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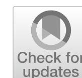

In-planta transient transformation of avocado (*Persea americana*) by vacuum agroinfiltration of aerial plant parts

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Abstract

Avocado, *Persea americana* Mill, is one of the most traded tropical fruits in the international market. To date, stable and transient transformation has only been achieved for of zygotic embryos and not of adult plant tissue, which limits functional genomics research. We provide the first transient *Agrobacterium*-mediated transformation methodology in avocado leaves that overcomes the recalcitrance to transformation of this species. We investigated the effect of *Agrobacterium* strain, leaf stage, wounding pre-treatment, the phytohormone jasmonic acid, and vacuum infiltration on transient transformation of avocado leaves. Using the *Agrobacterium* strain LBA4404 and the *RUBY* reporter a transformation frequency of up to 27% was obtained for avocado detached leaves. The transformation efficiency depended on the age of the leaf, with an intermediate stage of leaf development showing the highest efficiency of transient reporter gene expression. Microwounding pre-treatment facilitates agroinfiltration and coupled with leaf age are the primary factors influencing competence for transient transformation. Jasmonic acid did not significantly affect transient transformation in the absence of microwounding. However, microwounding and 250 µM of jasmonic acid acted synergistically to significantly enhance transient expression. Using this methodology with localized vacuum agroinfiltration, transient transformation of attached avocado leaves was achieved. This method unlocks the use of *Agrobacterium*-mediated transient transformation as a tool for explore gene function and metabolic pathways in both, detached and attached avocado leaves.

Key message

Leaf age, wounding and jasmonic acid counter the recalcitrant nature of avocado, unlocking the use of *Agrobacterium* mediated transient transformation as a tool for functional genetics in this plant. In addition, localized vacuum infiltration bypasses plant size constraints for *in-planta* transient transformation.

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Jorge Alberto Salazar-González, Manuela Castro-Medina, and Luis Enrique Bernardino-Rivera have contributed equally to this work.

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Keywords Avocado agroinfiltration · Transient transformation · *Agrobacterium* · Wounding · Jasmonic acid · Betalains · GFP · Vacuum infiltration · Detached leaf transient system · *In-planta* gene transformation

Introduction

The avocado, *Persea americana* Mill., is one of the most traded tropical fruits in the international market. Globally, avocado exports tripled between 2010 and 2020 (FAO 2021), and in terms of export value avocado became the leading commodity with 53% of global trade in major tropical fruits in 2021 (FAO 2022). Global demand and lucrative export prices have stimulated the expansion of this horticultural trees crop production.

Despite the great economic importance of the production of this horticultural tree crop, and the recent publication of its genome sequence (Rendón-Anaya et al. 2019), few molecular tools exist or have been utilized for avocado crop improvement (Tamayo-Ramos et al. 2022). Currently the molecular analyses are hindered by the lack of efficient and rapid transformation protocols. In the last two decades there has only been a single report using biolistics for transient transformation of embryogenic callus (Chaparro-Pulido et al. 2014), one report using *Agrobacterium rhizogenes* to produce composite avocado plants (Prabhu et al. 2017), and few reports detailing the use of *Agrobacterium* to generate stable transformants (Cruz-Hernández et al. 1998; Efendi 2003; Raharjo et al. 2003, 2008; Palomo-Ríos et al. 2011, 2012, 2017; Ahmed et al. 2012). These latter reports employed embryogenic masses derived from zygotic embryos, due to their high potential and competence for regeneration. However, as these zygotic embryos are derived from outcrossing, they are not the ideal starting material for the preservation of a scion's agronomic traits.

Whilst stable genetic transformation is essential for crop breeding applications, transient gene transformation, is a useful tool in plant cell and molecular biology research and provides results significantly faster compared to stable transformation. Transient gene expression allows the assessment of gene function and regulation by gene overexpression, protein subcellular localization assays, protein–protein interaction assays (co-immunoprecipitation assay and bimolecular fluorescence complementation assay), as well as protein-DNA interaction (dual luciferase assay) or gene silencing (Lu et al. 2003; Sparkes et al. 2006; Chen et al. 2008; Marion et al. 2008; Zhang et al. 2020). *Agrobacterium*-mediated transient transformation approaches have proven effective in a wide range of perennial species including aspen (Takata and Eriksson 2012), cacao (Fister et al. 2016), citrus (Acanda et al. 2021), plum (Yanchcva et al. 1994), apple (Maximova et al. 1998) and grapevine (Santos-Rosa et al. 2008). To our knowledge, there are no reports of *Agrobacterium*-mediated transient transformation of adult avocado plant tissue, perhaps due to avocado's recalcitrant nature, which limits functional genomics research and breeding (Palomo-Ríos et al. 2012). Tobacco is often used as a model for transient expression studies. However, since some molecular pathways are exclusive to certain species, a heterologous system may not always be appropriate (Manavella and Chan 2009). Therefore, a reliable method for transient gene transformation in avocado plant could significantly improve our ability to perform functional genetic assays in avocado rather than in model species. It could also be used to evaluate the transformation capabilities of different somatic tissues, which then in combination with tissue culture regeneration could identify potential alternatives to somatic embryos for stable avocado transformation.

This research explores different parameters to achieve *Agrobacterium*-mediated transient genetic transformation in avocado. First, detached leaves were used to evaluate different factors affecting avocado genetic transformation. Among the parameters tested on avocado was forced vacuum infiltration to infiltrate the *Agrobacterium* suspension (Bechtold and Pelletier 1998); different strains of *Agrobacterium tumefaciens* (Anderson and Moore 1979); leaf age given that recalcitrance to transformation has been shown to be inversely proportional to tissue age (Cervera et al. 1998); micro wounding of tissues (Potrykus 1990), and pre-culture on phytohormone cocktail-containing medium (Sangwan et al. 1991). Finally, with those optimal conditions identified on detached leaves, we tested *in-planta* transient gene transformation by vacuum agroinfiltration of leaves of mature avocado plants. We provide the first report of *Agrobacterium*-mediated transient transformation of detached and attached avocado leaves expressing the genes necessary for the overexpression of an entire biosynthetic pathway not natively found in this species, selecting critical parameters for its success and future application in the study of gene function and genetic improvement of one of the most important fruit crops.

Material and methods

Plant materials

Persea americana Mill. Hass cultivar grafted on Mexican race rootstock was obtained from the Fundación Salvador Sánchez Colín CICTAMEX, S.C. germplasm bank located at La Cruz Experimental Center at Coatepec Harinas in the

state of Mexico. Plants were grown under greenhouse conditions at CIATEJ facilities in Zapopan, Jalisco, Mexico (lat. 20°42′N, long. 103°28′W, altitude 1680 m).

Transformation plasmids and *Agrobacterium* cell cultures

Two plasmids were used in this study to monitor transient gene expression in avocado leaves; pJL-TRBO-G, carrying the Green Fluorescent Protein (GFP) reporter gene, was a gift from John Lindbo (Addgene plasmid # 80083) (Lindbo 2007), and *35S:RUBY*, carrying the enzymes required for betalain biosynthesis, was a gift from Yunde Zhao (Addgene plasmid # 160908) (He et al. 2020). Three widely used *A. tumefaciens* strains AGL1, LBA4404, and GV3101 (Intact Genomics, USA), were evaluated to assess the transient transformation efficiency of avocado leaves, strain EHA105 was not included because it has resistance to the antibiotic spectinomycin as well as the *RUBY* vector. Each expression vector was transformed into each of the Agrobacterial strain by electroporation. A single colony of transformed *A. tumefaciens* was plated on Luria–Bertani (LB) plates with the exception of LBA4404 (Yeast malt, YM), with the following antibiotics rifampicin (10 µg/ml) for all strains, carbenicillin (100 µg/ml) for AGL1, streptomycin (50 µg/ml) for LBA4404, gentamycin (30 µg/ml) for GV3101; and for plasmid selection, kanamycin (50 µg/ml) for pJL-TRBO-G, and spectinomycin (50 µg/ml) for *35S:RUBY*. Plates were incubated at 28 °C for 48 h in the dark. For agroinfection, colonies of transformed *Agrobacterium* cells were inoculated into LB broth or YM for LBA4404, supplemented with acetosyringone (AS, 20 µM) and grown 28 °C overnight at 250 RPM. Cultures were harvested by centrifugation at 5000 g for 10 min and pellets were resuspended in infiltration medium (4 g/l Murashige and Skoog Medium (salts and vitamins), 4 g/l sucrose, 20 mM MES, 5 mM $MgCl_2$, 150 µM AS, 0.1% v/v Plant Preservative Mixture (PPM), pH 5.3) (Acanda et al. 2021) before incubating for 4 h at 28 °C at 150 RPM. Cell suspensions were then adjusted to a final optical cell density OD_{600} of 0.6 with infiltration media. Each treatment applied to detached avocado leaves was also infiltrated into tobacco leaves with a needleless syringe as a positive control.

Agrobacterium mediated transient transformation

In previous studies, the expression levels of both reporters, TRBO-G and *RUBY*, is sufficient to be visually evaluated (Lindbo 2007; He et al. 2020). For each report gene, expression was visually ranked from 0 to 5, where zero is no expression, and five is high expression compared to the tobacco positive control by observing the leaves at 7 days post infection (DPI). This ranking system was used to initially evaluate the vector, the infiltration method, the *Agrobacterium* strain, bacterial density and vacuum conditions. Infiltration methods were initially evaluated using three different *Agrobacterium* strain at a cell density of 0.6 OD_{600} carrying the viral vector pJL-TRBO-G, or *35S:RUBY*, in five avocado detached leaves. Spontaneous infiltration was evaluated by dipping detached leaves into each bacterial suspension for 3, 16, or 24 h, while forced infiltration was tested by needleless syringe and vacuum infiltration. For the latter detached leaves ($n = 5$) were submerged into each *Agrobacterium* suspension and were put into a laboratory desiccator connected to a vacuum pump, until the vacuum reached −0.07 MPa; the vacuum was set for 5 min followed by a slow release of vacuum and this procedure repeated twice, in order to replace the air present in intercellular spaces of the leaf tissue with *Agrobacterium* suspension. Leaves were removed and blotted dry on paper towels to facilitate removal of excess bacterial suspension and air dried for an hour in the laminar airflow of a biosafety cabinet (Fujiuchi et al. 2016). Leaves were then incubated in a semi-wet chamber at 25 °C in the dark for 2 days, then light/dark period of 16/8 h. To evaluate the effect of wounding on transient gene expression, detached avocado leaves were mechanically wounded (60 wounds/cm^2) on the abaxial surface of the leaves using a microneedle roller (Supplementary Fig. 1), a device typically used for cosmetic purposes to generate micro-wounds (MW) to facilitate entrance of agrobacteria. To compare the effect of MW on the same avocado leaf, MW was only applied to one half of the leaf. To evaluate the effect of methyl jasmonate (JA) on transient gene expression, prior to vacuum infiltration 250 µM of JA was added to the agrobacterial suspension.

For *in-planta* transient transformation of avocado, *Agrobacterium*-mediated vacuum infiltration was applied to attached leaves of a branch, using the optimized conditions previously identified for detached leaves. The same 20 l vacuum desiccator was used, with a built-in analog vacuum gauge at the top of the unit. To allow access of a branch whilst enabling a complete seal, a flexible rubber hose was coated in vacuum grease to fit in the usual grooves on the vacuum chamber and any leaks were sealed using mouldable plastic (e.g., Nescofilm). Leaves were blotted dry on paper towels to facilitate removal of excess bacterial suspension. Attached leaves were then covered with a transparent plastic bag to maintain high humidity and prevent dehydration, plants were incubated at 25 °C in the dark for 2 days, then light/dark period of 16/8 h.

Spectrophotometric detection of Betalains

Agroinfiltrated leaves with *RUBY* reporter, MW and JA treatments were frozen in liquid nitrogen and ground with a mortar and pestle at 7 DPI. Betalains were extracted from

100 mg of ground tissue by resuspension with 300 µl 50% methanol, 1 mM ascorbic acid, 0.5% formic acid (Grützner et al. 2021). Samples were vortexed and then incubated on ice for 15 min. The samples were centrifuged at 13,000 RPM for 10 min at 4 °C and supernatant was recovered into a new tube. Spectrophotometric determination of betalains was measured at 535 nm (Polturak and Aharoni 2018) using the Infinite 200 PRO microplate reader (TECAN, Switzerland).

Statistical analysis

Absorbance data of betalain extracts were analyzed by one-way ANOVA using Prism 8 software (GraphPad, USA).

Imaging and confocal microscopy

Efficiency of *RUBY* expression in developmental stage, MW and JA treatments was determined in terms of the percentage of red-stained area compared to the total leaf area by image-J software (NIH, USA). For confocal microscopy a fresh unfixed section of leaf (Approx. 0.5 cm^2) was placed under a coverslip for imaging and observed at optical zoom 10X. The fluorescent signals were then detected using a Leica TCS SPE confocal microscope (Leica Microsystems Inc., USA) with a laser excitation at 488 nm and emission filter of 500–530 for GFP expression (Green), and emission filter of 675–725 for chlorophyll autofluorescence (red displayed). Images were taken and managed with the LAS X® software (Leica Microsystems Inc., USA). The GFP fluorescence signal was obtained from detached avocado leaves transiently transformed with the conditions that generated the highest betalain expression.

Protein extraction and Western Blot

Approximately 100 mg of avocado leaves infiltrated with *Agrobacterium* carrying pJL-TRBO-G following the method that generated the highest betalain expression; leaves were sampled at 7 DPI and flash frozen in liquid nitrogen. For protein extraction, leaf samples were ground into a fine powder and resuspended in 1 ml of TRIzol reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions for protein isolation. Total protein extracts were separated by SDS-PAGE in 10% polyacrylamide gels under denaturing conditions. Gels were blotted onto immobilon-P, 0.45 µm PVDF membranes (Merck Millipore, USA). After 3 h blocking with TBS-Tween 0.05% plus 5% fat-free milk, blots were incubated overnight with a monoclonal mouse antibody against GFP (Thermo Fisher Scientific, USA) 1:500 dilution. After washing the membrane three times for 5 min each in 1X TBST, blots were incubated with a goat anti-mouse IgG secondary antibody horseradish peroxidase-conjugated at a 1:5000 dilution (Thermo Fisher Scientific, USA) for 2 h at room temperature. Antibody binding was detected with the Pierce ECL Western blotting Substrate solution, following the manufacturer's instructions (Thermo Fisher Scientific, USA) and by means of an X-ray film, following standard procedures.

Results

Transient expression of GFP in avocado

In avocado, the use of GFP has been previously reported for transient biolistic transformation of embryogenic callus (Chaparro-Pulido et al. 2014), and for transformation of roots to develop composite avocado plants (Prabhu et al. 2017). In this study, several parameters, e.g., infiltration system, *A. tumefaciens* strain, *Agrobacterium* cell density, developmental stage of avocado leaves, pre-treatment with MW, were tested for transient transformation of avocado leaves using a viral vector pJL-TRBO carrying the GFP reporter, but with no success. After 7 DPI, infiltrated and control leaves were examined. However, both negative control (non-transformed) and the agroinfiltrated leaves showed only slight basal fluorescence, under a long wave UV light (365 nm), possibly due to the autofluorescence of the leaf (Buschmann and Lichtenthaler 1998). Supplementary Fig. 2 shows a representative image of negative control and agroinfiltrated leaves. This observation was consistent across different leaves under parameters described above (data not shown) and even after 14 DPI, thus, it was determined that this method was not suitable for rapidly assessing transformation efficiency in avocado. We therefore decided to evaluate the non-fluorescent reporter, *RUBY* that can be visualized without any special equipment.

Transient expression of the betalain biosynthetic pathway in avocado leaves

We assessed the effect of infiltration system on transient gene expression on avocado detached leaves, using the *35S:RUBY* reporter system. This expression vector comprises the cassette for expressing the enzymes P450 oxygenase CYP76AD1, L-DOPA 4,5-dioxygenase and glucosyltransferase, involved in the conversion of tyrosine into the red pigment, betalain (He et al. 2020). The *RUBY* reporter was selected because it is non-destructive visual reporter that allow simple and cheap macroscopic visual detection of the success of transient genetic transformation, from 3 days after agroinfiltration. We initially determined that avocado leaves are not suitable for agroinfection by co-culture of detached leaves with agrobacteria suspension at 0.6 OD_{600} for 3, 16, or 24 h as this method induced leaf necrosis after 3 DPI. Agroinfection using a needleless syringe, as is commonly

used for agroinfiltration of tobacco, was also found to be not suitable for avocado leaves. We therefore explored the use of vacuum infiltration of the bacterial suspension to evaluate the effect of *Agrobacterium* strain on transient gene expression. Since *A. tumefaciens* strains have different sensitivities to particular plant species, we started by testing three of the most commonly used laboratory strains (AGL1, LBA4404, and GV3101). Microwounding of one half of each leaf was performed prior to vacuum infiltration with five detached young leaves of avocado subjected to vacuum-based agroinfiltration at −0.07 MPa for 5 min with three vacuum breaks, with each strain suspension at 0.6 OD_{600}. Following vacuum infiltration, leaves were incubated in a semi-wet chamber at 25 °C, and *RUBY* expression was evaluated at 7 DPI. At this stage, the accumulation of betalains was visually evident, mainly around the microneedle-made wounds, as shown in Fig. 1. Different parameters (agrobacterial strains, bacterial density and vacuum conditions) were initial screened by macroscopic visual detection, on a scale we assigned from 0 to 5, by comparing the result in avocado leaf to the positive control in tobacco. Among the three *Agrobacterium* strains assessed, transient expression of *RUBY* was greatest with strain LBA4404 and lowest with AGL1 (Supplementary Table 1). This result shows that vacuum-based agroinfiltration is suitable for agroinfection of avocado leaves and that heterologous expression of the *RUBY* reporter functionally re-constitutes the betalain biosynthetic pathway in avocado leaves. However, these results also demonstrate that *Agrobacterium* strain influences transformation efficiency.

We next explored the optimal vacuum conditions and bacterial density for agroinfiltration of avocado leaves. Three different vacuum intensities (−0.02, −0.04, and −0.07 MPa), three different durations (1, 2, or 3 vacuum breaks), and three different cell densities (OD_{600} values of 0.3, 0.6, 1.0), were evaluated by monitoring betalain staining at 7 DPI. Betalain staining positively correlated with increasing vacuum intensity and the number of vacuum breaks suggesting the intensity and repetitions of vacuum infiltration has an effect on transformation efficiency (Supplementary Table 2). The vacuum infiltration conditions of −0.07 MPa, for 5 min with 3 vacuum breaks was then used to determine the effect of cell density on transient transformation using the LBA4404 *Agrobacterium* strain (OD_{600} 0.3, 0.6 and 1). Using the visual scoring system, it was determined that an OD_{600} of 0.6 resulted in higher betalain staining (Supplementary Table 3).

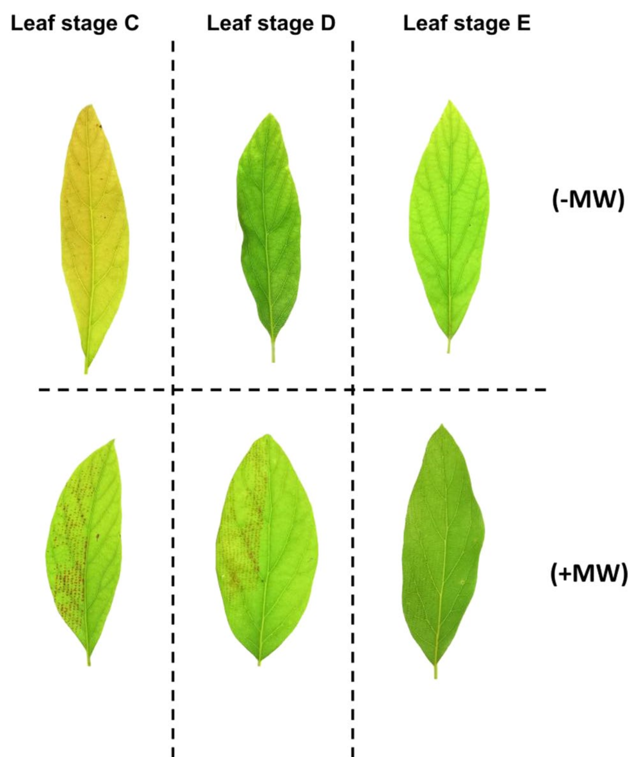

Fig. 1 Effect of leaf stage and microwounding on expression of *RUBY*. Representative image of one of five biological replicates of *RUBY* expression in avocado leaves at stages C-E and with microwounding applied to one half of the leaf (+MW), or without microwounding (−MW). Betalain (red) is located around the microneedle-made wounds. (Color figure online)

Effect of leaf age on transient gene expression

We next assessed the effect of leaf age on transient transformation using the optimized conditions previously identified (LBA4404 strain at 0.6 OD_{600}, leaf microwounding, vacuum infiltration at −0.07 MPa with 3 vacuum breaks). To assess the effect on *Agrobacterium*-mediated transformation of the developmental stage of avocado leaves, five successive developmental stages of leaves were selected based on macroscopic characteristics of size and color changes. The latter due to the greening pattern common in many tropical species due to the gradual increase in chlorophyll content (Whatley 1992). The first stage (A) corresponds to emerging leaves, the shortest in length in this classification, thin and bright brown in color; followed by leaves that undergo expansion but are of the same colour (B); then those that continue to expand in size but show an evident color transition towards green (C), then those that are light green and less thin than the previous ones (D) and finally the fully expanded, dark green and thick leaves (E) (Fig. 2). Leaves at the earliest stages (A and B) did not survive the infiltration treatments and so were excluded from our analysis. In contrast, after 7 DPI leaves at stage C and D displayed betalain in the periphery of the microneedle-made wounds but not in the non-wounded side of the leaves (Fig. 1), indicating the crucial impact of MW in transformation efficiency. Older leaves (stage E) consistently showed no evidence of reporter expression even after 14 DPI (Fig. 3).

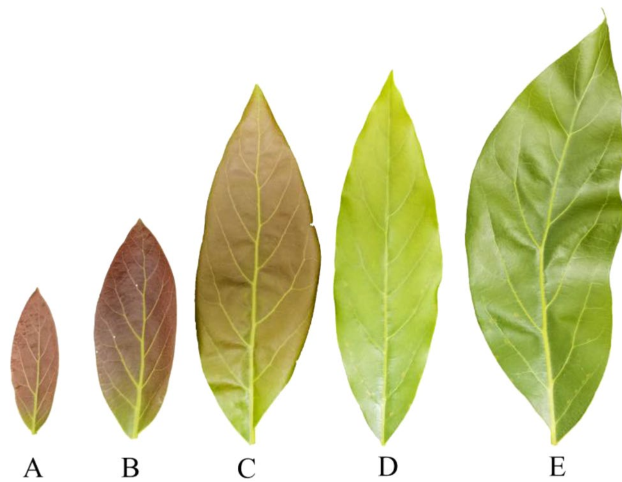

Fig. 2 Representative image of different avocado leaf stages used in the study: Detached leaves were grouped as follows, the first stage (**A**) corresponds to emerging leaves, the shortest in length in this classification, thin and bright brown in color; followed by leaves that undergo expansion but are of the same color (**B**); then those that continue to expand in size but show an evident color transition towards green (**C**), then those that are light green and less thin than the previous ones (**D**) and finally the fully expanded, dark green and thick leaves (**E**). Scale bar 2.5 cm. (Color figure online)

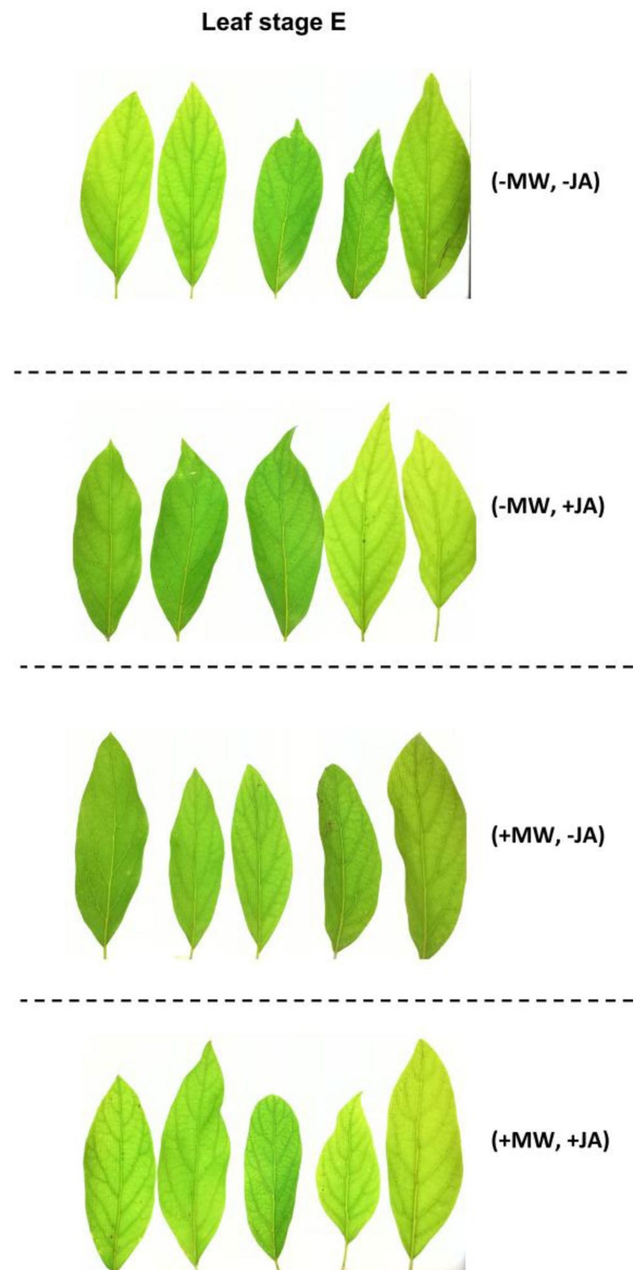

Fig. 3 Effect of leaf stage on expression of *RUBY*. Evaluation of Betalain presence in leaves at stage E. Leaves represent independent agroinfiltrations for each treatment. (MW, microwounding; JA, 250 µM Jasmonic acid). Betalain is not detected under any treatment

Effect of wounding pre-treatment and jasmonic acid

Previous work has shown that pre-treatment with the phytohormone JA can affect *Agrobacterium* mediated transformation (Jung et al. 2014). Having determined that MW and leaf age are critical factors, we therefore evaluated whether treatment with JA affects the efficiency of transient expression. Previous studies on detached sunflower leaves achieved a 4.6-fold increase in heterologous expression when JA 250 mM was added to *A. tumefaciens* cell suspensions and infiltrated into the plant leaves (Jung et al. 2014; Jung and McDonald 2016). Our observations were focused on leaves at stage C and D and using agroinfiltration of the *RUBY* reporter. Here, there was no significant betalain synthesis in leaves treated only with JA, and without MW, demonstrating that MW coupled with leaf age are the primary factors influencing the competence for transient transformation (Fig. 4).

Whilst JA did not appear to significantly affect transient transformation in the absence of MW, we did observe a synergistic effect when leaves were both MW and JA treated, which was consistent for both C and D leaf stages (Fig. 4). Image analysis showed that betalains accumulation was highest in stage D, followed by stage C leaves with MW and JA treatments, with betalains accumulation constituting 27.9% of total leaf area and 12.1%, respectively. In those treatments with MW but without JA the detected area of betalain staining is less than 10% being again higher for stage D, followed by C with 9.3% and 6%, respectively. Betalains are natural water-soluble pigments composed of betaxanthins and betacyanins, and show highest absorption at the 460–480 nm and 535–538 nm range (Polturak and Aharoni 2018). The image analysis data of leaf area accumulating betalains was supported by spectrophotometric quantification of leaf extracts (Fig. 5). Betalain was produced and accumulated at a significantly greater level in leaves at stage C and D with MW and JA (+ MW, + JA) treatment.

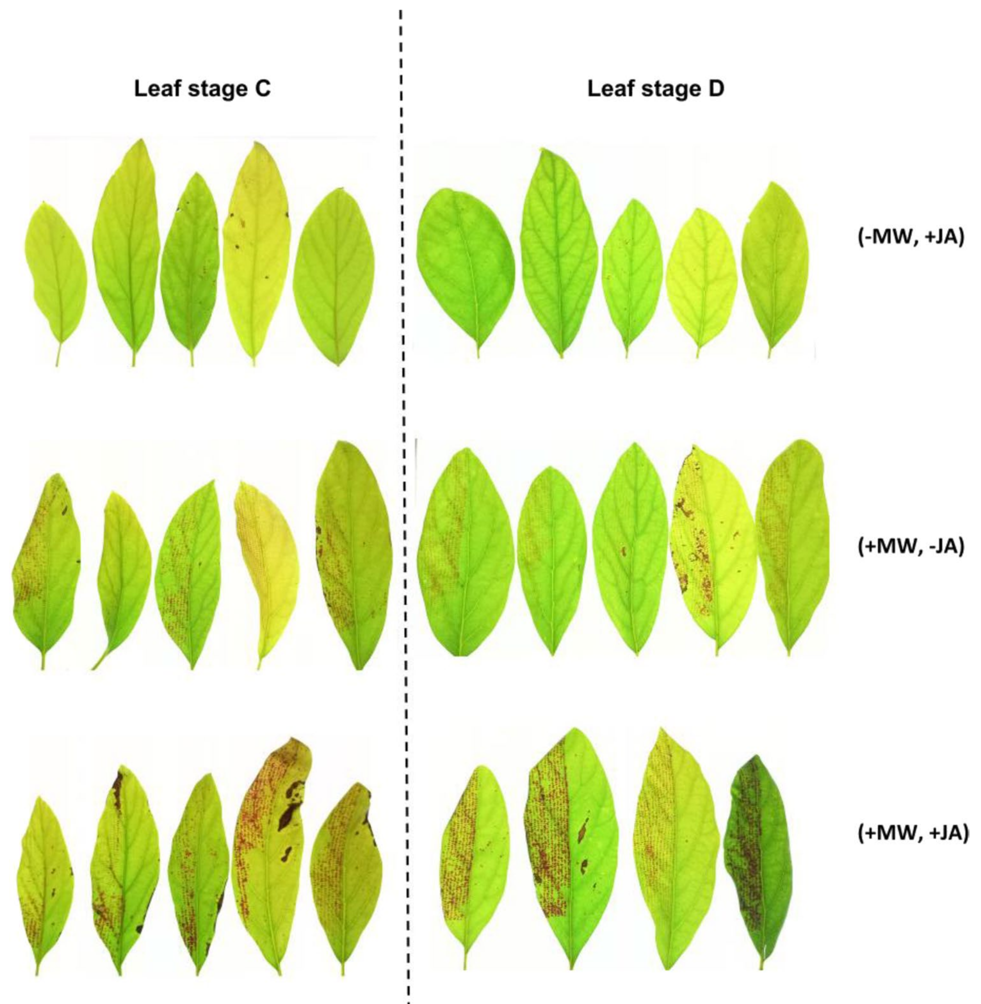

Fig. 4 Effect of MW and JA. Betalain accumulation following agroinfiltration of avocado leaves (stage C and D) with the *RUBY* reporter. Leaves represent independent agroinfiltrations for each treatment. Top row, no MW plus JA (250 µM) (−MW, +JA); middle row, MW without JA (+MW, −JA); and bottom row, MW plus JA (250 µM) treatment (+MW, +JA)

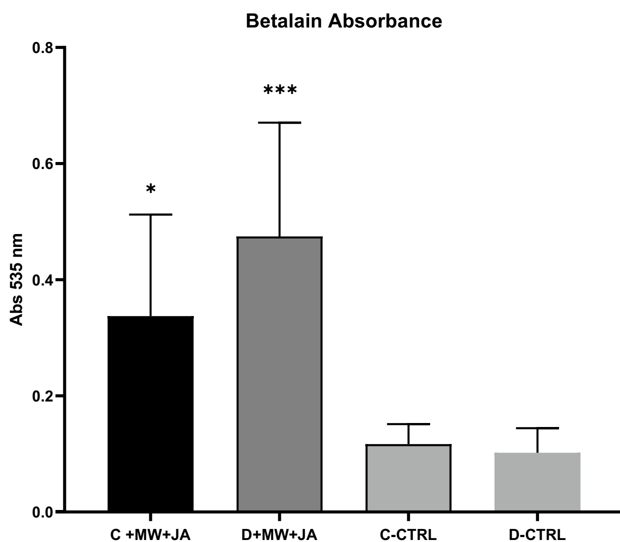

Fig. 5 Betalain determination by spectrophotometry. Extracts of the leaves (stage C or D) transformed with *RUBY* were analyzed by spectrophotometry at 535 nm to determine the presence of betalains (CTRL, WT untransformed avocado leaves; MW, microwounding; JA, 250 µM JA). *p-value < 0.05, ***p-value < 0.001

Confocal microscopy of GFP- expressing avocado leaves with synergic effect of MW and JA

Since betalain biosynthesis was successfully observed by agroinfiltration of D-stage avocado leaves with MW pretreatment and addition of JA, we tried again to assess GFP expression this time by confocal microscopy. After infiltration of agrobacteria harboring the pJL-TRBO-G into detached avocado leaves, we assessed GFP fluorescence as an indicator of successful *Agrobacterium*-mediated transformation. Apparent GFP fluorescence was mainly observed around some of the wounds (Fig. 6). The localization pattern of GFP around the wounds is similar to that observed with *RUBY*. Moreover, we also confirmed the accumulation of GFP protein by western blot of total protein extracts with monoclonal anti-GFP antibody. Western blot analysis revealed that MW in combination with JA treatment resulted in detectable GFP expression in some but not in all independent samples. No GFP was detected in leaves that were treated with JA but not MW (Fig. 7).

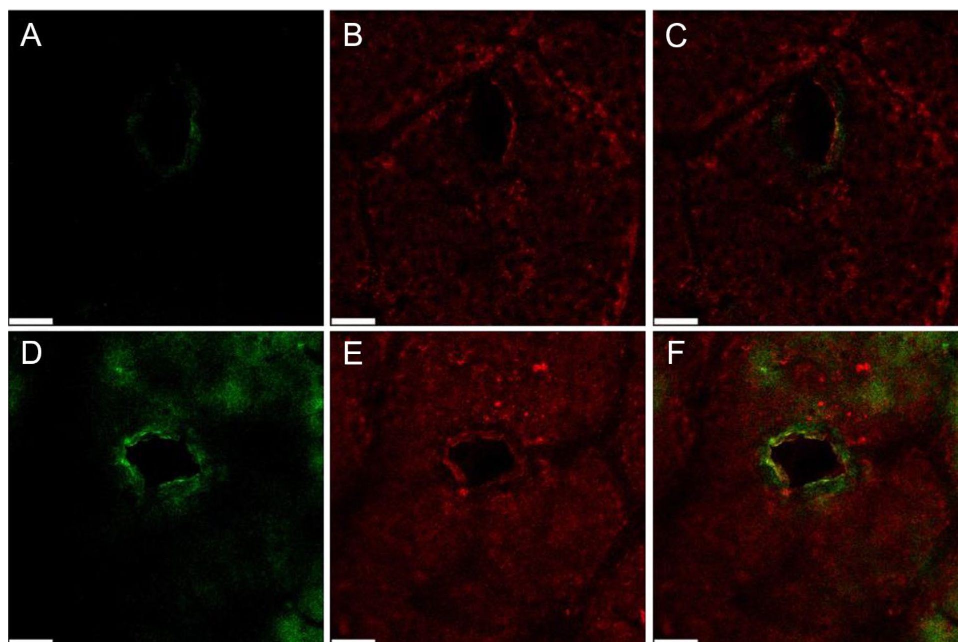

Fig. 6 Confocal microscopy of avocado leaves infiltrated with pJL-TRB-G. Fluorescence imaging of avocado leaves with MW (center, hole) and JA (250 µM) in negative control (**A–C**) and transformed with pJL-TRBO-G (**D–F**). GFP fluorescence (**A, D**), chlorophyll fluorescence (**B, E**), and merged images (**C, F**). Scale bar: 100 µm, Optical zoom: 10X

Fig. 7 Detection of GFP in agroinfiltrated avocado leaves. Total protein was extracted from independent leaves agroinfiltrated with pJL-TRBO-G. Lanes 1–4, independent plants were microwounded and treated with JA (250 µM); lanes 5–8 leaves were treated with JA only. Recombinant GFP was used as positive control (+)

Vacuum-based transformation *In-Planta*

Since conventional *in-planta* transient transformation techniques such as floral dip, and needleless syringe infiltration are not an alternative for many perennial species, we assessed whether the methods optimized for expression in detached leaves could be adapted for *in-planta* transient transformation. A major limitation here is that plant size prevents whole plant agroinfiltration with conventional infiltration vessels too small for this purpose. In this study, due to the larger size of the plant compared to the vacuum chamber, we applied the vacuum-based agroinfiltration to avocado attached leaves of a branch and not to the whole plant. To allow access of a plant branch inside the vacuum chamber a flexible rubber hose coated with vacuum grease was used to provide the vacuum seal between the upper and lower halves of the desiccator (Fig. 8a). The small gaps between the branch and the adapter must be sealed to allow vacuum formation inside the desiccator. This made it possible for several leaves attached to a branch of an avocado plant to be immersed in the *Agrobacterium* suspension and subjected to vacuum infiltration. Young leaves (stage C and D) attached to a branch of 1.7 m tall avocado plants were subjected to vacuum-based agroinfiltration at −0.07 MPa intensity together with 3 vacuum breaks, with LBA4404 strain at 0.6 OD_{600}, along with a pre-treatment of both MW and 250 µM JA. At 7 DPI, all infiltrated leaves showed betalain staining, with the same localization pattern as observed for detached leaves (Fig. 8b).

Discussion

In model plants such as tobacco transformed with GFP constructs, the use of a longwave UV lamp has been demonstrated to be a rapid means of evaluating transformation (Casper and Holt 1996)however, it has been reported that the detection of GFP in green tissues using this macroscopic method can lead to a substantial underestimation of expression levels. In this study, our inability to detect GFP fluorescence using this method could be due to the low transformation efficiency of the pJL-TRBO-G construct or interference by pigments within avocado leaves. Although it is a vector capable of expressing very high levels of foreign proteins in *N. benthamiana* plants, it has previously been reported to be unsuccessful in transforming detached whole sunflower, a plant that does not belong to the Solanaceae family (Jung et al. 2014). In addition, chlorophyll fluorescence has been shown to interfere with the detection of GFP fluorescence

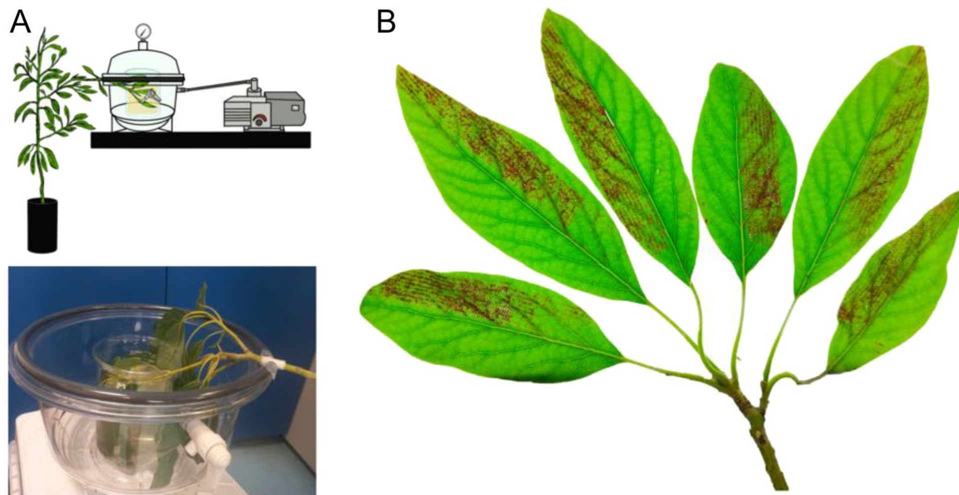

Fig. 8 *Agrobacterium*-mediated *in-planta* transient transformation of avocado with the *RUBY* reporter. **A** Vacuum-based agroinfiltration system with a vacuum pump and desiccator using a flexible rubber hose that has been coated in vacuum grease to fit in the usual grooves on the vacuum chamber, as a seal to allow access of a plant branch inside the vacuum chamber between the upper and lower halves of the desiccator and avoid branch crushing and vacuum loss; **B** Betalain accumulation in leaves attached to the branch of avocado plants at 7 DPI

in Medicago, rice and Arabidopsis, (Zhou et al. 2005). Our results indicate that GFP it is not a suitable reporter for rapid macroscopic evaluation by long wavelength UV illumination in avocado leaves and instead detection of this reporter requires access to specialized equipment.

The main advantage of *RUBY* is that visualizing the transformed area does not require specialized equipment such as fluorescence or confocal microscopes, contributing to the optimization of transient transformation methods in a quick and easy way, monitoring the plant throughout its life cycle. The *RUBY* reporter system has only recently been developed and has so far been used as a visible marker in Arabidopsis, rice (He et al. 2020), and Bamboo (Chen et al. 2021) and is promising to drive the evaluation of plants with thick waxy cuticles. The bright red color from betalain is easily contrasted from green leaf color and the brown caused by leaf damage and deterioration. However, this reporter does not allow detection at the subcellular level, as GFP does. The *RUBY* cassette was functionally expressed in avocado leaves, facilitating the observation of transient transformation, due to the production of functional enzymes for betalain synthesis, which led to the production of red spots on avocado leaves easily distinguishable with the naked eye from the third day after agroinfiltration.

Preliminary experiments indicated that avocado leaves are not suitable for agroinfection by co-culture with *Agrobacterium*, which is consistent with previous reports that reported tissue necrosis on leaf-discs of grape after co-cultivation with the LBA4404 *Agrobacterium* strain (Das et al. 2002). The vacuum-based agroinfiltration of detached leaves allowed testing conditions for transient expression in a more time and cost-effective manner than stable transformation. For avocado leaves where spontaneous infiltration or forced infiltration using needle-less syringe is not possible, vacuum infiltration allows controlled introduction of bacterial suspensions into leaf tissues, overcoming the influence of leaf structure on transformation efficiency of avocado leaves. One disadvantage is that this method requires specialized equipment such as a vacuum chamber with a pressure gauge, connected to a pump. Our results of increased expression of the *RUBY* reporter in avocado leaves by using −0.07 MPa vacuum intensity, and three vacuum breaks agrees with previous data possibly through increased of *Agrobacterium* cell suspension into leaf tissue (Joh et al. 2005; Simmons et al. 2009).

The macroscopic visual detection method of reporters provides a rapid way of screening for successful transient gene transformation events. Among the three *Agrobacterium* strains assessed, we observed that the LBA4404 is suitable for transient expression in avocado leaves. In two previous avocado transformation studies comparing different *Agrobacterium* strains, the LBA4404 strain did not give the best results, though this may be because the tissue they agroinfiltrated were globular somatic embryonic masses (Palomo-Ríos et al. 2012), and embryonic shoot axes (Ahmed et al. 2012).

We have determined that avocado leaves within a relatively narrow developmental time span are required for *Agrobacterium*-mediated transient genetic transformation, even with the addition of vacuum, MW and the application of JA. Working with mature leaves should be avoided until conditions for this leaf stage are optimized. It has

been demonstrated that the transformation of plant cells by *Agrobacterium* is inversely proportional to the age of the tissue (Wixom et al. 2018). The evaluation of different stages of leaf development of the tropical tree *Theobroma cacao* L. for transient transformation with *Agrobacterium* has shown that leaf age affected transformation efficiency (Fister et al. 2016). Some authors have proposed that physiological changes in the leaves when they mature hinder the infiltration of the *Agrobacterium* cell culture into the leaf parenchyma, therefore by reducing the diffusion of the *Agrobacterium* suspension and hence limiting the transformation potential of the tissue. The diffusive capability of syringe-infiltrated *Agrobacterium* suspension has been associated with the volume of the intercellular air spaces and the arrangement of the mesophyll cells inside the leaves (Zheng et al. 2021). A small intercellular and compartmentalized space, together with compact mesophyll cells could restrict the spread of the infiltrated suspension and therefore the transformation of cells. In *Rosa chinensis* it is thought that the stratum corneum and wax coat present in the outermost layer of mature leaves are responsible for the difficulty in infiltration and therefore the low transformation mediated by *Agrobacterium* (Lu et al. 2017). Another possible explanation for the interesting result for the null transformation efficiency of leaves in the E group is that we hypothesize that at the time of making the micro-holes with the MW, the dense waxy layer covered the hole created by the needle and this prevented agroinfiltration, due to the fact that 0% transformation efficiency in all leaves is quite peculiar in this leaf stage regardless of the treatments, leading to speculation as to whether the *Agrobacterium* suspension really did infiltrate.

Wounding is an integral step in *Agrobacterium*-mediated transformation and as well as providing an entry point for the bacteria, activates the production of *vir*-inducing molecules that facilitate the successful transformation of plants (Horsch et al. 1985). In avocado, the generation of micro-injuries on the leaf surface has a positive effect on transient gene expression, as has been reported for citrus (Acanda et al. 2021). Since other phenolic compounds such as vanillin and cinnamic acid have been previously reported to induce *vir* genes even more potently than acetosyringone (Cha et al. 2011), and given that these compounds are present in avocado (Castro-López et al. 2019), we hypothesized that the phenolic compounds released by the avocado leaf as a result of the MW pre-treatment, together with acetosyringone pre-treatment, may be behind the improved transformation efficiency.

Our result has shown for first time that MW pre-treatment and JA acted synergistically, and both were necessary to significantly improve avocado genetic transformation. Plant recalcitrance to *Agrobacterium* transformation is generally attributed to the activation of plant immune responses upon perception of the bacterium (Pitzschke 2013). JA is a phytohormone that signals the plant defense response to insect injury by activating induced systemic resistance (ISR), at the same time deactivating systemic acquired resistance (SAR), which is triggered by salicylic acid (SA) and defends the plant against biotrophic infections. Therefore, addition of JA to the *Agrobacterium* infiltration medium is hypothesized to suppress SAR, by complex crosstalk between JA and SA, making the avocado leaves more vulnerable to bacterial infections (Pieterse et al. 2009). Plants deficient in SA have shown increased susceptibility to *Agrobacterium*, while plants overproducing this metabolite show increased recalcitrance to infection (Yuan et al. 2007). A study of JA application prior to agroinfiltration of *Nicotiana benthamiana* leaves (Robert et al. 2015) suggests that JA has practical utility for enhancing recombinant protein expression by producing a significant depletion of large and small subunit of RuBisCO, and consequently an availability of metabolites and cellular resources for recombinant proteins. This synergistic response may be due to all the individual conditions mentioned above and as best of our knowledge has not been previously reported for avocado, opening an encouraging perspective for functional gene studies. It is unclear the effect of other concentrations promoted avocado leaves transformation, as this was not examined.

Agroinfiltration of attached leaves, rather than detached leaves, is ideal for further phenotypic experiments. Thus, the protocol of vacuum-based agroinfiltration of attached leaves to a plant larger than the vacuum chamber size unlocks the use of *Agrobacterium* mediated *in-planta* transient transformation as a tool for functional genetics in avocado. Using this method can open the way to explore gene function and metabolic pathways in this non-model plant and can be a starting point for other recalcitrant species to transformation. Compared to other methods of transient gene transformation, the vacuum-based agroinfiltration method for transient gene overexpression in avocado leaves is less time-consuming than protoplasts-based genetic transformation, is less expensive than biolistics, and does not have the compatibility, and gene of interest size limitations that viral vectors can have. To our knowledge, the vacuum-based agroinfiltration method has not been reported in a localized part of a plant that exceeds the size of the vacuum infiltration chamber, opening the possibility to apply i*n-planta* transient transformation without being limited by plant size. If this can be coupled to the regeneration of transformed cells either in leaves or other somatic cells, this would provide an opportunity to generate stable transgenic lines for clonally propagated cultivars, which could offer significant opportunity for trait improvement.

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Data availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest Authors have no conflict of interest to declare.

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