Leaf wounding and jasmonic acid act synergistically to enable efficient Agrobacterium-mediated transient transformation of Persea americana

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Abstract

Avocado, *Persea americana* Mill, is one of the most traded tropical fruits in the international market. Here, we report transient transformation of avocado leaves via agroinfiltration with the LBA4404 strain of *Agrobacterium tumefaciens* and constructs encoding the synthetic betalain gene biosynthesis cassette, *RUBY*, or GFP. The efficiency of transformation was dependent on leaf age, whilst microwounding and jasmonic acid treatments significantly enhanced transformation, acting synergistically to improve avocado transformation. This is the first report on Agrobacterium-mediated transient transformation on avocado leaves. It provides a useful tool in plant molecular and cellular biology research and has the potential to facilitate new capabilities to the genomics research community of this ancestral angiosperm.

Introduction

Commercially important cultivars of avocado are propagated clonally from shoot scions and traditional avocado breeding has been moderately successful but hampered due to avocados long juvenile period (3–15 years), complex reproductive biology and high degree of heterozygosity (Pliego-Alfaro et al., 2020). The application of biotechnology and particularly genetic transformation to breeding programs could overcome some of those limitations. For the last two decades there have been only a few reports of genetic transformation in avocado using Agrobacterium to generate stable transformants. However, one of the main challenges in avocado breeding has been the regeneration of transformed cells. Many of the stable transformation studies in avocado have employed embryogenic masses derived from zygotic embryos (derived from outcrossing), due to their high potential and competence for regeneration (Palomo-Ríos et al., 2012, 2017; Pliego-Alfaro & Murashige, 1988). The avocado industry requires optimized regeneration protocols for explants derived from tissues with an adult ontogenetic age, from scions in which their agronomic traits are known (Tamayo-Ramos et al., 2022).

Transient gene transformation, is a useful tool in plant cell and molecular biology research and provides results significantly faster than compared to stable transformation. Transient gene expression allows the assessment of gene function and regulation by 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 (H. Chen et al., 2008; Lu et al., 2003; Marion et al., 2008; Sparkes et al., 2006; Y. Zhang et al., 2020). Agrobacterium-mediated transient transformation approaches have proven effective in a wide range of perennial species including aspen (Takata & 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). Here, this approach has been proposed as a tool to facilitate functional analyses in avocado. To date, whilst there are reports of stable transformation of zygotic embryos, to our knowledge, there are no reports of transient transformation of adult plant tissue, perhaps due to avocados recalcitrant nature for regeneration and transformation, which limits functional genomics research and genetic improvement (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 & Chan, 2009). Therefore, a reliable method for transient gene transformation in avocado plant could significantly improve our ability to assess protein function, localization, and interaction, in avocado rather than in model species. It could also be used to evaluate the transformation capabilities of different somatic tissues, which 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. Among the main factors widely reported in previous studies of Agrobacterium-mediated transformation are that wounded tissues (Potrykus, 1990), and pre-culture on phytohormone cocktail-containing medium (Sangwan et al., 1991) affect susceptibility to Agrobacterium, and that recalcitrance to transformation is inversely proportional to tissue age (Cervera et al., 1998). Here, we used detached avocado leaves to evaluate these factors on transient genetic transformation. We provide the first report of transient genetic transformation in 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

Plasmid Construction and Agrobacterium Cell Cultures.

Two plasmids were used in this study, pJL-TRBO-G was a gift from John Lindbo (Addgene plasmid # 80083) (Lindbo, 2007), and *35S:RUBY* was a gift from Yunde Zhao (Addgene plasmid # 160908) (He et al., 2020). Each expression vector was transformed into *Agrobacterium tumefaciens* LBA4404 using an electroporation method. Transformed *A. tumefaciens* were plated on Yeast malt (YM) agar with streptomycin (50 µg/ml), rifampicin (10 µg/ml) and for plasmid selection; kanamycin (50 µg/mL) for pJL-TRBO and spectinomycin (50 µg/mL) for *35S:RUBY*, then incubated at 28°C for 48 hrs in the dark. For agroinfection a fresh single colony was grown in 3 mL of YM broth to avoid clumping of cells (B. Zhang, 2019), supplemented with acetosyringone (AS, 20 µM) at 250 RPM and 28°C overnight. Cultures were harvested by centrifugation at 3000 g for 10 minutes and pellets were resuspended with fresh washing medium (4 g/L Murashige and Skoog Medium (salts and vitamins), 4 g/L sucrose, 20 mM MES, 5 mM $MgCl_2$). Cells were again pelleted by centrifugation at 3000 g for 10 minutes and resuspended in infiltration medium (Washing medium + 150 µM AS, 0.1% v/v Plant Preservative Mixture (PPM), pH 5.3) (Acanda et al., 2021) before incubating for 4 hr at 28°C and 150 RPM. Cell suspensions were then adjusted to an OD_{600} ~0.6 with infiltration media. For treatments including methyl jasmonate (JA), 250 µM JA was added to the agrobacterial suspension, prior to vacuum infiltration (Jung et al., 2014).

Plant material and vacuum transformation

Persea americana Var Hass was obtained from CITAMEX. Detached leaves were harvested prior to vacuum infiltration and separated into five groups in order to assess the effect of leaf stage (Light-brown

(A), dull-brown (B), Transition (C), light-green (D) and dull-green (E); n=5, each group, Figure 1). To evaluate the effect of wounding avocado, detached leaves were mechanically wounded on the abaxial surface of the leaves using a roller with 540 microneedles (MW), each 1.5 mm, to facilitate entrance of agrobacteria (Acanda et al., 2021), Detached leaves, but not the exposed vascular bundles of the petiole, were submerged into Agrobacterium resuspended in infiltration medium and were put into a vacuum chamber until vacuum reach -0.07 MPa, the vacuum was set for 5 min following release of vacuum and repeated twice. 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.

Protein extraction and Western-blot

Approximately 100 mg of leaves infiltrated with pJL-TRBO-G GFP were ground into a fine powder with liquid nitrogen in a frozen mortar. 1 mL of TRIzol reagent (ThermoFisher) was added following manufacturer's instructions for protein isolation, and total protein extracts were quantified using the Micro BCA™ Protein Assay Kit (Thermo Fisher) following the manufacturer's instructions. 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 (MerckMillipore). 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 Scientific) 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 Scientific) for 2 h at room temperature (RT). Antibody binding was detected with the Pierce ECL Western blotting Substrate solution, following the manufacturer's instructions (Thermo Scientific) and by means of an X-ray film, following standard procedures.

Spectrophotometric detection of Betalains

Agroinfiltrated leaves were frozen in liquid nitrogen and ground with a mortar and pestle. Betalains were extracted from 100 mg of ground tissue by resuspension with 300 uL 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 & Aharoni, 2018) using the Infinite 200 PRO microplate reader (TECAN).

Imaging and Statistical analysis

Quantification of betalain in leaves was assessed by image-J software (NIH, USA) and Absorbance data of betalain extracts were analyzed by one-way ANOVA using GraphPad Prism 8 software.

Results And Discussion

Transient expression of GFP in avocado

Preliminary experiments determined that avocado leaves are not suitable for agroinfection using a needleless syringe, as is commonly used for agroinfiltration of tobacco. Vacuum infiltration of leaves was therefore performed to allow penetration of agrobacteria, carrying the pJL-TRBO-G vector, into the inter cell spaces (Simmons et al., 2009). After 8 days post infection (DPI), infiltrated and control leaves were examined under a long wave UV light (365 nm); this method was initially chosen as a rapid means of evaluating transformation and has been used routinely in model plants such as tobacco transformed with GFP constructs (Casper & Holt, 1996). However, both control (non-transformed) and the agroinfiltrated leaves showed only slight basal fluorescence, possibly due to the autofluorescence of the leaf (Buschmann & Lichtenthaler, 1998). Supplementary Fig. 1 shows a representative image of control and agroinfiltrated leaves. This observation was consistent across different leaves and thus it was determined that this was not suitable for rapidly evaluating transformation efficiency using fluorescent proteins in avocado. It has been reported that the detection of GFP in green tissues using macroscopic methods can lead to a substantial underestimation of expression levels. In observations made in medicago, rice and Arabidopsis, chlorophyll interferes with GFP fluorescence (Zhou et al., 2005).

To assess if GFP protein was expressed in infiltrated leaves, total protein was extracted and a western blot assay was performed, with antibodies specific to GFP. Microwounding and JA treatments were included to determine whether these affected transformation and transgene expression. WB analysis revealed that microwounding in combination with JA treatment resulted in detectable GFP expression in some but not all independent samples. No GFP was detected in leaves that were treated with JA but not microwounded (Figure 2). These results demonstrate that whilst GFP can be detected following agroinfiltration of avocado leaves, it is not a suitable reporter for rapid macroscopic evaluation by long wavelength UV illumination.

Transient expression of the betalain biosynthetic pathway in avocado leaves and the impact of leaf age

Due to the difficulty in assessing GFP expression easily and quickly, we decided to evaluate the non-fluorescent reporter, *RUBY*. This expression plasmid 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). Betalains were first discovered in angiosperm plants, where they are unique to the order Caryophyllales and are present in these plants instead of anthocyanins. Anthocyanins are derived from phenylalanine, while betalains are natural water-soluble pigments derived from tyrosine, and are composed of betaxanthins and betacyanins, the former yellow and the latter red to purple (Polturak & Aharoni, 2018). The bright red color from betalain is easily contrasted from green leaf color and brown caused by leaf damage and deterioration. The advantage of this reporter is that visualising the transformed area does not require specialized equipment such as fluorescence or confocal microscopes.

The *RUBY* construct was infiltrated into avocado leaves as described previously, using a combination of microwounding and vacuum infiltration of detached avocado leaves. Betalain synthesis produced red

spots on avocado leaves easily distinguishable with the naked eye from the third day after agroinfiltration. As we previously been shown for transient expression of the GFP reporter, microwounding was required for efficient transformation. Additionally, leaf age had a significant impact on reporter expression (Figure 3). Leaves at the earliest stages (A and B) did not survive the infiltration treatments and so were excluded from our analysis. In addition, older leaves (stage E) consistently showed little or no evidence of reporter expression. In contrast, leaves at stage C and D displayed consistent betalain staining in the periphery of the microneedle-made wounds, which was confirmed by pixel density analysis of the stained area around wound sites (stage C, 6%; stage D, 9%). Several authors have reported that the transformation of plant cells by Agrobacterium is inversely proportional to the age of the tissue (Wixom et al., 2018). 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 will 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). 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). 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 (Supplementary Fig. 2).

The use of the *RUBY* reporter system in avocado transformation therefore resolves the limitations of reporters requiring fluorescence or confocal microscopy. 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 (K. Chen et al., 2021) and is promising to drive the evaluation of plants with thick waxy cuticles.

Effect of wounding pre-treatment and jasmonic acid

Previous work has shown that pre-treatment with the phytohormone jasmonic acid (JA) can affect agrobacterium mediated transformation (Jung et al., 2014). Having determined that leaf age is a critical factor, we therefore evaluated whether treatment with JA affects the efficiency of transient expression. Our earlier experiments with pJL-TRBO-G had indicated that JA treatment alone was not sufficient for transient transgene expression (Figure 2). This observation was supported by new experiments focusing on leaves at stage C and D and using agroinfiltration of *RUBY*. Here, there was no significant betalain synthesis in leaves treated with JA, demonstrating that microwounding coupled with leaf age are the

primary factors influencing the competence for transient transformation (Figure 3). 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. 2013). 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 these, together with acetosyringone pre-treatment, may be behind the improved transformation efficiency in leaves that were microwounded.

Whilst JA did not appear to significantly affect transient transformation in the absence of microwounding, we did observe a synergistic effect when leaves were both microwounded and JA treated (Figure 4).(Cho & Winans, 2005) Image analysis was initially performed to quantify betalain staining and showed that staining was highest in stage D, followed by stage C leaves in the treatments with MW and JA with 27.9 and 12.1%, respectively. In the treatments without JA the area in which betalain is expressed is less than 10% being again higher for stage D, followed by C with 9.3 and 6%, respectively. This image analysis data was supported by spectrophotometric quantification of leaf extracts (Figure 5). Betalain was produced at a significantly greater level in leaves at stage C and D with MW and JA (+MW, +JA) treatment. Using this method to quantify transient expression, microwounding alone did not show significantly higher expression than control, highlighting the synergistic effect of both microwounding and JA treatment.

Our result of increased overexpression of the heterologous genes by including JA in the agroinfiltrated cell suspension in avocado leaves agrees with previous data for transient expression in detached whole sunflower (*Helianthus annuus* L.) leaves using vacuum infiltration (Jung et al., 2014). 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 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 of the individual conditions mentioned above and as best of our knowledge has not been previously reported, opening an encouraging perspective for functional gene studies.

Conclusion

This research introduces a new species to the list of transiently genetically transformed species, but more importantly, we present simple and rapid treatments for overcoming the recalcitrance of avocado leaves to agrobacterium infection. The detached leaf transient avocado method is economical compared to other methods such as biolistics and enables the study of metabolic pathways or gene expression in the plant, which opens the possibility of synthetic biology studies in this plant of high commercial interest, without the need of the high cost and time-consumming regeneration of a stable transgenic plant or the regulatory frameworks associated with stable transgenics. Our data revealed that the LBA4404 strain having the Ach5 chromosomal background and utilizing octopine is suitable for transient expression in avocado. The *RUBY* reporter proved to be a useful, simple and fast tool for the evaluation of avocado transient transformation. Whereas GFP expression can be detected following agroinfiltration, its detection requires more time consuming and specialized methods. In this study we found that leaf age is a critical factor for transformation success. We have determined that avocado leaves within a relatively narrow developmental time span are required for Agrobacterium-mediated transient genetic transformation, even with the help of vacuum, wounding and the application of JA. Working with mature leaves should be avoided until conditions for this leaf stage are optimized. We also found that a microwounding pre-treatment and jasmonic acid acted synergistically, and both were necessary to significantly improve avocado genetic transformation. To our knowledge, this is the first report of Agrobacterium-mediated transient transformation in avocado leaves, as well as the first report of overexpression of an entire metabolic pathway that is not native to this plant. This system is efficient in heterologous gene expression in this ancestral angiosperm and provides a novel alternative to the avocado genomics research community by allowing better progress in the analysis of gene expression through a transient transformation approach. 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.

Declarations

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Contributions

JASG, MCM, and EMT performed the experiments and analysed the data. RUL and SAC were responsible for the idea of the article, the funding acquisition and project administration. All authors contributed to the writing-reviewing and editing.

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Data availability statement

Enquiries about data availability should be directed to the authors.

Conflict of interest

Authors have no conflict of interest to declare.

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Figures

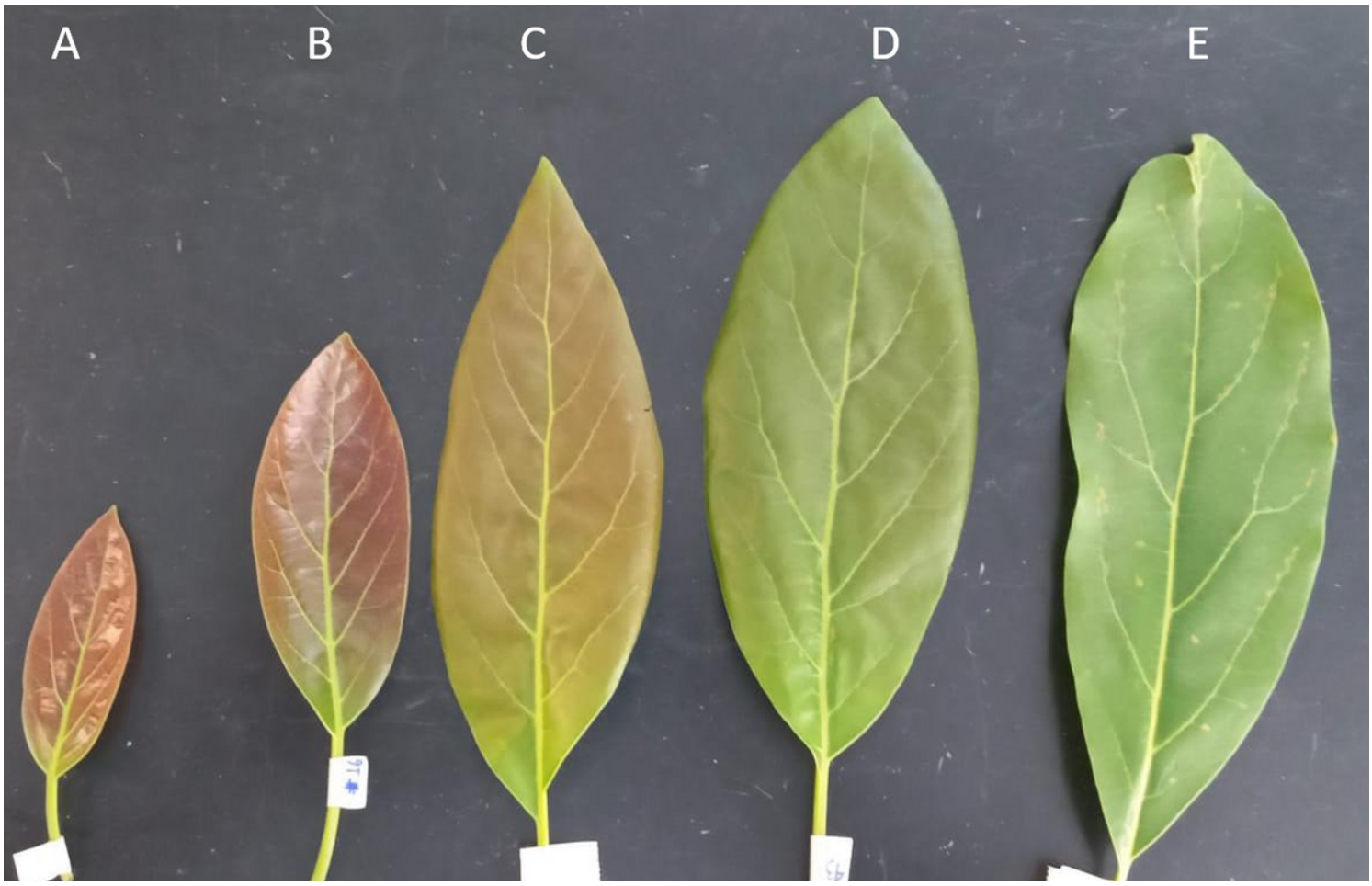

Figure 1

Representative image of different avocado leaf stages used in the study: Detached leaves were grouped as follows (Light-brown (A), dull-brown (B), Transition (C), light-green (D) and dull-green (E).

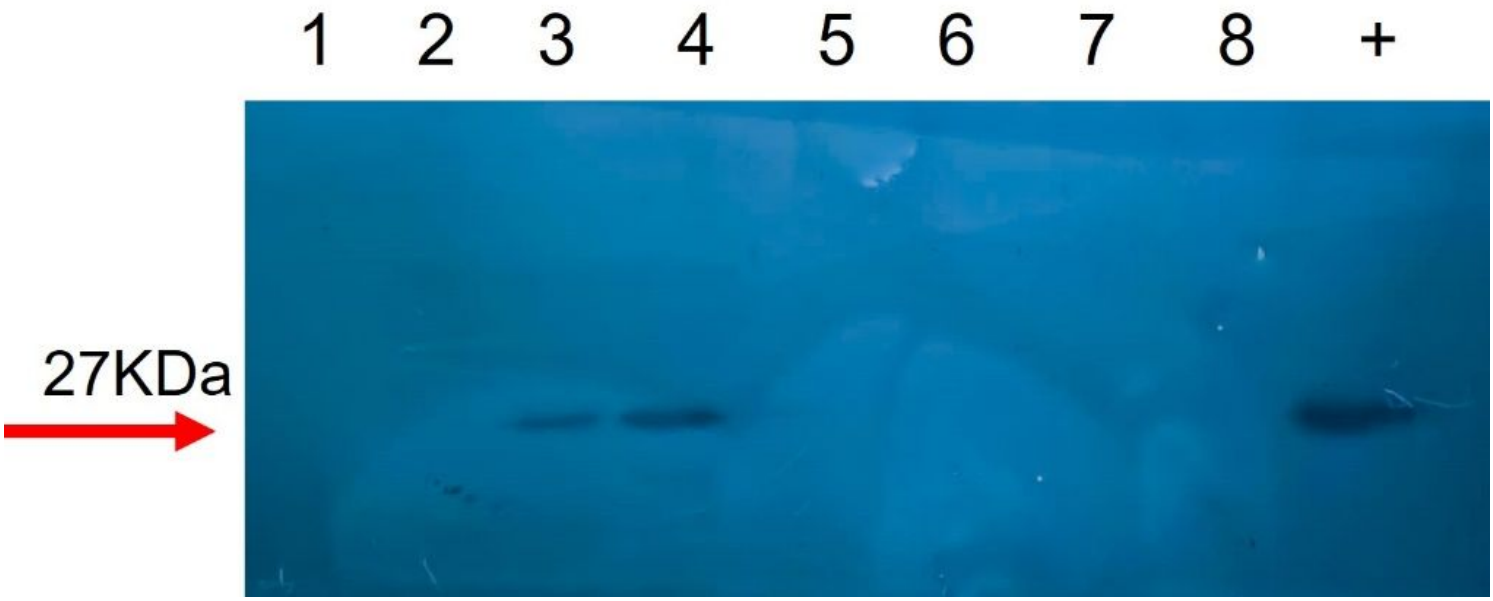

Figure 2

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

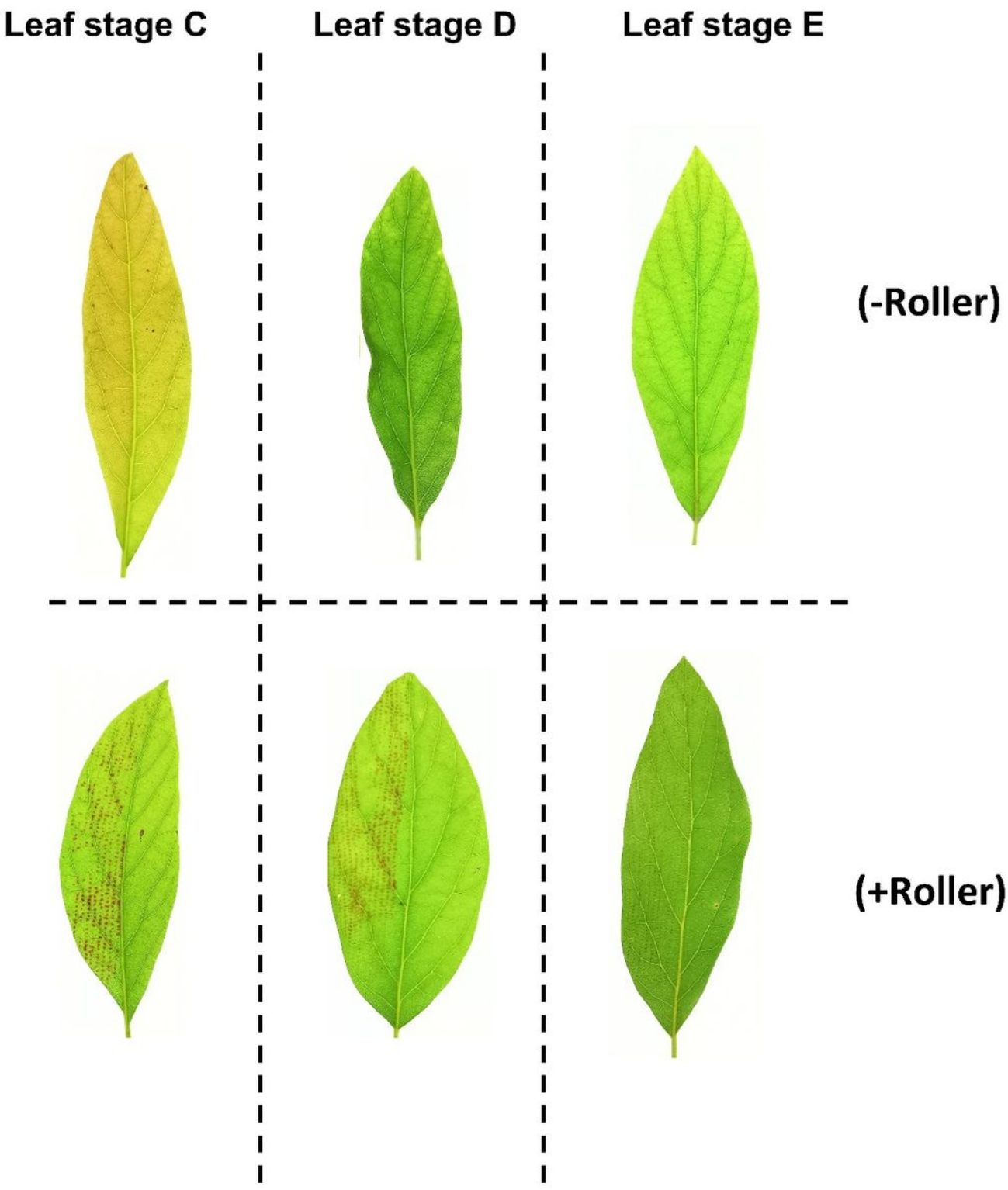

Figure 3

Effect of leaf stage on expression of *RUBY.* Representative image of one of five biological replicates of *RUBY* expression in avocado leaves at stages C-E and with (+MW) or without (-MW) microwounding. Betalain is located arround the wounds, it can be observed that in stage E there is no presence of betalain under any condition.

Figure 4

Effect of wound pretreatment and JA. Betalain staining following agroinfiltration of avocado leaves (stage C and D) with the *RUBY* reporter. Leaves represent independent agroinfiltrations for each treatment. Top row, no microwounding plus JA (250μM); middle row, microwounding and bottom row, microwounding plus JA (250μM) treatment.

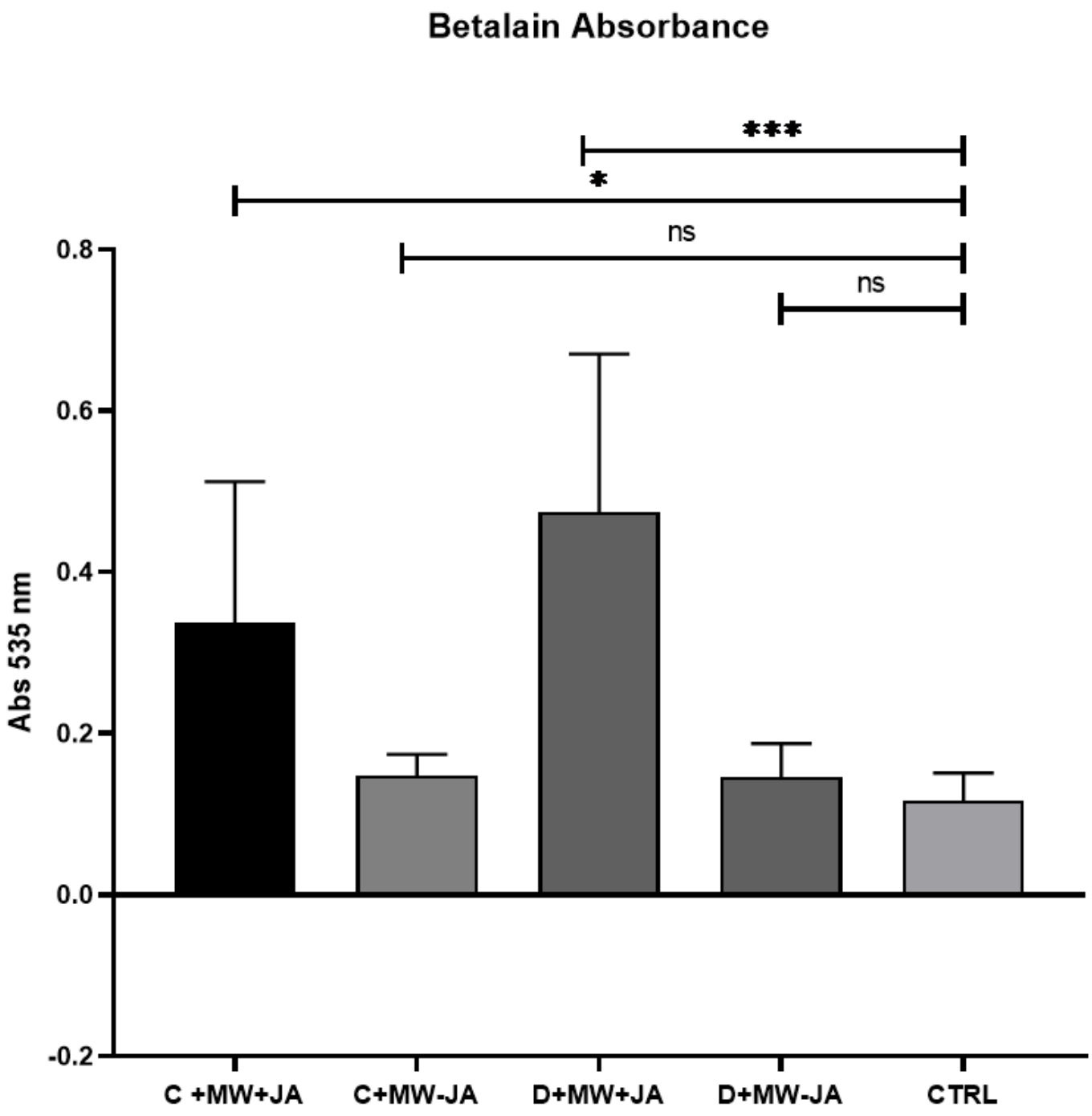

Figure 5

Betalain determination by spectrophotometry. Extracts of the leaves transformed with *RUBY* were analyzed by spectrophotometry at 535 nm to determine the presence of betalains (*P<0.05, ***P<0.01).

Supplementary Files

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- Supplementaryfigures.docx