# Transient transformation of sunflower leaf discs via an *Agrobacterium*-mediated method: applications for gene expression and silencing studies

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**The sunflower belongs to the *Compositae* family and is an economically important crop because of the quality of its oil. Unfortunately, molecular analyses are limited due to the lack of genomic information, mutant libraries and efficient and rapid transformation protocols. In a wide variety of species, *Agrobacterium*-mediated transient transformation is a useful tool that can provide valuable insight into many biological processes. However, this technology has not been routinely applied to the sunflower because of difficulties with infiltration. Here, we present an optimized protocol for *Agrobacterium*–mediated transient transformation of leaf discs. Using this procedure, we were able to quickly overexpress or silence a given gene, enabling us to study several biochemical processes and characterize sunflower regulatory sequences. One of the major advantages of this approach is that in only 1 work-week it is possible to acquire considerable molecular information while avoiding the use of controversial heterologous systems. Transforming heterologous species is frequently unacceptable, as the conservation of molecular events in many cases is not well documented.**

## INTRODUCTION

The transformation of plants has become a crucial tool for understanding gene function. However, obtaining stable transformants is not yet feasible for many species. Even in those species for which routine protocols are available, such as *Arabidopsis*, the process is time-consuming and labor-intensive[1,2]. Several months are needed to obtain homozygous transgenic *Arabidopsis* plants; longer periods are needed for species like maize, rice and soybean[3–6].

The sunflower is a crop in the *Compositae* family that is mostly used to produce high quality oil. Although this crop is of great economic importance in many countries, genomic tools have not yet been developed. As a result, our understanding of the metabolic and signal transduction pathways in this organism is limited. A few protocols have already been described for obtaining stable transgenic sunflower plants[7–13]. *Agrobacterium*-mediated transformation involves the use of wounded plant material or macerated tissues (the metabolites that are released during these treatments are required for bacterial virulence) and the regeneration of transformed cells that receive the T-DNA insertion; low efficiencies result[7]. An improvement in the transformation frequency has been achieved by combining microprojectile bombardment with *Agrobaterium* infection[7]. A comparison of the transformation efficiencies of direct gene transfer into protoplasts, particle bombardment and co-culture with *Agrobacterium* revealed that only the co-culture technique is able to generate a stable transgenic callus[8]. Unfortunately, the same study showed that the transformed cells are not able to regenerate[8]. This method was later improved by using a combination of sonication and macerating enzymes[9]. A different *Agrobacteria*-mediated transformation protocol that does not involve tissue regeneration was shown to produce fertile transgenic plants at a rate of 2% (ref. 10). Additional protocols for obtaining stable transformants using various sunflower genotypes have been shown to have slightly higher efficiencies[11]. All of these protocols use *NPTII*, β-glucuronidase (*GUS*) and *GFP*, or other reporter genes to verify transformation[8–14]. The selection of transformants, regeneration of tissue, growth of sunflower plants (with a long life cycle) and generation of homozygous lines are time-consuming, especially compared with the time required to obtain homozygous transgenic *Arabidopsis* plants. It is possible that these obstacles have prevented these techniques from being used to elucidate signal transduction or metabolic pathways in this crop.

The transient expression of genes, an alternative method for obtaining molecular information, has been successfully used in certain model systems and agronomic crops[15–19]. This kind of analysis has provided valuable insight into biological processes, such as small RNA function, promoter activities, gene regulation, subcellular protein localization, protein–protein interactions and protein stability[15–20].

The advantage of transient assays over stable transformations is undoubtedly the rapid generation of experimental results. For example, the activity/expression of a given transgene can usually be assayed within a few days of transfection[15,21]. For most plants, transient transformation techniques involve *Agrobacterium* infiltration. In these methods, *Agrobacterium* suspensions are injected into the leaf parenchyma and allowed to infect the plant cells. In the sunflower, however, the structure of the leaf and the thickness of the epidermis make it difficult for the bacterial culture to enter the parenchyma, resulting in low transformation efficiencies. To overcome this challenge, experimental protocols with protoplasts have been developed[8]. Transient gene expression has also been used in stable transformation experiments involving the bombardment of explants[14] or shoot apices[9,14]. Protoplasts can be transformed by direct gene transfer, bombardment or *Agrobacteria* infection with rather good efficiencies, as assayed by expression of the reporter genes *GUS, GFP* and/or *NPTII*. Obtaining a good preparation of protoplasts, however, is not simple and requires highly sterile conditions and special handling. On the other hand, bombardment requires costly equipment that is not always available.

These experimental difficulties in transforming sunflower cells, either stably or transiently, have limited the knowledge of this crop's genomics and metabolism. The molecular studies that have been performed until now have been carried out by analysis of gene or protein expression/activity in sunflower plants[22–24], stable transformation of a heterologous system such as *Arabidopsis*[25–31], or transient transformation of leaves of the heterologous *Nicotiana benthamiana* using *Agrobacterium tumefaciens*. Recently, genetic studies were carried out with cDNA-amplified fragment length polymorphism[32–34] and genomic scans to select candidate genes associated with quantitative trait loci (QTL)[35]. These approaches, as well as microarray analyses, were carried out using available expressed sequence tag (EST) information and have provided valuable data for improving cultivated sunflower crops[32–34]. However, each of these approaches has serious limitations, an analysis of gene expression or protein activity only provides information on the relative abundance of that individual gene or protein. The analysis of stable or transient heterologous transformants requires conservation of the biochemical mechanisms, which is difficult to validate. Thus, any experimental results that are obtained from a heterologous species cannot necessarily be applied to the sunflower. Furthermore, ectopic expression can generate artificial and complex phenotypes that have no biological relevance. In addition, loss of function phenotypes cannot be studied using heterologous transformation. Unfortunately, no sunflower mutant libraries are available for this kind of analysis either.

Here, we describe a simple and rapid protocol for the efficient transient transformation of sunflower leaf discs. This protocol can be easily carried out in any ordinarily equipped laboratory. Briefly, sunflower leaf-discs can be simply obtained by cutting healthy leaves with a hole puncher and then transfected with *Agrobacteria* previously transformed with a suitable construct bearing the gene of interest or a small interfering RNA (siRNA). The key that makes this technique highly efficient is the application/disrupting of vacuum to the discs immersed in the bacteria suspension in a simple dessicator. The success of the infiltration can be monitored, depending on the lab equipment, by fluorescence of green fluorescent protein (GFP) or absorbance of the GUS product in control discs transformed in parallel with suitable constructs. This technique was successfully tested in our laboratory and found to generate strong transient expressions of sunflower transcripts and proteins[28–31].

This technique will allow us to obtain new molecular information about the signal transduction pathways that exist in this crop. We have also used this protocol to induce RNA silencing in sunflower leaves by introducing inverted-repeated sequences that lead to the formation of siRNAs corresponding to selected target genes[28–31]. With this method we were able to study the pathways that are regulated by several sunflower transcription factors, such as HAHB4, a member of the HD-Zip family. We were able to both overexpress and inhibit the expression of this transcription factor and others. Target gene expression levels were measured in the transformed tissue and correlated with the data obtained in stably transformed *Arabidopsis thaliana* plants[28–30]. In addition, the crosstalk between hormone-regulated pathways involving HAHB4 could be successfully elucidated[30]. We were also able to characterize the *cis*-acting elements that are present in the transcription factor promoter regions by carrying out experiments both under normal conditions and during biotic or abiotic stress[30,31].

The protocol described here is potentially suitable for a wide variety of molecular studies, including gene regulation, protein localization, tagged protein expression, chromatin immunoprecipitation, protein–protein interactions and protein stability. Considering its simplicity, we suggest that this protocol with slight modifications might be used to transform other species that present similar challenges to transformation. The main difference between this protocol and previously informed ones for other species[19], that makes it suitable for the sunflower (and other plants presenting difficulties in infiltration), is that the excision of leaf discs combined with vacuum application leads to an efficient infiltration, which could not be achieved by the direct infiltration of leaves or other tissues. It is important to note that appropriate controls are required to ensure that changes observed in the transcriptome, proteome or metabolome are due to the introduced transgene and not by artifacts from the methodology. In addition, in the quantification of hormones or metabolites involved in the plant stress response, appropriate controls must be included and literature data on the normal levels of these compounds should be consulted before interpreting the results.

An additional limitation of this protocol, shared with others for transient transformation, is that only specific tissues/organs in a given developmental stage are suitable (in this case, leaf discs). This fact leads to the need of a careful interpretation of the results, as many transcripts, proteins or metabolites may not be naturally present in such tissues/organs. Misinterpretations due to the non physiological condition of the excised discs can be avoided by choosing appropriate controls, but conclusions from transient transformation must be adequately examined regarding the normal expression of the tested molecules in the tissue used and directly discarded if the changing molecules are not normally expressed. For example, a transcript/protein specifically expressed in roots and not in other organs should not be tested with this protocol. Although these inconveniences exist, this technology is unique in its ability to produce efficient transient expression or silencing of genes/proteins in sunflower leaves. Thus, we believe it will be a fundamental tool for molecular analyses in this crop.

## Experimental design

**Choice of appropriate vectors.** Any plasmid that can replicate in *A. tummefaciens* and encodes a cDNA or a gene controlled by a plant-recognised promoter/terminator can be used in this technique (e.g., the CaMV 35S promoter/OCS terminator). If a promoter that has not already been tested in sunflower leaves is used, we recommend including a control construct that bears this promoter directing the expression of a known reporter gene. To knock out a given gene, antisense, siRNAs or artificial micro-RNAs (amiRNA) can be used[36]. We strongly recommend the use of siRNAs (inverted-loop-repeat sequence) to achieve gene silencing as siRNAs have been shown to spread across 10–15 cells through plasmodesmal channels[18] and induce silencing in untransformed cells. Transformation efficiencies can be quantified by measuring a constitutively expressed antibiotic resistant gene, which is included in the selected plasmid. This reporter is especially useful for experiments designed to measure transcript levels by qPCR.

**Design of controls.** To assess the efficiency of the assay, positive controls should be included with *Agrobacterium* cells transformed with a construct bearing a proven strong promoter and a reporter gene. In our experience, cells that were transformed with a plasmid encoding the

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*GUS* or a triple Yellow Fluorescent Protein (YFP) plus a nuclear localization signal (NLS) driven by the 35S CaMV promoter were found to be appropriate[28–31]. In addition, this positive control is useful for discarding artifacts that are produced by the transformation itself. For the same purpose, it is useful to include *Agrobacteria* cells transformed with an empty vector as an additional control. It is also recommended that each control and each experiment be carried out with at least six discs.

**Overexpression experiments.** When carrying out transformations for the overexpression of a given gene, it is highly recommended that *Agrobacterium* cells containing a plasmid encoding a silencing suppressor be included in the transformation suspension (Step 5). In our experience, the *Tomato Bushy Stunt Virus* P19 silencing suppressor[37] has proven to be appropriate for this task.

## MATERIALS

### REAGENTS

▲ **CRITICAL** All stock solutions must be prepared with sterilized dd$H_2O$ in autoclaved tubes or flasks.

- Sunflower seeds (Advanta, cat. no. CF33 cv.)
- $MgCl_2$ (Sigma, cat. no. M8266)
- 70% (vol/vol) Ethanol (Merck, 1085430250) ! **CAUTION** Ethanol is flammable. Store the solution at 4 °C.
- 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid, (X-Gluc) (Fermentas, cat. no. R0852)
- $NaH_2PO_4$ (Sigma, cat. no. S8282)
- $Na_2HPO_4$ (Sigma, cat. no. S7907)
- *N*, *N*′-Dimethyl formamide (Sigma, cat. no. D4551) double-distilled water (dd$H_2O$). Once sterilized by autoclaving, store at room temperature (21–23 °C)
- TRIzol reagent (Invitrogen, cat. no. 15596018) ! **CAUTION** This reagent is toxic and causes burns. Avoid skin contact and ingestion.
- Tryptone
- Yeast extract
- NaCl
- Rifampicin (Sigma, R3501)
- Streptomycin (Sigma, cat. no. S6501)
- Kanamycin (for pBI series)
- Acetosiringone (Roth, cat. no. 6003.1). Store at 4 °C
- Silwet L-77 (Lehle Seeds, cat. no. VIS-02)
- KCl
- $KH_2PO_4$
- Cefotaxime (Sigma, cat. no. C7039). Store at −20 °C
- MS medium (Sigma, cat. no. M5524). Once sterilized by autoclaving, store at room temperature until use
- Triton X-100 (Sigma, cat. no. T8787)
- Dimethyl sulfoxide (Sigma, cat. no. D4540)
- Plasmids: pBI121, accession no. AF485783 (ABRC stock centre); pHannibal, accession no. AJ311872 (CSIRO Plant Industry); and pGreenII, accession no. EF590266 (ABRC stock centre)
- Soil: a soil mixture for horticultural use that is commercially available and well drained. Adjust the major nutrients by adding a commercial fertilizer
- *Agrobacterium* strain LBA 4404 or others like GV 310, EHA 3105 can be used[38,39]

### EQUIPMENT

- Standard desiccator connected to a water vacuum pump
- Orbital thermostatized and non-thermostatized shakers
- 250 ml Erlenmeyer
- 27 cm × 27 cm or larger soil pots for sunflower culture
- Epiuorescence or confocal microscope
- 37 °C incubator. An ordinary 37 °C oven could be suitable
- Growth chamber or greenhouse suitable for long or short day conditions
- Laminar flow hood
- 50 ml conical polypropylene tubes (BD Biosciences or similar)
- Sharp 11 mm hole puncher
- Cover glass

### REAGENT SETUP

**Plant material and growth conditions** *Helianthus annuus* L. are grown in a growth chamber at 26–28 °C under long-day photoperiods (16 h of illumination generated with a mixture of cool-white and GroLux fluorescent lamps) at an intensity of ~150 μE $m^{-2}$ $s^{-1}$ in 26 cm diameter × 28 cm height soil pots. Two- to three-week-old plants are used in the transformation assays. The plants must be healthy and well maintained. Leaves that are harvested from plants grown under inappropriate conditions lead to poor transformation efficiencies. As in other infiltration protocols, young expanded leaves prove to be better material[40] (**Fig. 1a**).

**Bacteria culture** *Agrobacterium tumefaciens* strain LBA4404 is used, wild type or transformed, with the plasmids mentioned above. Be sure to have the appropriate *Agrobacteria* cultures before starting the plant transformation protocol. The addition of acetosyringone ensures that a highly infectious *Agrobacterium* culture and high transformation efficiencies are achieved. Acetosyringone is a natural metabolite that is released when the plant is wounded. The external addition of this reagent facilitates T-DNA insertion into the plant cell. ▲ **CRITICAL** To allow time for the *Agrobacteria* transformation, clone selection, storage of culture stocks, plating and growth of transformed and wild type lines, it is necessary to start the experiment 1 week before starting the plant transformation protocol. If the transformed *Agrobacteria* is already available, 2 days should be a sufficient amount of time to grow the cultures.

*Agrobacterium* selection markers: for LBA4404 strain, 50 μg $ml^{-1}$ rifampicin plus 100 μg $ml^{-1}$ streptomycin. Also add the specific antibiotic for the selected plasmid (e.g., 100 μg $ml^{-1}$ kanamycin for pBI series). Store the solutions at −20 °C and defrost them just before use.

**Transformed *Agrobacteria*** Transformation of *Agrobacteria* can be carried out according to the electroporation procedure described in the Biorad Technical Bulletin. ▲ **CRITICAL** Preparation of competent cells takes 2 days, and additional 2 days are needed to visualize the transformants. *Agrobacteria* must be grown at 28 °C. ■ **PAUSE POINT** Once transformed, bacterial stocks can be stored for long periods at −80 °C in 50% (vol/vol) glycerol or on petri dishes (LB-agar–antibiotics) at 4 °C for 1 month.

**X-Gluc solution** Prepare 100 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in *N*,*N*′-dimethyl formamide. ▲ **CRITICAL** Store this solution at −20 °C in the dark and discard it if it turns slightly pink. ! **CAUTION** Dimethyl formamide is toxic and should be stored and handled in a chemical hood.

**β-Glucuronidase assay buffer** Prepare 100 mM phosphate buffer (19.5 ml 0.2 M $NaH_2PO_4$ and 30.5 ml 0.2 M $Na_2HPO_4$ 100 $ml^{-1}$ buffer) adjusted to pH 7.0 with KOH. Just before use, add 0.1% (vol/vol) Triton X-100. ▲ **CRITICAL** Phosphate buffer can be stored indefinitely at room temperature (20–25 °C). Solution containing 0.1% Triton X-100 needs to be prepared fresh for each experiment.

**GUS assay substrate** Immediately before use, add to the assay buffer enough X-Gluc solution (100 mM) to make a solution of 1 mM final concentration.

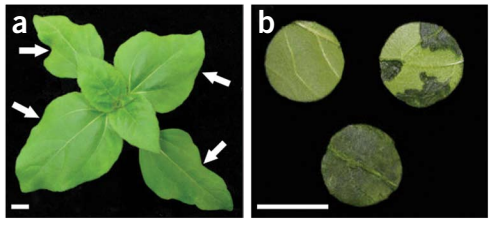


**Figure 1** | Plant material used for transient transformation. (**a**) A 3-week-old healthy sunflower plant that has developed in a suitable manner, grown as described in Step 1. The arrows indicate the leaves that were used to obtain discs in Step 7. (**b**) Abaxial side of the infiltrated sunflower discs after *Agrobacterium* transformation in Step 10. Upper left disc: uninfiltrated disc. Upper right disc: an improperly infiltrated disc. Lower disc: a fully infiltrated disc. Scale bar represents 10 mm.

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**100 mM acetosyringone** Dissolve 392.4 mg acetosyringone in 10 ml of dimethyl sulfoxide and then mix it with 10 ml of distilled water. Filter-sterilize the final solution and store in the dark at −20 °C for up to one year.
**Infiltration buffer** Prepare a solution containing 10 mM MgCl. Sterilize this solution by autoclaving and store it indefinitely at room temperature. Just before use add acetosyringone to a final concentration of 100 μM.

Liquid LB medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter, adjusted to pH 7.5). Once sterilized by autoclaving, store at room temperature for several months.
**Murashige and Skoog (MS) medium** Dissolve 4.4 g of MS salts per liter, pH 5.7; sterilize by autoclaving and store it at 4 °C for several months.

**Oligonucleotides design for transcript quantification by qRT-PCR** The primers used to carry out the qRT-PCR of putative target genes were designed using the online Primer3 software (http://fokker.wi.mit.edu/primer3/input-040.htm). These primers must be 18–20 bp long with a melting temperature between 57 and 62 °C; they must amplify a DNA segment that is between 90–150 bp. We recommend designing them to match a segment near the 3′-untranslated region. The ESTs used to design the primers were obtained from the TGI databases (http://compbio.dfci.harvard.edu/tgi/). Oligonucleotides targeting the Actin 2/8 genes were used as reference genes (HA–ACTF: 5′-GGTAACATCGTGCTCAGTGGTGG-3′; HA–ACTR: 5′-AACCACCTTGATCTTCATGCTGC-3′).

## PROCEDURE

### Plant growth ● TIMING 2–3 weeks

**1|** Grow sunflower plants, one seed per pot, in a controlled growth chamber or greenhouse under a long-day photoperiod (16-h light and 8-h dark at 25 °C) with an illumination of 130–150 μE $m^{-2}$ $s^{-1}$ and 40–65% relative humidity for 2–3 weeks.
▲ CRITICAL STEP Be sure to use young healthy leaves for the transformation (**Fig. 1a**). Alternatively, plants can be grown under short photoperiods (8-h light, 16-h dark) to obtain larger leaves.

### Growth of *Agrobacterium* strain and leaf disc transformation ● TIMING 6 d

**2|** Inoculate a single colony of the *Agrobacterium* strain harboring the gene of interest in a binary vector into 5 ml of liquid LB medium containing the appropriate selection antibiotics. Incubate the culture for 16 h at 28 °C with vigorous shaking (180–200 rpm).

**3|** Use 0.5 ml of the culture from Step 2 to inoculate 50 ml of LB supplemented with the appropriate antibiotics plus 5-μM acetosyringone. Incubate for 16 h at 28 °C with vigorous shaking.
▲ CRITICAL STEP The addition of acetosyringone in this step as well as in Step 5 is crucial to achieving a highly infectious *Agrobacterium* culture and high transformation efficiencies.

**4|** Collect the bacteria by centrifuging the culture in pre-sterilized polypropylene tubes at 2,000*g* for 10 min at room temperature. Discard the supernatant.

**5|** Gently resuspend the cells in 10 ml of infiltration buffer. Once resuspended, use them to prepare 50 ml of a 0.5 OD (600 nm) bacterial suspension in infiltration buffer. Incubate the culture for 4–6 h at room temperature with gentle shaking. If the experiment involves the overexpression of a gene, add to the same tube 10% vol/vol of a 0.5 OD bacterial suspension containing a p19-encoding plasmid prepared in a separate tube at the same time. The bacteria transformed with a p19-encoding plasmid suppress the silencing of the overexpressed gene. The encoded protein captures 21–25 mer small RNAs, inhibiting their silencing function.

**6|** Add 10 μl $liter^{-1}$ Silwet L-77 to each bacterial suspension.
▲ CRITICAL STEP The addition of Silwet L-77 facilitates the infiltration process, giving rise to a higher number of fully infiltrated discs.

**7|** Obtain sunflower leaf discs by cutting the tissue with a sharp 11 mm hole puncher. Care must be taken to avoid the central vascular tissue to ensure that homogeneous leaf discs are obtained. Immediately after cutting, immerse the discs in 45 ml of bacterial suspension contained in a 50-ml polypropylene tube.
▲ CRITICAL STEP Infiltrate at least six times more discs than will be used in the experiment in order to allow for the selection of the best infiltrated discs (**Fig. 1b**). Some discs may get damaged during the procedure or fail to be infiltrated; we recommend discarding these discs to ensure a successful experiment, as cutting discs is relatively quick and does not require expensive materials.

**8|** Place the tubes containing the discs in the desiccator and apply vacuum for 1 h.
▲ CRITICAL STEP To improve infiltration efficiency, abruptly break the vacuum every 15 min by quickly unplugging the vacuum source from the desiccator. If the infiltration is working, the tissue will change color to a darker green (**Fig. 1b**).
▲ CRITICAL STEP Be sure to place the polypropylene tubes in a heavy rack to prevent them from falling when the vacuum is abruptly broken.

**9|** Break the vacuum and allow the leaf discs to sit in contact with the bacterial suspension for an additional 20 min.

**10|** Wash the discs six times with the PBS solution, manually applying vigorous shaking to remove excess *Agrobacterium*.

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**11|** Select the best infiltrated discs by visual inspection and transfer them to a 250-ml Erlenmeyer flask containing 50 ml of MS. See **Figure 1** for guidance: well infiltrated discs are darker on their surface and clearly distinguishable from those that are not well infiltrated.

**12|** Incubate for 24 h in a long-day growth chamber with gentle agitation (120 rpm) at 23 °C.

**13|** Replace the medium with fresh MS supplemented with 250 μg $ml^{-1}$ cefotaxime and incubate for 2 extra days. Cefotaxime is an antibiotic that kills *Agrobaterium*, diminishing the background that could be caused by these cells. It does not affect the plant cells.

**14|** Wash the discs twice with $ddH_2O$.

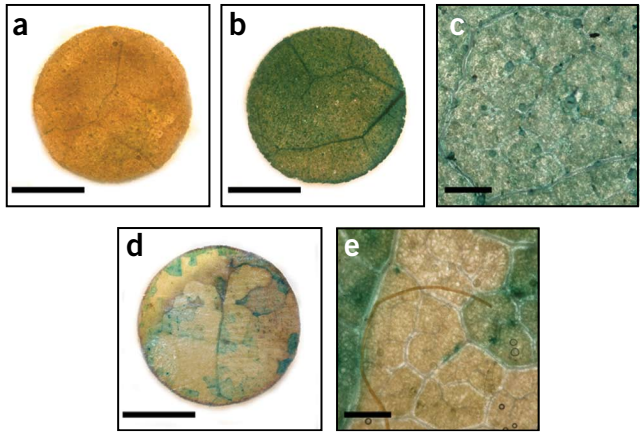


**Figure 2 |** β-Glucuronidase (GUS) expression in transformed sunflower leaf discs. GUS staining of sunflower discs transformed with: empty vector (**a**), *35S:GUS* (**b**,**c**) or *35S:GUS* not properly infiltrated (**d**,**e**). Note: GUS staining is only visualized in the infiltrated areas. Black scale bars represent 5 mm (**a**,**b**,**d**) or 250 μm (**c**,**e**).

**15|** Proceed in one of the following ways, depending on the goal of the experiment. Follow options A and B to test the transformation efficiency using reporter genes: (A) for *GUS* and (B) for *GFP*. Option C should be followed if the purpose of the experiment is to determine the transcript levels of the selected genes. Option D should be followed if the aim of the experiment is to test the activity of a promoter.

**(A) Testing *GUS* reporter activity to evaluate transformation efficiency** ● **TIMING 2 d**

(i) Submerge the discs in the GUS assay substrate.

(ii) Apply vacuum for 30 min.

(iii) Incubate at 37 °C in the dark (37 °C oven) until the blue color of the *GUS* product appears (usually, overnight).

(iv) Transfer the leaf discs to a tube containing absolute ethanol to stop the reaction. Keep the tissue in ethanol until it loses the chlorophyll green color.

■ **PAUSE POINT** Leaf discs can be stored at 4 °C in absolute ethanol for several weeks before observation under a microscope or dissecting microscope (**Fig. 2**).

**? TROUBLESHOOTING**

(v) For observation by microscopy, mount each leaf disc with a drop of water and cover with a cover glass. Observe the tissue immediately to avoid excessive stress due to mounting. The color signals derived from the expression of the tested protein will be visible in the transformed area.

**(B) Testing fluorescent reporter activity to evaluate transformation efficiency** ● **TIMING 3 h**

(i) Mount the tissue on a slide glass with a water drop and cover it with a cover glass.

(ii) Immediately observe the mounted tissue in an epiuorescence or confocal microscope to avoid dehydration of the sample. The uorescence signal will be observable in transformed cells. If *YFP:NLS* is used, the signal is detected in the nucleus. See **Figure 3**.

**? TROUBLESHOOTING**

**(C) Determination of transcript levels in the transformed tissue** ● **TIMING 2–3 d**

(i) If the experiment involves a hormone treatment, transfer the discs that are obtained in Step 14 to fresh MS containing the selected chemical compound/hormone and incubate them with gentle agitation for the chosen period/s of time under long-day conditions in a growth chamber.

(ii) Harvest the discs individually and freeze them immediately with liquid nitrogen.

■ **PAUSE POINT** Once frozen, the samples can be stored for several months at −80 °C until RNA extraction.

(iii) Extract the RNA using the TRIzol technique following the manufacturer's instructions.

■ **PAUSE POINT** RNA can be stored at −80 °C in 70% vol/vol ethanol for several months.

(iv) Check RNA concentration with a UV spectrophotometer at 260/280 nm and integrity by electrophoresis in agarose.

(v) Quantify transcript levels using a northern blot or qRT-PCR (see refs. 41 and 42 for general guidelines).

**? TROUBLESHOOTING**

**(D) Measurement of promoter activity** ● **TIMING 2 d**

(i) Apply the desired stimulus to the leaf discs (i.e., light/dark, hormones, abiotic or biotic stress) for the selected period of time.

(ii) Follow Step 15, (options A or B) depending on the selected reporter gene, to evaluate the promoter activity.

**? TROUBLESHOOTING**

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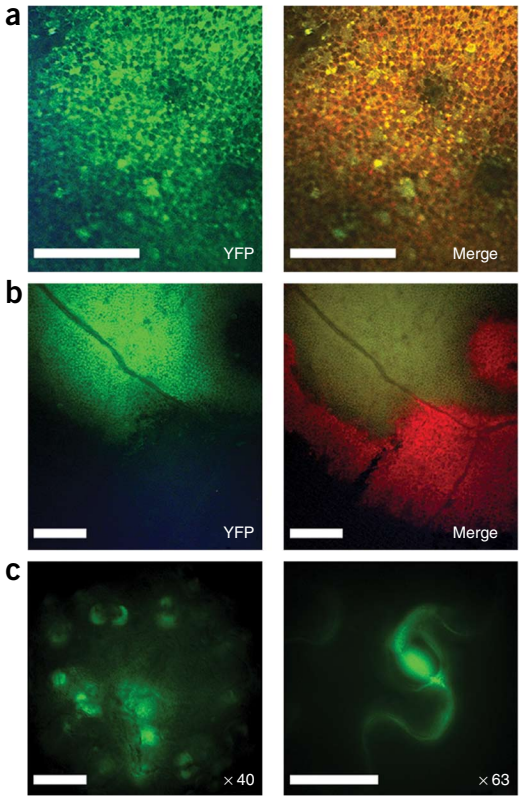


**Figure 3** | YFP expression in transformed sunflower leaf discs. (**a**,**b**) YFP fluorescence of sunflower discs that had been fully infiltrated with the *YFP:NLS* construct (**a**) or partially infiltrated with the same construct (**b**). Left panels show the YFP fluorescence, whereas right panels are the merge of the YFP signal and the plastid autouorescence (red). The image was obtained with an epiuorescence dissecting scope with appropriate filters for detecting YFP and chlorophyll fluorescence. The size bar represents 1 mm. (**c**) The nuclear localization of the reporter gene can be observed in the cells located under the epidermis. The images were obtained with an epiuorescence microscope under ×40 or ×63 amplification as indicated. Leaf discs were examined for YFP expression with a Leica MZ FLIII microscope (Leica Microsystem GmbH, Wetzlar, Germany) fitted with wide- and band-pass YFP filters and a AxioCam HRc (Carl Zeiss GmbH, Jena, Germany) digital camera with AxioVison software (version 3.1; Zeiss). **a** and **b** size bar 1 mm, **c** size bar represents 250 μm.

## ● TIMING

Step 1, Growth of sunflower plants: 2–3 weeks
Steps 2–14, Growth of *Agrobacterium* and transformation: 6 d
Step 15 (options A or B), Evaluation of transformation efficiency: 2 d or 3 h
Step 15 (options C or D), Quantification of transcript levels or promoter activity: 2–3 d or 2 d

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1** | Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 15 A(iv) or B(ii) | Poor infiltration efficiency | Transformed leaves are too old | Repeat the experiment with younger and well-expanded leaves at different developmental stages. The optimal developmental stage may vary slightly depending on the genotype |
| | | Enough vacuum not applied | Use a more powerful vacuum pump. Do not forget to abruptly break the vacuum at least four times during the infiltration step |
| 15 C(v) or D(ii) | Positive controls were successful, but the gene of interest is not expressed | Incorrect promoter | Be sure that the promoter used is active in sunflower tissues (several sunflower promoters and the CaMV 35S promoter have been already tested, refs. 21–23,43) |
| | | The gene was silenced | Co-transform with a construct expressing the *Tomato Bushy Stunt Virus* P19 silencing suppressor (ref. 24) |

## ANTICIPATED RESULTS

The described protocol makes it possible to transform sunflower leaf tissue, in order to express or silence a given gene. It is efficient enough to obtain a suitable number of undamaged transformed cells for statistical analyses. Typically, it is possible to obtain ten discs from each leaf and more than 80% are well infiltrated. Under optimal conditions, a plant with two leaf pairs would yield 40 discs. When the transcript levels of the expressed gene are low, six to seven replicates are needed to obtain sufficient data; when these levels are high, three replicates are usually sufficient to obtain a *P*-value of $<0.05$ (refs. 28–31). Considering the number of controls that must be carried out, including infiltrations with bacteria expressing reporter genes and untransformed *Agrobacteria*, three plants would be needed to carry out an experiment that would evaluate the insertion

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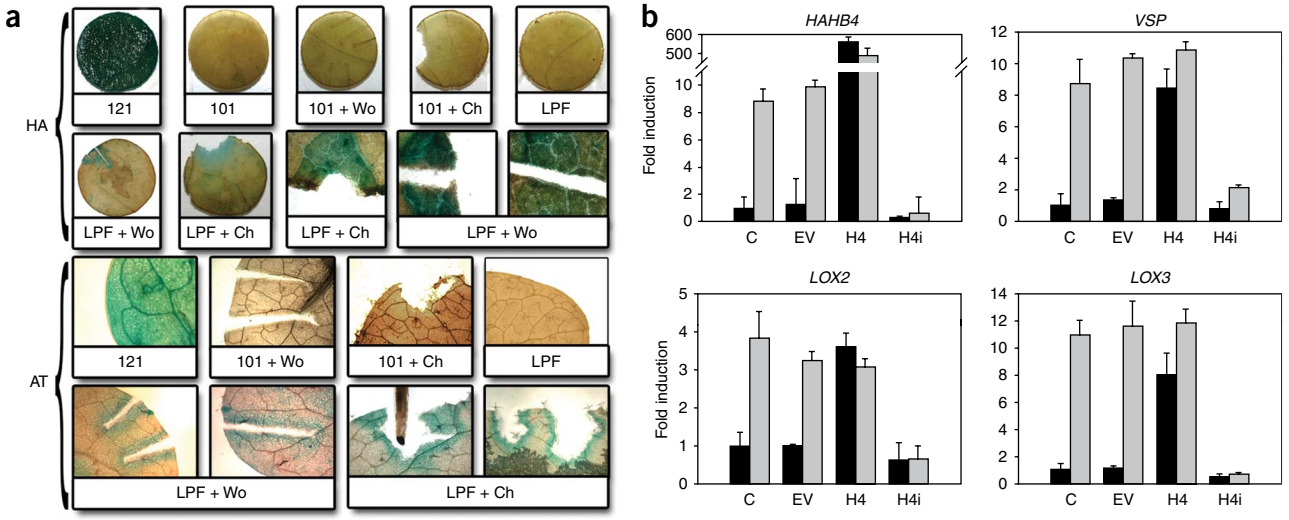


**Figure 4** | Regulation of the expression of the *HAHB4* transcription factor and its target genes visualized after sunflower discs transient transformation. β-Glucuronidase (GUS) histochemistry of transiently transformed sunflower leaf-discs (HA) or stable transformed *Arabidopsis* (AT) plants. Both species were transformed with the negative control vector pBI101 (*GUS* without promoter, 101 bp), the positive control vector pBI121 (*35S:GUS*, 121 bp) or the *HAHB4* promoter (1,221 bp) fused to the *GUS* cDNA (LPF, HAHB4 Long Promoter Form) (**a**). The leaf discs were subsequently subjected to mechanical wounding (Wo) or damage caused by chewing insects (Ch) (*Spilosoma virginica* for sunflower and *Bradysia impatiens* for *Arabidopsis*). Transcript levels of putative HAHB4 target genes in transiently transformed sunflower discs (**b**). Sunflower leaves were transformed with a $MgCl_2$ solution (C), empty vector (EV), *35S:HAHB4* (H4) or an RNAi construct silencing endogenous *HAHB4* (H4i). Transcript levels of different wound-response genes were measured by qRT-PCR after treating the plants with 200 μM methyl-jasmonate (gray bars) or placing them in control conditions (black bars). Control sunflower plants were cultured at 28 °C, control *Arabidopsis* plants at 24 °C, both were cultured in chambers under a long photoperiod of 16 h light and 8 h in darkness. *ACTIN* genes (*ACTIN2* plus *ACTIN8*) were used as an internal control. Fold change is expressed as the result of dividing the values obtained for each transcript after the different treatments by the values obtained for the same transcript in control conditions. Standard deviations were calculated from at least three independent experiments. Differences were considered significant when the *P*-values were <0.05 (Student's *t*-test). VSP: vegetative storage protein; LOX2: lipoxygenase 2; and LOX3: lipoxygenase 3. This figure was adapted and reproduced with permission from reference 30.

of a single gene. It should be noted that the amount of RNA that can be isolated from a single disc is enough to carry out qRT-PCR measurements with several oligonucleotides pairs[28–31]. This high recovery of RNA allows for at least three or more biological replicates (one from each plant) to be carried out for each treatment. We have observed that there is almost no variability between replicates of the controls with the reporter genes *GUS* or *YFP*.

This technique has been successfully used in our laboratory for two different types of experiments: evaluating the effect of overexpression/silencing sunflower transcription factors[28,30] and analyzing the activity of certain promoters under a variety of external conditions[29,31].

In the first type of experiment (transcription factor overexpression/silencing), we analyzed the expression of target genes with qRT-PCR and levels of protein products with enzymatic activity assays[31]. Additionally, we were able to establish the interaction between the signal transduction pathways in which these transcription factors are involved with those regulated by phytohormones. In this sense, the sunflower transcription factor HAHB4 was shown to have a crucial role in ethylene signaling pathways, inducing a marked senescence delay in *Arabidopsis* transgenic plants. This delay was correlated with the repression exerted by this transcription factor on genes related to ethylene synthesis (*ACO* and *SAM*) and ethylene signaling (*ERF2* and *ERF5*)[28], as well as on the sunflower homologues of these genes. Another example illustrating the reliability of this protocol in establishing relationships between signal transduction pathways related with phytohormones in which *HAHB4* is involved, is fully described in reference 30. We have demonstrated that *HAHB4* has a key role in the response to biotic stress factors. This gene was shown to upregulate the synthesis of the phytohormones jasmonic acid (JA) and ethylene (ET). Conversely, *HAHB4* decreased ET sensitivity and SA accumulation. The transient transformation of sunflower leaf discs has permitted us to validate the observations carried out in stable transformed *Arabidopsis* plants (used as a heterologous system) regarding transcript levels of putative HAHB4 target genes. In this sense we were able to determine that several genes involved in JA biosynthesis and defense-related processes were upregulated. In addition, this technique allowed us to quantify the activities of the encoded sunflower proteins lipoxygenase, hydroperoxide lyase and trypsin inhibitors (TPI) in both *HAHB4*-silenced (reduced activities) and *HAHB4*-overexpressing tissue (enhanced activities)[30]. **Figure 4** (adapted from ref. 30) illustrates these experiments; transcript levels of *VSP*, *LOX2* and *LOX3* were quantified in leaf discs transformed with an empty vector and with constructs able to overexpress or knockout *HAHB4* after treating (or not) the leaves with JA (**Fig. 4b**).

In the second type of experiment, we established the functionality of *cis*-acting elements that are present in sunflower promoters. Using either wild type or mutated promoter-*GUS* fusion constructs, we were able to quantify transcript levels

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of *GUS* and the enzymatic activity of this gene product under abiotic stresses. Promoter fusion analysis, both in stable transformed *Arabidopsis* and transiently transformed sunflowers, demonstrated that the induction of *HAHB4* expression by chewing or wounding treatments is regulated at the transcriptional level[31]. **Figure 4a**, adapted from reference 30, shows that the results obtained in both systems (heterologous and homologous) were very similar and allowed us to conclude that the regulation of this gene by biotic stresses and related hormones occurs at the transcriptional level and is conserved between these two species.

For example, we demonstrated that *HAHB4* downregulates the expression of genes encoding chlorophyll a/b-binding proteins as well as a large group of photosynthesis-related genes, as it was demonstrated[29]. In accordance, a reduction in chlorophyll a/b-binding proteins content was observed in stable transformed *Arabidopsis* plants. Moreover, *cis*-acting elements located in the *HAHB4* promoter were identified as responsible for the regulation of *HAHB4* by darkness[29].

Additional functional studies were carried out in order to demonstrate the activity of four ABREs (ABA Responsive Element) located in this promoter[31]. Site-directed mutagenesis on the isolated promoter followed by plant transformation, both *Arabidopsis* and sunflower, analysis of the transgenic plants/tissues by histochemistry and real time RT-PCR have shown results indicating that just one ABRE out of the four is responsible for ABA (Abscisic Acid), NaCl and drought regulation[31]. These reports show that transient transformation of sunflower leaf discs enables us to elucidate molecular mechanisms involving genes, enzymes, metabolites and hormones previously unknown in the sunflower.

There are alternative techniques that could be used for this type of experiment; however, no data relevant to our work have yet been generated with these alternative techniques, so we are not able to make comparisons between methods. Previously published protocols have been evaluated in terms of transformation efficiencies using reporter genes, whereas we used the present technique to carry out a functional characterization of different sunflower transcription factors and promoter *cis*-acting elements.

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