An improved method for rapid analysis of promoters using modified sonication-assisted transient assay

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Abstract

We present here a modified, sonication-assisted transient transformation assay for rapid analysis of cis-regulatory elements. We tested promoter elements from *MIR159B* locus of *Brassica juncea* by generating stable transgenic lines and compared the transcriptional activity of GUS reporter with that of the transient assay method. To obtain reliable and repeatable results, and to omit false-positive data, we optimized several parameters including sonication duration and cycle and concentration of *Agrobacterium tumefaciens* measured as optical density (O.D.) at 600 nm. To the best of our knowledge, this is the first report of promoter characterization of *MIR159B* from *Brassica juncea*, and comparative analysis of stable and transient lines. Our analysis shows that the protocol described herein allows understanding promoter activity/transcriptional control in tissues other than leaf or protoplast which have remained the mainstay for transient analysis thus far. We tested reporter gene GUS under the control of constitutive promoter, CaMV 35S, and *MIR159b* from *Brassica juncea*. We optimized the duration of sonication (5-, 10- and 15-min cycle), bacterial density (measured as O.D at 600 nm = 0.6/0.8/1.0) and Agro-infection time (5, 10, 15 min), and co-cultivation (12-, and 24-h). Sonication cycle of 10-min, followed by Agro-infection and co-cultivation with *Agrobacterium tumefaciens* with O.D. 600 nm = 0.8 and for 12 h was found to be optimum. We could successfully express reporter genes in deep-seated tissues such as floral organs and pollen grains where it was previously not possible to perform transient assay. Constitutive GUS activity was observed when reporter was placed under control of the constitutive promoter of CaMV 35S. Reporter GUS when placed under transcriptional control of *MIR159b* promoter from *Brassica juncea* showed reporter activity in floral tissues, in mature pollen grains. Comparative analysis of reporter activity from stable transgenic lines at T2 generation with that of transient assay system reveals identical to near-identical reporter activity. Transient assay could be successfully performed in tissues collected not only from *Arabidopsis thaliana*, but also from *Brassica juncea* and *Brassica nigra* to demonstrate its wide applicability. Our modified method thus has the potential of quick and rapid analysis of promoter activity and allows us to record the developmental dynamics and spatio-temporal expression pattern driven by specific promoters. Suitable modification and controls should also allow analysis of hormonal regulation and identification of *trans*-factors via DNA–protein interactions. Furthermore, this method can also be extended to study promoters under various environmental conditions that otherwise do not allow growth and complete life cycle of healthy plants and can be modified to test reporter activity in other non-model plants or plants with long life cycle.

Keywords Sonication · Promoter · Transient assay · *Brassica* · *MIR159*

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Introduction

Analysis of transcriptional regulatory mechanisms guiding gene expression is a critical first-step towards understanding gene regulatory network (Gilbert 2002; Harmer 2009). Spatio-temporal pattern associated with transcriptional regulation can be analysed when promoter sequences associated with the gene/s of interest are analysed using either stable lines generated via promoter:reporter fusions, or using transient assay. Additional detailed characterization

of molecular components of transcriptional regulation are revealed when *trans* factor proteins bound to specific DNA motifs found on promoters are identified using Yeast One-Hybrid (Ouwerkerk and Meijer 2001) or Chip-CHiP (Ren 2000), as has been demonstrated in *Arabidopsis thaliana* (De Smet et al. 2013) and rice (Kuijt et al. 2014). However, prior to investing time and effort in generating stable transgenic lines, it is often worthwhile to test promoter function using a suitable transient assay system such as in *Nicotiana benthamiana* leaf system (Leuzinger et al. 2013), *Arabidopsis thaliana* seedlings through co-cultivation (Li et al. 2009; Wu et al. 2014), *Arabidopsis* leaf protoplast (Abel and Theologis 1994), *Arabidopsis* roots through hydroponics (Levy et al. 2005), *Agro*-infiltration through electroporation or PEG (Hauptmann et al. 1987; Yoo et al. 2007; Lee and Yang 2006), liposome-mediated direct DNA transfer to protoplast (Caboche 1990), biolistic gun-mediated DNA transfer to the plant tissue (Morikawa et al. 1989), and biolistic transformation of *Arabidopsis* leaves (Ueki et al. 2008). Few researchers have also reported sonication-mediated introduction of DNA into protoplasts to perform transient assay (Joersbo and Brunstedt 1992). All these methods rely on performing transient assay on a select tissue such as leaf or protoplast and thus none of these methods recapitulates the true cellular or tissue-specific conditions that are necessary for understanding the accurate spatio-temporal gene expression pattern driven by *cis*-regulatory elements such as promoters. Most of the strategies outlined above for transient analysis, thus, at best address the feasibility of testing whether a specific DNA segment is capable of acting as a promoter without any information on spatio-temporal expression dynamics of promoters (Yang et al. 2000).

Transient assays described in the literature thus far suffer from two major drawbacks that are: (1) the difficulty in the introduction of recombinant plasmid either as naked DNA during the biolistic method, PEG mediated or via *Agrobacterium tumefaciens* in *Agro*-infiltration; and (2) the difficulty associated with tissue handling prevents transient assay being performed in materials other than protoplast or leaf. For instance, the present delivery methods do not allow efficient transfer of plasmid DNA into small plant tissues or into tissue that is deep seated, such as emerging floral buds or pollen grains enclosed within anther locule or ovule present within the gynoecium.

MIR159 is a three-member gene family in *Arabidopsis thaliana* (*MIR159a*, *MIR159b* and *MIR159c*) and has undergone expansion in *Brassica juncea* owing to *Brassica* lineage-specific triplication and the allotetraploid nature of *Brassica juncea* (Kagale et al. 2014; Jain and Das 2016). However, *MIR159b* is present as one copy in both *A. thaliana* and *B. juncea* permitting comparative analysis between orthologs. Previous analysis has focussed on understanding the role of *MIR159b* from *A. thaliana* (Allen et al. 2010)

and no report exists for *B. juncea*. Analysis of *MIR159b* was thus undertaken to understand the role of *MIR159b* promoter from *B. juncea* in transcriptional regulation during development and to compare its transcriptional activity with that of previously reported *A. thaliana*. To improve the reliability of transient assay for analysis of promoter elements and to develop a rapid method for promoter analysis so as to obtain an accurate or near-accurate spatio-temporal expression pattern, we tested sonication-assisted introduction of a full-length and deletion series of *B. juncea*-*MIR159b*-promoter:reporter transcriptional fusion construct harboured in *A. tumefaciens*, into various plant parts. We also generated stable transgenic lines of *Arabidopsis thaliana* with promoter associated with *MIR159B* from *Brassica juncea* and collected spatio-temporal reporter activity. This, to the best of our information, is the first report of analysis of promoter activity of *MIR159B* from *Brassica juncea*. A comparative analysis of reporter activity between stable transgenic lines and the transient assay system reveals that the modified transient assay method recapitulates the native expression pattern obtained with stable transgenic lines generated using *B. juncea*-*MIR159B* promoter:reporter transcriptional fusion constructs. This improved method, thus, allows us to record the developmental dynamics and spatio-temporal expression pattern driven by specific promoters, and in future should also allow analysis of hormonal regulation and identification of *trans*-factors via DNA–protein interactions. Furthermore, this method can also be extended to study the promoters under various abiotic and biotic stresses and hormonal regimes, and plants with long life cycle. The applicability of this method needs to be tested in a variety of other non-model plants.

Materials and methods

A 1.5 kb sequence of promoter associated with *MIR159B* from *Arabidopsis thaliana* Col-0 (TAIR version 10) was used to perform BLASTN analysis against *Brassica rapa* chromosome version 1.5 at BRAD (Cheng et al. 2011) using default parameters (subject = *B. rapa* chromosome v 1.5; low complexity filter = on; expect = 10; description = 100; alignment = 50). Subject sequence with high scoring pair (HSP) value was retrieved from *Brassica rapa* chromosome version 1.5 database. Locus-specific primer pair was designed so as to amplify the 1 kb and 0.6 kb sequences from *Brassica juncea* var. Varuna.

PCR was performed using genomic DNA of *B. juncea* var. Varuna isolated from juvenile leaves using previously described protocol (Kumari et al. 2013; Kusumanjali et al. 2012). A 1 kb fragment of the putative promoter was amplified using the primer pairs 5'-GGTCTGGATGGTCTGCGAAA-3' (fwd) with 5'-CAAAACAAGAGACAACATA

GGATC-3' (rev); and as a 5'- nested 0.6 kb fragment using 5'-ATCCATCCTTCAATCCTGACC-3' (fwd) with 5'-CAA AACAAGAGACAAGTAGGATC-3' (rev). PCR was carried out using first a denaturation phase of 94 °C for 4 min, then 30 cycles at 94 °C for 45 s, 58 °C for 30 s, and 72 °C for 1 min with a final 10 min extension step at 72 °C. The amplicons (1000 and 600 bp) were separated on 1.0% agarose gel, excised and fragment purified using commercially available GeneAid™ PCR/Gel purification kit following the manufacturer's recommendation and standard practices (Sambrook and Russell 2001; GeneAid™, Taiwan). The amplicons of 1.0 and 0.6 kb were first cloned in pGEM-T-Easy (Promega, USA) using the manufacturer's recommendation and as per our published protocol (Kumari et al. 2013), and plasmids from three independent colonies were sequenced for confirmation (Sambrook and Russell 2001).

Binary plasmid construction

Full-length (1.0 kb) and a 5'-nested deletion (0.6 kb) of the putative promoter from *B. juncea* MIR159B was cloned upstream of the GUS reporter gene at *Sma*I site in pCAMBIA1391Z backbone (www.cambia.org) using standard procedure (Sambrook and Russell 2001). The recombinant binary plasmid was transformed first into chemically competent *E. coli* DH5α; their orientation of the insert was checked through PCR employing primer pair of promoter forward (as mentioned above) in combination with 5'-GGTGTAGAGCATTACGCTGC-3' (GUS reverse) and selected clones were validated through sequencing. The binary plasmid pCambia 1304, wherein the GUS reporter is driven by 35S-CaMV promoter was used for standardization. The plasmid was isolated from sequence-verified clones and then introduced into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method; bacterial transformants were selected on LB medium supplemented with rifampicin (25 µg/ml), gentamicin (25 µg/ml) and kanamycin (50 µg/ml) as per standard procedure (Sambrook and Russell 2001).

Plant growth and transformation

Arabidopsis thaliana Col-0 seeds were surface sterilized in seed sterilization solution (95% ethanol, with 0.05% Triton X-100, final concentration) before they were sprinkled on a plate containing the Murashige–Skoog (MS) media supplemented with 0.8% agar, 3% sucrose, and at pH 5.8 prepared as per the manufacturer's recommendation (Duchefa Biochemie). Seeds were first kept for stratification at 4 °C for 48 h and then allowed to germinate in a 16 h light/8 h dark cycle at 22 °C. One-week-old seedlings were transferred to soil:soilrite mix and plants were allowed to grow at 16 h

light/8 h dark cycle at 22 °C till seed maturation. *Arabidopsis thaliana* plants were transformed using the protocol of Clough and Bent (1998) using the floral dip method. Seeds of transgenic plants were screened on MS+ agar supplemented with hygromycin (15 mg/L).

Unopened floral buds, open flower, leaf, stem and root of mature *Brassica juncea* and *B. nigra* field-grown plants (at flowering stage), and cotyledonary leaves of 6-day old seedling germinated under culture conditions were collected in 0.5 ml Murashige–Skoog (MS) liquid medium containing 3% sucrose and used for sonication experiments.

Sonication and co-cultivation

Ultrasonic Water Bath (Grant Scientific) with frequency of 50–60 Hz was used for sonication treatment. Various plant tissues such as inflorescence, siliques, leaf and roots of *Arabidopsis thaliana* Col-0 were harvested and immersed in 0.5 ml Murashige–Skoog (MS) liquid medium containing 3% sucrose in a sterile 2.0 ml Eppendorf tube. The Eppendorf tube containing the material was subjected to sonication cycles. Each sonication treatment cycle consisted of three phases of “sonication–rest–sonication”, with each phase being of equal duration. Sonicated tissues were then immediately transferred to an overnight *Agrobacterium tumefaciens* culture having the required O.D for co-cultivation (12 or 24 h) in the dark. After co-cultivation, the tissue was processed for GUS expression analysis.

GUS histochemical assay

Histochemical assays for GUS activity was performed as described by Jefferson et al. (1987). 50 ml 1 M sodium phosphate buffer (pH 7.0) containing 50 mg X-Gluc, 50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 50 µl Triton X-100 was used as the GUS staining buffer. Tissues were incubated in GUS staining buffer at 37 °C, in the dark for 12 h. Chlorophyll was removed from tissue by destaining with a combination of glacial acetic acid and ethanol mixed in a 1:1 ratio. Tissues were imaged under Carl-Zeiss Stemi 305 Stereo microscope and Carl-Zeiss Scope A1 microscope (Carl Zeiss, Germany).

Result

To develop sonication-assisted *Agro*-infiltration as a reliable method for transient expression in intact tissue, we standardized parameters including duration of sonication, bacterial cell density and co-cultivation time. All standardizations were performed using GUS reporter driven by

35S-CaMV promoter in a pCambia1304 vector (Figs. 1, 2, 3) and the effectiveness of this method was tested on a range of tissues which are not commonly used to perform the transient assay, i.e. mature flower, root and silique; and leaf from *Arabidopsis thaliana*, *Brassica juncea* and *Brassica nigra* (Figs. 4a–c, 5; Supplementary Fig. 2).

Effect of sonication

As sonication involves agitation using high-energy sound wave, it was important to identify the duration for which sonication treatment can be given without causing extensive physical damage to the plant parts. All sonication treatments were given at 50–60 Hz cycle. Each treatment

Fig. 1 Sonication-assisted transient expression analysis of CaMV35S promoter:GUS reporter. Leaf and inflorescence of *Arabidopsis thaliana* Col-0 was subjected to a sonication–rest–sonication cycle of 5 min with different bacterial densities and co-cultivated for different

time periods: O.D. 0.6 and 12 h (a and b) or 24 h (g and h); O.D. 0.8 and 12 h (c and d) or 24 h (i and j); and O.D. 1.0 with 12 h (e and f) or 24 h (k and j). Scale bar represents 200 μ m

Fig. 2 Sonication-assisted transient expression analysis of CaMV35S promoter:GUS reporter. Leaf and inflorescence of *Arabidopsis thaliana* Col-0 was subjected to sonication–rest–sonication cycle of 10 min with different bacterial densities and co-cultivated for differ-

ent time periods: O.D. 0.6 and 12 h (**a** and **b**) or 24 h (**g** and **h**); O.D. 0.8 and 12 h (**c** and **d**) or 24 h (**i** and **j**); and O.D. 1.0 with 12 h (**e** and **f**) or 24 h (**k** and **j**). Scale bar represents 200 μ m

cycle consisted of three phases of “sonication–rest–sonication” with each phase of equal duration. Four different sonication durations were tested and “sonication–rest–sonication” phases were given for 1, 5, 10 min or 15 min (for example: 5:5:5 refers to sonication for 5 min, followed by 5 min rest and then 5 min of sonication) before co-cultivation followed by GUS expression analysis.

The duration of 1 min for sonication was found to be short because only small spots of GUS expression at random places could be observed (data not shown); sonication–rest–sonication cycle of 5 min also gave non-uniform, random GUS reporter expression in a limited area (Fig. 1 (0.6a and b); (0.8c and d); (1.0e and f). At the other extreme, a sonication–rest–sonication cycle of 15 min each

Fig. 3 Sonication-assisted transient expression analysis of CaMV35S promoter:GUS reporter. Leaf and inflorescence of *Arabidopsis thaliana* Col-0 was subjected to sonication–rest–sonication cycle of 15 min with different bacterial densities and co-cultivated for differ-

ent time periods: O.D. 0.6 and 12 h (**a** and **b**) or 24 h (**g** and **h**); O.D. 0.8 and 12 h (**c** and **d**) or 24 h (**i** and **j**); and O.D. 1.0 with 12 h (**e** and **f**) or 24 h (**k** and **j**). Scale bar represents 200 μ m

was found to cause extensive damage to the leaf tissue, although high constitutive reporter activity was detected [Fig. 3 (0.6a and b); (0.8c and d); (1.0e and f)]. Under the present experimental conditions, a duration of 10 min each for “sonication–rest–sonication” was found to be optimal as uniform reporter activity was detected with minimal damage to the tissue [Fig. 2 (0.6a and b);(0.8c and d);(1.0e and f)].

Effect of bacterial density

Generation of stable transgenic lines through the floral dip method requires *A. tumefaciens* at an optimal O.D. of 0.8 (Clough and Bent 1998). Optimal bacterial density for reporter activity using transient assay was thus evaluated by adjusting the optical density of *Agrobacterium tumefaciens* suspension at 600 nm to 0.6, 0.8 and 1.0 for co-cultivation. A

Fig. 4 Sonication-assisted transient assay can be used in a variety of tissues. Sonication-assisted transient expression analysis of CaMV35S promoter::GUS reporter was performed in open flower, leaf, root and silique, tissues of *Arabidopsis thaliana* which are not

commonly used for transient assay, using 10 min of sonication–rest–sonication cycle and 12 h of co-cultivation with *A. tumefaciens* at O.D.=0.8

comparison of the three conditions revealed a direct correlation between bacterial O.D. and intensity of GUS reporter activity. Analysis of reporter activity revealed intense GUS activity throughout all plant tissues tested when co-cultivated at O.D. of 1.0 (Figs. 1e, f; 2e, f, 3e, f), and least activity at O.D. 0.6 (Figs. 1a, b, 2a, b, 3a, b). In comparison, GUS expression after co-cultivation with bacterial density at O.D 0.8 showed moderate reporter activity that also allowed analysis of distribution of GUS activity in various parts (Figs. 1b, c, 2b, c, 3b, c).

Effect of co-cultivation time

To determine the optimal co-cultivation period, one cycle of sonication/rest/sonication for 5, 10 and 15 min was performed and was followed with co-cultivation using *A. tumefaciens* at an O.D. of 0.6, 0.8 and 1 for 12 and 24 h at 25 °C. GUS expression was detectable with all tested conditions. However, a comparative analysis of co-cultivation periods showed that sonication followed by 24 h of co-cultivation led to tissue softening, shrinkage and disintegration, and impaired handling. We therefore performed all further co-cultivation experiments for 12 h in the dark [Fig. 1 (0.6g and h); (0.8i and j); (1.0k and l), Fig. 2 (0.6g and h); (0.8i and j); (1.0k and l) and Fig. 3 (0.6g and h); (0.8i and j); (1.0 K and l)].

Sonication-assisted transient assay is tissue independent and can be used in other plants

We performed sonication-assisted transient assay with a 10 min sonication cycle, bacterial O.D.=0.8, and 12 h co-cultivation in flower, root and silique, tissues that are otherwise not routinely employed for analysis, and observed near ubiquitous reporter activity (Fig. 4).

In a parallel experiment, we also tested the suitability of the transient assay in non-model plants with longer life cycle than *A. thaliana*, namely, *Brassica juncea* and *Brassica nigra*. Sonication-assisted transient expression analysis of CaMV35S promoter::GUS reporter was performed in open flower, cotyledon, mature leaf, stem and root of *Brassica juncea* var Varuna and in unopened floral buds and open flowers of *Brassica nigra*, which are not commonly used for transient assay, using 10 min of sonication–rest–sonication cycle and 12 h of co-cultivation with *A. tumefaciens* at O.D.=0.8 and obtained near ubiquitous reporter activity as expected (Fig. 5, Supplementary Fig. 2).

The native binary vector, pCambia1391Z that harbours a promoterless GUS as reporter was used as negative control in *A. thaliana* (10 min sonication cycle, bacterial O.D.=0.8, and 12 h co-cultivation) and did not give any reporter activity (Fig. 6a–d).

Comparative analysis of promoter of MIR159B using transient assay and stable transgenic lines

We tested the validity of the “sonication-assisted transient assay method” using a comparative analysis of reporter activity driven by promoter associated with MIR159B gene from *Brassica juncea* cv. Varuna (henceforth designated as Prom-Bj-MIR159B) through stable transgenic lines and in transient assays. Dissection of promoter function was performed using a 5'-nested deletion (0.6 kb) and a full-length promoter (1.0 kb) that were cloned upstream of the GUS reporter in pCambia1391Z vector backbone. We have previously tested and shown that native pCambia1391Z does not confer any reporter activity in the absence of the promoter (Fig. 6). Expression of reporter gene β -glucuronidase (GUS) in T2 lines of transgenic *Arabidopsis thaliana* plants carrying prom-MIR159B-1 kb:GUS fusion revealed GUS

Brassica juncea

Fig. 5 Sonication-assisted transient assay can be used in *Brassica* species. Sonication-assisted transient expression analysis of CaMV35S promoter::GUS reporter was performed in open flower, cotyledon, mature leaf, stem and root of *Brassica juncea* var Varuna,

which are not commonly used for transient assay, using 10 min of sonication–rest–sonication cycle and 12 h of co-cultivation with *A. tumefaciens* at O.D. = 0.8. Scale bar represents 200 μm

expression in flower bud of stage 11 (Fig. 7d, e; Alvarez-Buylla et al. 2010); and transgenic plants carrying prom-MIR159B-0.6b:GUS fusion revealed GUS expression in flower buds of stage 9–12 (Fig. 7g, j; h, k). No GUS activity was detected in any other tissues like leaf (Fig. 7c, f; i, j), siliques, mature flowers in both full-length and 5'-nested deletion promoters [prom-MIR159B-1 kb:GUS (Fig. 7f; Supplementary Fig. 1) and prom-MIR159B-0.5 kb:GUS (Fig. 7i; Supplementary Fig. 1)].

A comparison of the results from transgenic lines, and obtained upon sonication-assisted transient method with both prom-MIR159-1 kb:GUS and prom-MIR159B-0.5 kb:GUS fusion constructs revealed identical reporter activity with no significant visible difference in the site and stage of expression, namely in prom-MIR159-1 kb:GUS fusion expression in flower bud of stage 11 (Fig. 7d, e) and prom-MIR159B-0.5 kb:GUS fusion expression in flower bud of stage 9–12 (Fig. 7j,

Fig. 6 Promoterless GUS as present in pCambia1391Z does not show reporter activity in transient assay in different tissues (inflorescence, leaf, root, silique) of *Arabidopsis thaliana*

Fig. 7 Comparative analysis of reporter activity obtained from stable transgenic lines and after transient assay using promoter associated with MIR159B from *Brassica juncea* reveals identical reporter activity. Full-length promoter of 1.0 kb (Bj-p_{159B}-A06-1 kb) and

a 5'-nested deletion of 0.6 kb (Bj-p_{159B}-A06-0.6) promoters were cloned upstream of GUS in pCambia1391Z and used for transient assay in flower and leaf (10 min sonication cycle, 12 h co-cultivation and bacterial O.D.=0.8) and to generate stable transgenic lines

h). No GUS activity was detected in any other tissues such as leaf, siliques and mature flowers with both prom-MIR159B-1 kb:GUS and prom-MIR159B-0.5 kb:GUS (Supplementary Fig. 1).

Discussion and conclusions

Sonication-assisted transient expression method proved to be effective on at least two major accounts. Firstly, the savings observed in terms of time was substantial. Accurate reporter activity could be generated within a matter of days after sonication, not counting the period of 4–5 weeks taken to grow the test plant. This is in contrast to at least two generations of life cycle of T1 and T2 transgenic plants (with each cycle being of at least 6 weeks duration). Secondly, the sonication-assisted method allowed us to evaluate reporter activity in a wide range of plant tissues, in contrast to earlier used methods that relied on only leaves, whole seedling or protoplast as exemplified in syringe infiltration of *Agrobacterium* suspension in tobacco leaves (Leuzinger et al. 2013), *Arabidopsis* leaf-derived protoplasts (Abel and Theologis 1994), co-cultivation of *Arabidopsis thaliana* seedling with *Agrobacterium* suspension (Li et al. 2009; Wu et al. 2014), Agro-infiltration of *A. thaliana* leaves (Lee and Yang 2006), in roots using hydroponics (Levy et al. 2005), transfer of naked plasmid DNA to superficially located plant tissue with gene gun (Morikawa et al. 1989) and liposome and PEG-mediated DNA transfer (Caboche 1990; Yoo et al. 2007). Sonication-assisted transient expression method also does not require any dedicated equipment or chemicals and thus proves to be rapid and cost-effective.

Previously, MIR159 has been demonstrated to play a role in anther development (Plackett et al. 2011; Wang et al. 2012), and analysis of *Arabidopsis thaliana* MIR159b promoter revealed reporter activity in anther and seedling (Allen et al. 2010). The reporter activity obtained in the present study using both transient and stable transgenic lines also exhibit reporter activity in anther and is in agreement with previous literature. Sonication-assisted transient expression assay thus provided accurate, tissue-specific expression of promoter when the results obtained with this method were compared to those obtained from data of transgenic lines of the T2 generation.

We thus conclude that this modified method will allow cataloguing of spatio-temporal expression pattern driven by specific promoters that correlate with developmental dynamics in a rapid and cost-effective manner. This method will also prove useful in plant systems that are recalcitrant and not amenable to transformation to generate stable transgenic lines. Future studies should focus on extrapolating this basic sonication-assisted methodology to analyse hormonal regulation of promoter activity and in identification of

trans-factors via DNA–protein interactions. This assay needs to be extended to evaluate promoter activity under various abiotic and biotic stresses under laboratory conditions.

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Author contributions CC, DC and SD were involved in planning of the study. CC and GJ performed the experiments and collected data. CC, DC and SD analysed the data and wrote the manuscript. All authors have read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interest that may be construed as conflict of interest.

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