenylpropanoid biosynthetic pathway genes by various transcription factors. Both systems led to comparable results on the regulation of the promoters tested. However, the agrobacterium-mediated co-transformation assay needs significantly less time, requires only mixing of cultured plant cells with agrobacteria, is less labour-intensive and allows handling of multiple assays in parallel, making it suitable for medium- to high-throughput analyses. In addition, the binary vectors used are the same for both cell-based assays and stable plant transformations.

Keywords: cell suspension culture, protoplast, Arabidopsis thaliana, hypervirulent agrobacteria, transcription factor, target promoter.

Introduction

Transient expression systems are widely used in plant sciences for various applications, including protein expression and purification (Andrews and Curtis, 2005; Doran, 2000; Ferrando *et al.*, 2000), sub-cellular localization of fluorescent fusion proteins (Earley *et al.*, 2006; Koroleva *et al.*, 2005), or the study of promoters and/or promoter–transcription factor interactions (Baudry *et al.*, 2004; Hellens *et al.*, 2005). The systems are not only fast, compared with stable transformation, but also unaffected by position effects related to the site of integration. This is a major advantage when analysing the activity of promoters. Stable transformation of promoter–reporter systems requires the analysis of numerous lines, as the expression of the transgene is highly dependent on the integration site of the transfer DNA and the number of inserted transgenes. Transient expression systems take advantage of the expression burst of DNA introduced into plant cells prior to degradation or the stable integration into

the genome. Hence transient expression is suitable for analysis of the unbiased activity and function of promoter elements, and their induction or repression by transcription factors. The most commonly used systems for these applications are based on protoplast transfection or agrobacterium infiltration into leaves. The protoplast system employs isolated plasmid DNA introduced via PEG-mediated DNA uptake. Consequently various plasmids can be introduced at the same time. The major disadvantage of this system is the labour-intensive preparation of protoplasts, and the need for large quantities of isolated plasmid DNA. Furthermore, the transfection rate can be variable and dependent on the plasmids used (Sprenger-Haussels and Weisshaar, 2000). Large binary vectors used for stable transformations often result in a poor transfection rate. Consequently, smaller constructs, generated to achieve a high transfection rate, have to be employed, resulting in the use of different

constructs for *in vivo* and *in planta* analysis. The other system, which is based on leaf infiltration using *Agrobacterium tumefaciens*, has mostly been reported for tobacco (*Nicotiana benthamiana*). It is fast and allows simultaneous transfection with several strains. However, its efficiency can vary drastically, not only depending on the age of the leaf or the plant genotype, but also from one experiment to another. When using the β-glucuronidase (*uidA*, GUS) reporter system, there is the additional problem of working under non-aseptic conditions. Large amounts of microorganisms present in the infiltrated leaves might lead to a misinterpretation of the results, as many microorganisms across the kingdoms do express endogenous GUS activity (Andrews and Curtis, 2005).

Here we present a new, easy method for the transient co-expression of transcription factors and promoter–reporter constructs. The method combines the advantages of monoseptic cell-culture systems and agrobacterium-mediated transformation, which was successfully used with large Gateway-compatible binary transformation vectors. The recently reported hypervirulent strain LBA4404.pBBR1MCS *virGN54D* and LBA4404pBBR1MCS-5virGN54D of *A. tumefaciens* allows the high efficiency transformation of cultured *Arabidopsis thaliana* cells, without the need for selecting stably transformed lines (Koroleva *et al.*, 2005; van der Fits *et al.*, 2000). To demonstrate the potential of this method for the analysis of transcriptional activation/repression, we compared it with a commonly used protoplast transfection assay, taking advantage of the well characterized regulation of the phenylpropanoid biosynthetic pathway.

Results and discussion

### Experimental set-up of the cell-culture system

The system combines a high-efficiency transfection method (Koroleva *et al.*, 2005) with transactivation assays, as reported by Hartmann *et al.* (1998). For co-culture experiments, freshly diluted cultured *A. thaliana* cells were distributed to multi-well culture plates, ensuring homogeneous conditions for all parallel experiments. The agrobacteria were grown overnight in liquid culture, washed once and re-suspended in the plant cell culture medium. A strain expressing the anti-silencing protein 19 K (Voinnet *et al.*, 2003) was added to each sample to ensure a high transgene-expression level over several days. The hypervirulent agrobacteria carrying the effector or reporter constructs were added according to the desired set-up. The use of multi-well plates and transfection by simply adding the agrobacteria was less time-consuming than the preparation of protoplasts, and allows the parallel handling of numerous assays. Most consistent results were achieved using 3-mL set-ups in six-well plates.

However, initial screenings were successfully carried out using 1-mL samples in 24-well plates, which allowed a higher throughput but led to a higher variability. The use of the GUS reporter system allowed us to test samples not only using quantitative fluorescence assays but also qualitative histochemical staining by simply adding X-Gluc staining solution to aliquots of the co-culture mixture. The transformation efficiency, estimated by co-transformation with GFP, varied between 50% and 80%. The transactivation potential of a given transcription factor was intrinsically dependent on the nature of this factor, rather than on the transformation efficiency.

Promoter activities recorded by the protoplast and cell-culture systems

For comparison of the two systems, we used identical effector ORF constructs and known minimal promoter sequences fused to the *uidA* ORF. The fragments were introduced into different transformation vectors. Minimal plasmids, lacking plant-selection markers, were used for protoplast transfection to ensure a high transfection rate. Furthermore, these plasmids had to be transferred to methylation-deficient *Escherichia coli* strains, as unmethylated plasmids show a reduced GUS background activity when introduced into protoplasts (Torres *et al.*, 1993). For the cell-culture system, we used large Gateway-compatible binary vectors, also used in conventional plant transformations, the only modification being an intron inserted into the *uidA* ORF of the reporter constructs to prevent *GUS* expression in agrobacteria. The background GUS activity was very low in both systems, allowing sensitive measurements (Figure 1). The activity determined in extracts of cells transformed only with the 35S-GUS construct was taken as a reference (set as 1) for the relative GUS activity in the transactivation experiments. Whereas the 35S-GUS samples showed highest activity in the protoplast system, this was not the case for the agrobacterium-mediated cell-culture transformation. A reason for this may be the different efficiency of the vectors used. Even though this leads to a different scale for the transactivation experiments, the results obtained with both systems show the same mode of regulation for all three different types of transcriptional regulation tested.

First, the combined interaction of two transcription factors to activate a target gene was analysed using TRANSPARENT TESTA2 (TT2)/MYB123 and TT8/BHLH42, which need to interact for the activation of the *DIHYDROFLAVONOL REDUCTASE (DFR)* promoter (Baudry *et al.*, 2004). As shown in Figure 1, the background activity of the *DFR* promoter was low in both systems, and addition of only one effector (TT2/MYB123 or TT8/BHLH42 alone) had no effect on the level of relative GUS activity. Only the simultaneous addition of both effector constructs led to an activation of the *DFR* reporter
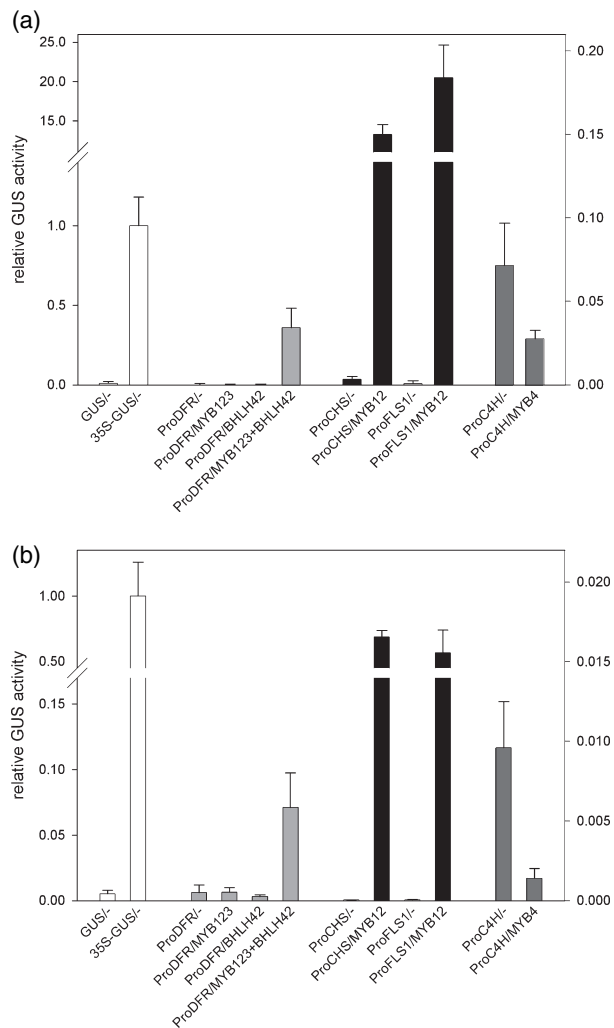
Figure 1. Relative GUS activity of the reporter constructs in transient co-transfection assays. The 35S-GUS control was taken as a reference. The *y*-axis on the right side is for the two outer right bars only.
(a) Results of the agrobacterium-mediated co-transformation (*n* = 6, ±SD).
(b) Results obtained by protoplast transfection (*n* = 6, ±SD).

construct, in accordance with results reported by Baudry *et al.* (2004).

Second, the potential of a single transcription factor to activate multiple target genes was represented by MYB12 activating the flavonol pathway genes *CHALCONE SYN-THASE* (*CHS*) and *FLAVONOL SYNTHASE1 (FLS1;* Mehrtens *et al.*, 2005). The transactivation potential of MYB12 towards the promoters of *CHS* and *FLS1* could be demonstrated clearly in both systems (Figure 1). Both reporter constructs showed a high induction on addition of the MYB12 effector construct, which in both systems was significantly higher than the activation of the *DFR* promoter by either TT2/MYB123 or TT8/BHLH42. The effect of MYB12 as a positive regulator of flavonol biosynthesis could be further confirmed by a light yellow staining of the plant cells, expres-

sing the 35S-driven MYB12 effector construct compared with wild-type cells (Figure 2). Extracts of these cells showed an increased flavonol content as revealed by thin-layer chromatography (data not shown), indicating an intact secondary metabolism of the plant cells. Such striking accumulation of flavonols was not observed in the proto-plast system. Thus this method also has potential for studying the effect of transcription factor(s) on the metabol-ome (and also the transcriptome) of *A. thaliana* in a high-throughput fashion in microtitre plates.

Third, as an example of gene repression, the negative regulator of *CINNAMATE-4-HYDROXYLASE (C4H),* MYB4, was analysed (Jin *et al.*, 2000; Hemm *et al.*, 2001). Although basal expression of the *C4H* reporter is low in both cell systems, a repression by MYB4 could be clearly seen in both the protoplast system and the cell-culture system, further validating the agrobacterium-mediated transfection system as a simple alternative method (Figure 1).

Figure 2 shows corresponding results as obtained by histochemical GUS staining of the plant cells directly in culture plates. As already revealed by the quantitative GUS analysis, the blue staining indicates strong activation of the *CHS* and *FLS1* promoter by MYB12, appearing already about 1 h after substrate addition. Fainter GUS staining visible after a few hours indicated light activation of the *DFR* promoter by TT2/TT8. Cells transformed with the *ProC4H-GUS* construct displayed blue GUS activity-derived staining only after overnight incubation with the staining solution. Consequently, the appearance and intensity of the GUS staining allows a preliminary assessment of the experiments prior to fluorimetric (quantitative) GUS analysis.

Comparison of the two systems and concluding remarks

As outlined in Figure 3, both the protoplast system and the cell-culture system follow a similar workflow. However, the major differences also constitute the advantages of the agrobacterium-mediated system. The employed constructs can also be used directly for stable plant transformation, avoiding the necessity of different vectors for *in vivo* and *in planta* studies. A further drawback of the protoplast system is the actual preparation of protoplasts and plasmid DNA. It is not only time-consuming, but also labour-intensive. Moreover, removal of the cell wall presents a significant stress for the plant cell, likely to affect the overall meta-bolism of the cell. In contrast, the agrobacterium-mediated system makes use of intact plant cells which are just mixed with agrobacteria. Furthermore, the simple and fast pro-tocol for co-transformation allows us to handle multiple assays in parallel, making the system ideal for its applica-tion in medium- or high-throughput assays. Although protocols for high-throughput protoplast systems have been described, they have not been used extensively to date (Hilson, 2006).
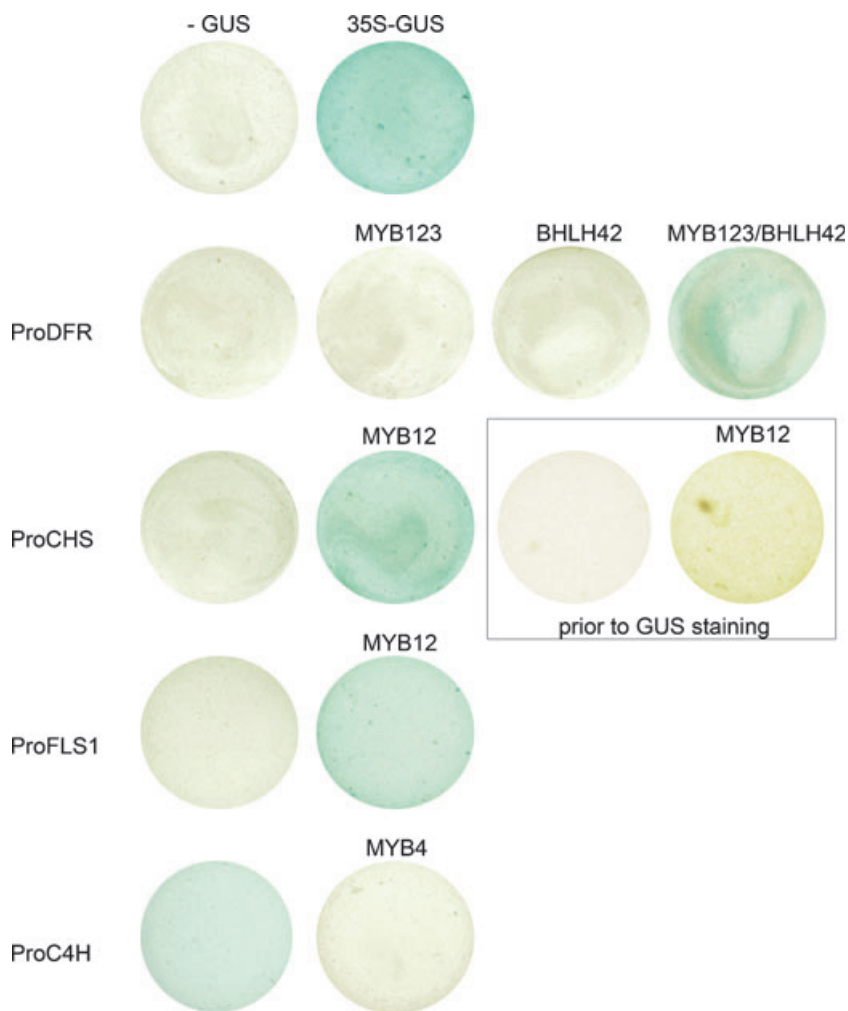
Figure 2. Histochemical GUS staining of the agrobacterium-transformed cultured *Arabidopsis thaliana* cells.
Pictures of one well for each experimental set-up are shown. The reporter constructs are given on the left side, the effector constructs are indicated on top of the respective well image. Unstained cells transformed with the 35S-MYB12 construct activating the flavonol biosynthesis are shown in the box. They displayed a light yellow staining compared with untransformed cells (see text).
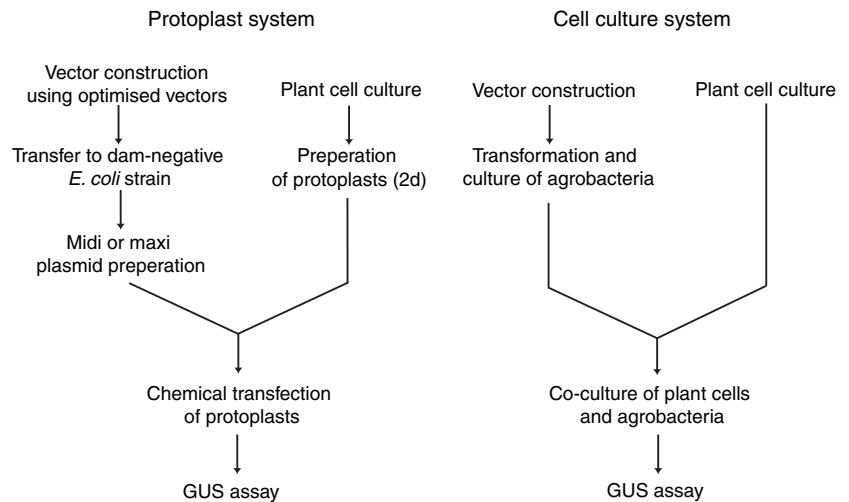
Overexpression of a transcription factor of interest in cultured *A. thaliana* cells might interfere with a given stoichiometry between different factors. This limitation is intrinsic to the experimental approach, and also affects studies in transgenic plants (*in planta* analyses). However, the triple assay (TT2/MYB123 and TT8/BHLH42 as effectors, DFR as target; Figure 1) worked well in both systems, although the relation of effectors could only be optimized in the protoplast system. Also, the results with MYB4 as a repressor of C4H promoter activity in the transformation system did not require effector titration as performed before (Jin *et al.*, 2000). Potentially, an additional advantage of the transformation system is that transcription takes place in an integrated situation when compared with protoplast transfections that result in improved, or at least more robust, readouts.

The method presented here offers a fast and flexible alternative to the protoplast system. It is less time-consuming, allows work with multiple assays in parallel, and is suitable for assessing the transactivation potential of transcription factors towards multiple putative target promoters, as revealed, for example, by analysing micro-array data for co-regulated transcripts. The preparation of multiple vectors for screening is time-limiting. However, several projects aiming to characterize transcription factor families provide Gateway-compatible ORF clone collections of *A. thaliana* transcription factors (Gong *et al.*, 2004; Paz-Ares, 2002), which could be screened with the method presented here to find novel regulators of given target genes. It should be noted that the analysis of transcription factor–promoter interactions in mutant backgrounds that is possible with mesophyll protoplasts is also an option, but if cell-culture lines with the required genetic background are not available, this option seems not to be feasible.

Together, although the systems differ regarding the cells and plasmids used, the results obtained with both methods allowed us to draw the same conclusions regarding promoter activity and regulation. We therefore propose this simple method as a fast and effective alternative to the protoplast or other systems for analysing the transactivation potential of transcription factors toward their target promoters.

Protoplast system

Vector construction using optimised vectors

Transfer to dam-negative *E. coli* strain

Midi or maxi plasmid preperation

Plant cell culture

Preperation of protoplasts (2d)

Chemical transfection of protoplasts

GUS assay

Cell culture system

Vector construction

Transformation and culture of agrobacteria

Plant cell culture

Co-culture of plant cells and agrobacteria

GUS assay

Experimental procedures

Generation of gateway entry clones

Most reporter Entry clones were generated by PCR on genomic DNA using specific primers with attB extensions followed by BP-reaction with pDONR201 (Invitrogen Life Technologies, http://www.invitrogen.com). The primers RS813 (5′-attB1-AA-GCTTTGTGCATAACTTTTTTTGTC-GTCTCG-3′, includes *Hin*DIII site) and RS814 (5′-attB2-CCATGGTAGTTTGTGTATCCGCAATG-ATATTG, includes *Nco*I site) were used to amplify a 1024-bp *C4H* promoter fragment (*ProC4H$_{-1024}$*), and a 738-bp *FLS1* promoter fragment (*ProFLS$_{-738}$*) was generated with the primers RS739 (5′-attB1-AAGCTTCCTATACTGTAGTTTTTCCTTTTTC-3′, includes *Hin*DIII site) and RS740 (5′-attB2-CCATGGTTTTTTTTGGTAG-TTTGCGTTGCCGGA-3′, includes *Nco*I site). *ProDFR$_{-265}$*, a 265-bp *DFR* promoter fragment, was amplified using the primers DFR-PROM-fwd (5′-attB1-AAGCTTCACACCTAAGGAAATAATAAAAT-CAAC-3′, includes *Hin*DIII site) and DFR-PROM-rev (5′-attB2-CCATGGTTGTGGTTATATGATAGATTGTGT-3′, includes *Nco*I site). A 705-bp *CHS* promoter fragment (*ProCHS$_{-705}$*) was generated by PCR using the primers RS741 (5′-attB1-AAGCTTATTTTCAGAC-AGATATCACTATGAT-3′, includes *Hin*DIII site) and RS742 (5′-attB2-CCATGGTAGTATACACCAACTTGGGTTTATTAG-3′, includes *Nco*I site) and transferred into pDONR201. The effector Entry clones encoding TT2/MYB123 and TT8/BHLH42 have previously been described by Baudry *et al.* (2004). The MYB4 Entry clone was generated by PCR on cDNA using the primers RS491 (5′-attB1-CCATGGGAAGGTCACCGTGCTGTGAG-3′, includes *Nco*I site) and RS492 (5′-attB2-ATTATTTCATCTCCAAGCTTCGAAAGC-3′) and transfer of the resulting PCR product into pDONR201. The MYB12 coding sequence was amplified from cDNA using the primers RS161 (5′-AATTCCAGCTGACCACCATGGGAAGAG-CGCCATGTTGCGAG-3′) and RS162 (5′-GATCCCCGGGAATTGC-CATGTCATGACAGAAGCCAAGCGACCAA-3′). Tags at the primers 5′-ends were used in a second amplification using the primers MJ144 (5′-attB1-GCCAATTCCAGCTGACCACCATG-3′) and MJ143 (5′-attB1-GCGATCCCCGGGAATTGCCATG-3′). The resulting prod-uct was inserted via BP-reaction into pDONR201.

Protoplast co-transfection and vectors

The At7 cell culture, protoplast isolation, co-transfection and determination of standardized GUS activity were carried out as described by Hartmann *et al.* (1998). All reporter constructs are based on the vector pBT10GUS (Sprenger-Haussels and Weisshaar, 2000). The constructs used for measuring the expression from the *CHS* (164 bp) and *FLS1* (153-bp) promoters have been described by Hartmann *et al.* (1998, 2005). The 1024-bp *C4H* promoter–reporter construct has been described by Jin *et al.* (2000), and the *DFR* (520-bp) promoter–reporter by Mehrtens *et al.* (2005). Also, all effector constructs have been described previously: MYB4 (Jin *et al.*, 2000; in pJIT60, Guellineau and Mullineaux, 1993); TT2/MYB123 and TT8/BHLH42 (in the Gateway-compatible vector pBTdest, Baudry *et al.*, 2004); and MYB12 (Mehrtens *et al.*, 2005; in pBT8, Sprenger-Haus-sels and Weisshaar, 2000).

Binary vectors for cell co-culture experiments

The effector Entry clones were recombined via LR reaction with pGWB2; the reporter Entry clones were recombined with pGWB3i, which was generated by inserting the 189-bp intron from pPCV 6NFHyg GUS Int (Vancanneyt *et al.*, 1990) into the GUS ORF of pGWB3 at the *Sna*BI restriction site by blunt-end ligation. The negative and positive control for GUS expression were generated by PCR amplification of the intron-tagged GUS ORF from pGWB3i and subsequent transfer into pDONR207 by BP recombination. The final destination clones were generated by LR recombination with pGWB1 and pGWB2, for the promoterless and the 35S control, respectively.

Cell culture and transformation

Arabidopsis thaliana cells were grown in the dark at 22°C with gentle shaking at 160 r.p.m. The cells were inoculated weekly at a 1:5 dilution into fresh medium (4.3 g l^{-1} MS basal salts (Duchefa, http://www.duchefa.com), 4 ml l^{-1} Gamborg's vitamin solution (Sigma, http://www.sigmaaldrich.com), 1 mg l^{-1} 2,4-dichlorophen-oxyacetic acid (2,4-D), 30 g l^{-1} sucrose pH 5.8).

The agrobacteria strains used were the hypervirulent strain LBA4404.pBBR1MCS *virGN54D* for the effector and reporter vectors (van der Fits *et al.*, 2000) and the anti-silencing strain 19 K (Voinnet *et al.*, 2003). Agrobacteria from fresh plates were grown overnight in YEB medium with the respective antibiotics. Cells were harvested by centrifugation, washed once in the plant cell culture medium and re-suspended in 25% of the initial culture volume. 25 µl of the 19 K strain and 25 µl of the reporter and/or effector strains were added to 3 ml 1:5 diluted plant cell culture in six-well sterile culture plates (Corning Inc., http://www.corning.com). After 3–4 days of co-culture (dark, 22°C, 160 r.p.m.), 1 ml of each sample was centrifuged and the pellet was stored at –80°C until GUS analysis. The remaining cells were treated with 500 µl X-Gluc staining solution (50 mM NaH_2PO_4, pH 7, 1 mM X-Gluc) for 1 h to overnight at 37°C without shaking.

Sample preparation and GUS analysis

The pelleted cells from 1 ml co-culture were homogenized in 500 µl assay buffer (50 mM NaH_2PO_4 pH 7, 1 mM EDTA, 0.1% (v/v) Triton X-100) with an Eppendorf pestle and subsequent vortexing. Cell debris was removed by centrifugation (4°C, 15 000 *g*, 15 min) and the supernatant was used for determination of GUS activity (Jefferson *et al.*, 1987) and protein quantification (BCA kit, Pierce Biotechnology, http://www.piercenet.com) with BSA as a standard.

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